Detection of Lysophosphatidic Acid in Serum; Towards a Cost Effective Test for Ovarian Cancer

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Abstract

Ovarian cancer is a disease that affects a quarter of a million new women and causes over 140,000 deaths worldwide annually. This is primarily due to how difficult the disease is to detect, with few symptoms present in the early stages of the disease and difficult to physically feel masses. Currently detection of ovarian cancer requires time consuming and expensive imaging studies such as transvaginal ultrasound and MRI scans, which are only performed if it is already suspected that a woman has the disease. The only currently accepted blood test for ovarian cancer detects the biomarker CA-125, but this test is only sensitive to 50% of cases and as a result is rarely used for signaling the presence of the cancer. There is another biomarker for ovarian cancer called lysophosphatidic acid (LPA) which has a sensitivity and specificity of over 90% for the disease, making it promising for use in testing for ovarian cancer. There are currently no low cost or easy to perform blood tests for LPA, preventing its use as a general test that can be performed at yearly physicals. However there is a duel protein system comprised of Gelsolin and Actin which is interrupted by interactions with LPA, which can therefore be used to develop a low cost blood test for the marker. These proteins could be bound to a biosensing surface, and the release of actin as a result of LPA would generate a measurable signal. Studying the fouling behaviour of LPA and serum on transverse shear mode biosensors, also known as
quartz crystal microbalances, showed that the background fouling signal was too large for a test to be developed on this class of devices. Instead a test based on colorometric sensing of dye modified actin was conceived and evaluated. This test comprised of the protein gelsolin being bound to solid phase in a way capable of holding onto the protein in serum samples. The gelsolin was also be bound to a fluorescent dye modified actin that could be measured using colorimetric absorbance or fluorescence. These proteins were successfully created, and shown to create a complex that could be broken by LPA in a concentration dependent manner. Immobilization of this protein complex was performed on silica gel, using Ni-NTA which was attached to the surface via a trichlorosilane linker functionalized with an acid chloride head group, which was first synthesized in these experiments. Several conditions in the development of the surface Ni-NTA adlayer and subsequent protein immobilization were evaluated, and it was found that such things as a large pore size in the silica gel, removal of excess toluene from the surface following adlayer addition, and adequate washing and drying of the silica were all of crucial importance to the performance of this test in serum. Development of this test showed promise in quantifying LPA at biologically relevant concentrations, however more work needs to be done to lower the limit of detection to a point below the cut-off level for ovarian cancer. Crucially this test is very cheap to produce, and requires little human labour to perform which are characteristics required for its wide scale adoption in ovarian cancer screening.
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List of Abbreviations

ab-NTA - Nα,Nα-bis(carboxymethyl)-L-lysine
APCA - 3-(2-allyloxy)propanoic acid
APC - 3-(2-allyloxy)propanoyl chloride
CA-125 – Cancer Antigen 125
CAG - contact angle goniometry
CT – Computer Tomography
DCM - Dichloromethane
DMF - Dimethylformamide
ELISA - Enzyme-linked immunosorbent assay
EOC - Epithelial ovarian cancer
FTIR - Fourier transform infrared spectroscopy
HSP-10 - Heat Shock Protein 10
HTS – trichloro(hexyl)silane
LPA – Lysophosphatidic acid
MALDI - Matrix assisted laser desorption ionization
MEG-Cl - 3-(3-(trichlorosilyl)propoxy)propanoyl chloride
MEG-OH - 2-(2-mercaptoethoxy)ethan-1-ol
MRI – Magnetic Resonance Imaging
NMR - Nuclear Magnetic Resonance
NSA - Non-specific adsorption
OC – Ovarian Cancer
OTS – trichloro(octadecyl)silane
PAGE - Polyacrylamide gel electrophoresis
PBS - Phosphate buffered saline
PET - Polyethylene terephthalate
PFP-TTTA - Perfluorophenyl 12-(trichlorosilyl)dodecanoate
QCM - Quartz Crystal Microbalance
SAM – Surface Assembled Monolayer
SDS - Sodium Dodecyl sulfate
TBAP - Tert-butyl-3-(2-allyloxy)propanoate
TFA - Trifluoroacetic acid
TSM - Transverse Shear Mode Wave Device
UV - Ultraviolet
UV-Vis - Ultraviolet-visible absorbance spectroscopy
XPS - X-ray photoelectron spectroscopy
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Appendix B - TSM Visual Basic Code

Appendix C - Synthetic Schemes
Chapter 1

1 Introduction

1.1 Ovarian Cancer

In women over 50 cancers are one of the leading causes of death, at over 15%, making it a serious problem around the world. Among female cancers, one of the most dangerous is ovarian cancer. Ovarian cancer is a general term used to identify any cancers that originate or find their main tumors in women’s ovaries or fallopian tubes. The majority of ovarian cancer cases result from tumor origination in the surface epithelial cells of the ovary, known as epithelial ovarian cancer (EOC). Though ovarian cancer is less common than several other female cancers, such as breast cancer, it has the highest fatality to case ratio of all gynecological cancers making it a serious issue especially for post-menopausal women.

Specifically ovarian cancer accounts for over 225,000 new cases and over 140,000 deaths worldwide each year. Although incidence rates have shown a decrease in developed countries over the last few decades the overall number of new cases is increasing as a result of a growing and aging population. Though the majority of ovarian cancer cases are diagnosed in older women, the disease is also present to some extent in younger populations, with girls in their teens being diagnosed and even dying of the disease each year. Despite increased survival rates for other cancers over the last few decades, ovarian cancers survival rate has remained roughly steady since 1995, with a 5 year survival rate of less than 40% of those women who survive their first year. In fact, survival rates for the disease have improved little since the 1970s despite advances in cancer treatment and surgery.

At more than 70% the majority of women are diagnosed with late stage ovarian cancer. Unfortunately the five year survival rate for women diagnosed at a late stage, versus those diagnosed in stages I or II, is extremely low with less than 40% of late stage women surviving 5 years compared to 90% for those diagnosed in early stages. This decreased survival rate is mostly due to the unusually rapid rate of ovarian cancer metastasis, which is due to a lack of anatomical barrier to prevent seeding of the tumor into the peritoneal cavity. As a result it is crucial to improve diagnosis in the early stages of the disease to increase the survival rate for those women who suffer from it.
There is also a large hereditary component to ovarian cancer susceptibility, with more than 10% of women who develop ovarian cancer also having mutations in breast cancer gene 1 (BRCA1) or breast cancer gene 2 (BRCA2).\textsuperscript{21–24} As such a family history of ovarian cancer is one of the greatest predictors of the disease.\textsuperscript{25–27} For these gene mutations BRCA1 mutations were responsible for more cases in women under 50 years of age, while BRCA2 mutations were responsible for more cases in older women.\textsuperscript{22} Despite the increase in ovarian cancer rates for women who carry these mutations, these women also have an increased survival rate than ovarian cancer patients without BRCA mutations.\textsuperscript{28,29}

### 1.2 Symptomology, and Existing Detection and Screening

Ovarian cancer often presents with few symptoms at an early stage and vague symptoms at later stages. These symptoms include bloating, nausea, difficulty eating, back pain, and urinary urgency among others, which tend to persist and worsen as the cancer advances.\textsuperscript{30–35} As stated, these symptoms don’t tend to appear until the diseases later stages, making early detection more difficult as women with the disease have little to no physical indication of its presence. As such the predictive value of symptoms in early stage detection is around 1%, meaning physical symptoms can rarely be used to detect the disease in its early stages,\textsuperscript{36} but can be useful in signaling the disease at later stages.\textsuperscript{37} The depth of tumors in the body also makes physical detection more difficult as the masses cannot be easily felt from the outside unlike with breast cancer. Thus it generally isn’t even looked for by women until their symptoms have progressed, which is generally when the cancer has reached later stages.

The common way of detecting ovarian cancer is through imaging techniques mentioned earlier; transvaginal ultrasound, computer tomography scans (CT), and magnetic resonance imaging (MRI). As well there is a blood test that exists which looks for the ovarian cancer biomarker CA-125. This current blood test for CA-125 is not ideal for reasons that will be discussed in the next section, and is only useful when combined with imaging studies for the detection of ovarian cancer at any stage.

Ovarian cancer presents as adnexal masses, which are relatively common and rarely malignant.\textsuperscript{38,39} Only in approximately 1% of women who have these adnexal masses do they turn out to be malignant, and for the remaining majority it is important to avoid unnecessary
As such it is important to establish the nature of these masses by imaging techniques before proceeding with biopsies and further testing.41–43

The most common technique for imaging ovarian masses is transvaginal ultrasonography, which allows for basic imaging of the masses. Early stage and benign masses share different physical characteristics to later stage and malignant masses, which can be seen by ultrasound. These characteristics of late stage tumors include more papillary projections, more commonly being multilocus, larger in size, and containing more solid mass than early stage tumors.44–47 A simple scoring system has been developed to determine if the tumor is benign or malignant, and decide on future steps.48 The system uses the presence of any malignant traits of the mass, such as papillary growth, as well as CA-125 levels to determine if the mass is malignant. If mass is found to exhibit morphologies between benign and malignant then consultation with an expert sonographer and further imaging is required.

Differences also exist between benign and malignant masses when imaged by magnetic resonance imaging, and positron emission tomography/computed tomography.42,43,49,50 Due to the presence of solid and cystic areas within malignant masses, as well as necrosis within the solid tissue, they appear to be hyperintense on diffusion-weighted images, with only intermediate intensity on T2-weighted images, whereas benign masses tend to be hypointense on both of these images.39 These techniques have a much higher specificity and accuracy for determining a masses nature than ultrasound, with a specificity of 84% for MRI versus 40% for ultrasound, and an accuracy of 89% for MRI versus 64% for ultrasound.51 They are however less accessible and more expensive than ultrasound, and are therefore generally used after a mass has been located by ultrasound and determined not to be benign. CT scans may be preferable to MRI due to their lower expense and greater availability.52,53

However, these ultrasounds and other imaging techniques are expensive and time consuming to perform and require a trained physician for the duration of the test. It has also been found that only 1% of screened women present with unusual ovarian morphology, of which only a fraction have ovarian cancer, making its high cost further impractical. As well they do little to detect ovarian cancer in the early stages of the disease, with only 25% of women diagnosed in stages I and II when the outcome of the disease is much better.54 A simple and inexpensive blood test that can flag potential ovarian cancer patients for further imaging would be ideal.
1.3 Ovarian Cancer Biomarkers

Biomarkers are compounds that when found in biological samples are indicative of certain disease states, or the presence of certain cell types and mutations. Ideally there would be a biomarker that is present or elevated in all ovarian cancer patients at all stages, and that is easy to detect. Markers that are present in only a subset of patients or that require the use of multiple markers in expensive testing are not as useful, though they are better than nothing. An ideal biomarker may not exist, but there have been several biomarkers identified for ovarian cancer.

When biomarkers for ovarian cancer are discussed the first that is typically mentioned is cancer antigen 125 (CA-125), which was mentioned earlier. This marker is the basis for the only current ovarian cancer monitoring protocol. CA-125 has been used to monitor ovarian cancer patients for over three decades, but is not viable on its own for the detection of ovarian cancer. This is because it is only present at elevated levels in approximately half of patients at an early stage, and 92% of late stage patients. So on its own it cannot be used to identify most early stage ovarian cancer patients when detection is most useful. It also isn’t applicable for monitoring all patients, as 8% in the later stages of the disease won’t show elevated levels. It is also present at elevated levels in benign conditions such as pregnancy, or menstruation giving it a high rate of false positives when used for detection.55

Although CA-125 alone is not suitable for the detection for ovarian cancer, studies combining CA-125 screening with other biomarkers have shown a positive increase in detection ability. Su and colleagues used a multiple logistic regression model, with values for CA-125, ApoA-I, transferring, and TTR (which are all prospective biomarkers for ovarian cancer) for early detection of ovarian cancer. This model provided a sensitivity of 89% and a specificity of 97% for detection of early-stage ovarian cancer. This test however uses multiple screening methods, and is therefore expensive to perform.56

Since CA-125 isn’t a catch all biomarker, as it isn’t expressed in all ovarian cancer patients at all stages, researchers have been looking for other potential markers. One such marker is HE4, which is found to be elevated in two thirds of early and late stage ovarian cancer patients, has very high sensitivity, and is even present in a third of non CA-125 producing tumours.55 However studies have found that only serous and endometrioid EOCs overexpressed HE4, and
that other EOCs rarely overexpressed this protein. As such HE4 in combination with CA-125 is most useful in classifying patient risk in those with serous and endometrioid EOCs.

Another biomarker, which has been mentioned in the media, is mesothelin. Mesothelin is found to be elevated in three quarters of ovarian cancer patients, but it is also found to be elevated for those suffering from other cancers and those with mesothelioma. As a result Mesothelin is not a catch all biomarker, as is desired. Therefore, in order for mesothelin to be used as a biomarker for the detection of ovarian cancer, one would need to combine it with other biomarker tests such as CA-125. For now mesothelin is most useful in monitoring disease progress for ovarian cancer patients currently undergoing treatment.

Though several other potential markers have been identified, such as Osteopontin, HSP-27, HSP-60, calreticulin, vimentin, and fibrinogen-γ, none of them are expressed in most if not all of ovarian cancer patients. They all suffer from the same limitation that they are not consistently overexpressed until later stages of the disease, when detection is less important.

Additionally circulating miRNA strands have been identified in relation to ovarian cancer presence. This study identified 22 miRNA strands that were differentially expressed between ovarian cancer patients and healthy controls. In order for these markers to be useful in disease detection, many of these miRNA markers would have to be detected simultaneously for diagnosis leading to greater cost and labour required for the test. As well these miRNA patterns may be expressed in other disease states, which have not been studied.

One potential biomarker that may overcome the limitations of those mentioned previously is Heat shock protein 10 (HSP-10), which is a protein that has had some initial study completed for its potential as an ovarian cancer biomarker. Using Western immunoblotting the study analyzed levels of HSP-10 in the sera of 10 patients with stage III ovarian cancer, and 9 healthy controls. They found HSP-10 was present in all ovarian cancer patients, and no controls, suggesting HSP-10 would make for a suitable biomarker for ovarian cancer detection. However, it may not be ideal for detecting ovarian cancer at early stages as at this point it is still unknown if HSP-10 is found in the sera of patients with stage I ovarian cancer. Further studies need to be performed before HSP-10 can be confirmed as a prospective biomarker for the detection of ovarian cancer.
Lysophosphatidic acid (LPA) (Fig. 1), which is a signaling lipid, is another potential ovarian cancer biomarker. In separate studies this signaling lipid was found to be elevated in 90% of stage I ovarian cancer patients, and 100% of later stage patients, and has been linked as a potential biomarker for ovarian cancer with sensitivity and specificity of over 90%. It was also found that LPA elevation correlated to the stage of the disease with stage III and IV patients presenting higher LPA serum concentrations than stage I and II patients. These elevated levels make LPA a promising marker in the early detection of ovarian cancer. However, as with the other biomarkers discussed, LPA levels were found to be elevated in a subset of healthy controls and patients with benign gynecological diseases, approximately 10% and 25% respectively. Although this is an issue present with other biomarkers, the presence of LPA in nearly all cases of ovarian cancer and its resultantly high sensitivity and specificity make it a much better biomarker to detect. A detection method for ovarian cancer utilizing LPA as a marker would be useful in identifying ovarian cancer patients in at risk populations, such as women over 50 and women with family histories of ovarian cancer, but would not be useful as a screening tool for the entire population of women given the number of false positives it would produce.

1.4 Current Lysophosphatidic Acid Detection

Commonly LPA is detected and quantified in samples using standard analytical methods. This includes the use of capillary electrophoresis and ultraviolet detection, gas chromatography paired with FAA or Mass Spectrometry, thin layer chromatography paired with mass spectrometry, liquid chromatography paired with mass spectrometry or absorbance spectroscopy, and MALDI mass spectrometry. Although these techniques are very sensitive towards LPA, they almost always require lipid extraction or another work-up of the serum samples before use. They also are labour intensive, time consuming, and use expensive
instrumentation to quantify LPA. As such these methods find little use outside of academic labs, and new methods that use lower cost instrumentation and are rapid and easy to perform are required for LPA to be included in mass screening for ovarian cancer.

There have been several other methods developed for the detection and quantification of LPA that aim to overcome these challenges. One such method involves lipid extraction of a biological sample, followed radio labeling of LPA by recombinant LPA acyltransferase and \(^{14}\)C oleoyl-CoA (coenzyme A).\(^{91}\) Once labeled the lipids are separated by TLC, and radioactive spots are identified using iodine vapour and scraped off. LPA concentration is then measured by forming a scintillation cocktail and measuring the solution's scintillation. The advantage of this test is primarily its low limit of detection at 0.2 pmol, and a large limit of linearity allowing for a broad range of concentrations to be accurately measured. However the test itself takes multiple days to perform, and is highly labour intensive. As a result it is unlikely to be adopted in a clinical setting despite its strong performance.

Another method of detection utilizes an enzyme linked fluorometric assay, which uses hydrogen peroxide and monoglyceride lipase to generate a measureable fluorescence signal.\(^{81,92}\) This method also presents with a low limit of detection in the nanomolar range, making it very sensitive when quantifying LPA. It does however still require lipid extraction form initial serum samples, adding to the labour and time required to perform the test. Another potential downside is the specificity of this test. The enzyme this test relies on, MGL, is known to react with a wide variety of lipids which could interfere with the ability of the test to accurately measure LPA.\(^{93}\)

Another interesting method for quantifying LPA in biological samples is a colorometric assay based around an enzyme cycle which produces and amplifies hydrogen peroxide.\(^{94}\) This assay has a higher limit of detection than the previously mentioned assays at 0.03 μM, but the ability for it to be used on a low volume of serum without the need for lipid extraction makes it much less labour intensive and time consuming to perform. The primary enzyme for the cycle is lysophospholipase, which primarily produces LPA from lysophosphatidylcholine and is specific to LPA.\(^{95}\) However this test is not routinely used for clinical quantification of LPA, possibly owing to difficulty in producing lysophospholipase, or instability in testing solutions.
More recently another colorometric test for LPA has been developed that uses interactions between LPA and copolythiophene to generate a measurable colorometric and fluorometric signal.\textsuperscript{96} This is not as sensitive as the previously mentioned tests, with a limit of detection of 0.6 $\mu$M, but is simple to perform and low cost. The downside however is a limited understanding of copolythiophene's interactions with other compounds found in serum and plasma. Due to this it is difficult to know if this test is truly sensitive to LPA, or if any derived signal is caused by competing compounds.

For several years ELISA assays for LPA have been commercially available, however they are not commonly used in screening for ovarian cancer.\textsuperscript{97} This is most likely due to the labour intensive nature of performing ELISA tests, making it prohibitive to perform any large scale screening for the disease.

Even with all of these detection methods for LPA there is still no commonly used screening for this important biomarker. This is likely due to the issues mentioned above: labour intensive, time consuming, and high cost methods are unlikely to be used in any large scale screening. As such a new method for quantifying LPA in serum needs to be developed that overcomes these hurdles. The test has to work in serum without the need for lipid extraction. It needs to be rapid and easy to perform. Finally, it needs to be low cost.

\subsection{1.5 Biosensors as Possible Detection Methods}

Biosensors are types of analytical devices that combine a physiochemical detector with a biological component to produce a measurable signal in response to specific biological stimulus. There are several different types of biosensors some of which are commonly used in medical diagnostics, such as those based on electrochemical detection, or those based on light detection.\textsuperscript{98} The most famous diagnostic biosensor is the commonly used glucose sensor for diabetes, which is actually an oxygen electrode combined with the enzyme glucose oxidase to detect glucose.\textsuperscript{99–101}

Biosensor based detection methods for diseases are potentially a very powerful technology, but still fall prey to some problems such as fouling. Fouling, or non-specific adsorption, is the deposition of undesired materials, such as lipids and proteins from tissue and fluid samples, on a surface.\textsuperscript{102–106} For biosensors this problem of deposition prevents an instrument from being
selective to the desired molecule of detection, and reduces the instrument sensitivity as a result of additional undesired molecules being present. It is difficult to correct for the fouling of a surface as the amount of fouling varies greatly from sample to sample. Current approaches to the problem of fouling involve the creation of a surface assembled monolayer (SAM) that prevents undesired molecules from adsorbing to the surface.\textsuperscript{107}

Once the problem of fouling has been solved, a biosensor could be used for the detection of ovarian cancer by developing an appropriate sensing scheme. Biosensors operate by using a molecular probe for the target molecule of interest to selectively bind the molecule of interest to the surface. These probes must be selective for the target molecule, stable on the surface, and preferably bind the target molecule reversibly allowing for the biosensor to be used multiple times. These probes can be antibodies grown against the target molecule, aptamers, which are oligonucleotides engineered to bind the target molecule selectively using the SELEX procedure,\textsuperscript{108} proteins, or other selectively binding molecules. As was discussed earlier LPA would make a good prospective biomarker for ovarian cancer, and the first step in producing a functional biosensor for the disease is the identification or development of probes for this molecule.

Once a potential probe, such as an engineered aptamer, has been developed for the biomarker of choice it must be attached to the surface of the biosensor. Biosensors may be based on several different technologies, such as electrochemistry, acoustic wave physics, or colorimetry, which may or may not also require the presence of fluorescent labels. Probes can be linked down with organic linking molecules that are bound to the sensor surface through a functional group, such as a silane group onto hydroxyl coated surfaces.\textsuperscript{109} These linking molecules would need to be bound to the surface within an anti-fouling layer, which also allows spacing between probe molecules. This spacing is especially important when the biomarker or probe are large and need suitable space for the binding event to occur.

Once the probe and an anti-fouling layer have been added to the device surface the device can be tested and calibrated using several different concentrations of biomarker solution, and eventually fluid or tissue samples. In the case of ovarian cancer, one promising branch of biosensors are those based on acoustic wave physics, such as transverse shear mode waves (TSM, also known as QCM) or EMPAS. These devices operate by introducing a shear mode wave in a quartz disc
using an electromagnetic field.\textsuperscript{110,111} The frequency at which the disc vibrates depends on the surface chemistry, and fluid slip. One of these devices with a suitable probe will vibrate at measurably different frequencies depending on the amount and nature of the material present on the surface, which should change depending on the amount of analyte to be detected that has bound to the surface probe. Another possible avenue of detection is colorimetric or fluorometric detection, some examples of which can be found in the previous section.

1.6 Introduction to Transverse Shear Mode Devices

Mentioned earlier quartz crystal microbalances are a type of sensing platform that take advantage of the piezoelectric properties of quartz. These devices are also sometimes referred to as transverse shear mode devices. The devices operate by passing an alternating current through gold electrodes which are on opposite sides of a thin quartz disc, which causes the quartz between the electrodes to vibrate at its resonant frequency. This vibrational frequency is affected by the amount and nature of material on the gold surface.\textsuperscript{111} Though traditionally utilized for gas phase sensing, QCM devices have been adapted to sensing in liquid phase environments by using flow through cells that channel the liquid over only one surface of the quartz, leaving the other in gas.\textsuperscript{112} There has been significant interest in using these devices in biosensing due to their relative ease of use and low cost compared to other analytical methods.\textsuperscript{113} This sensing can be achieved by binding a probe molecule to the surface of the gold, which binds selectively to the analyte of interest causing a measurable change in vibrational frequency (\textbf{Fig. 2}). However due to the complicated nature of bio fluids non-specific adsorption or fouling of compounds other than those of interest makes these devices difficult to use in these fluids.\textsuperscript{107}
Figure 2 – Use of an antibody or other probe molecule to detect an analyte of interest in a complex solution, and the affect it has on the vibrational frequency of the quartz disc.

In order to adapt QCM devices for use in biosensing, one first must deal with this issue of fouling. The most common method for dealing with fouling is the use of a surface assembled monolayer, which prevents the adsorption of unwanted compounds.\textsuperscript{107} Ideally these monolayers provide a great barrier to adsorption, which at the same time being thin enough so as not to dampen the signal caused by the binding of an analyte of interest to the probe. One promising monolayer for use with QCM devices is 2-(2-mercaptoethoxy)ethan-1-ol (MEG-OH), which is a thin molecule that forms an antifouling layer on gold surfaces (Fig. 3).\textsuperscript{114}

Figure 3 – Assembly of MEG-OH on a gold surface to create an anti-fouling SAM.

QCM devices could allow for the development of a detection method that can rapidly, cheaply, and easily detect LPA compound from serum samples which would allow many women to be tested for ovarian cancer, and increase the number of women for which the disease is detected
during early stages. The effect on QCM disc frequency that samples of serum and LPA at various concentrations have in the presence of a MEG-OH SAM was evaluated in chapter 4 to determine the consequences on surface fouling, and whether it is feasible to develop this platform further for detection in serum. It is predicted that LPA will have little effect on surface fouling in the presence of a SAM, but serum may cause fouling issues due to the many potentially fouling compounds contained within it.\textsuperscript{115–118} This hypothesis was tested and is discussed in chapter 4.

1.7 Probes for Lysophosphatidic acid

As was mentioned in the previous section in order to quantify LPA through a biosensing platform a suitable probe must first be found. Although SELEX does provide a potential way of making a surface probe by developing an aptamer, turning first to nature may prove beneficial since LPA is a known signaling lipid.

There are several membrane proteins that bind to LPA and act as receptors for various cellular processes.\textsuperscript{119} These receptors include LPA\textsubscript{1} which is responsible for cellular proliferation, survival, stress fiber formation, and neurite retraction.\textsuperscript{120,121} These processes are important for brain development, pulmonary fibrosis, and neuropathic pain demonstrating the important role LPA plays in healthy people.\textsuperscript{122–125} Additional membrane receptors are LPA\textsubscript{2} and LPA\textsubscript{3}, responsible for cell rounding and neurite elongation respectively.\textsuperscript{126} There are also LPA\textsubscript{4} and LPA\textsubscript{5} present, which are both responsible for neurite retraction and cell rounding.\textsuperscript{127–129}

Although these proteins could make for a suitable probe for LPA they may lose functionality when removed from a cellular membrane and deposited on a biosensor surface as a result of no longer being surrounded by the lipid membrane, which is required for maintaining most membrane proteins’ structure.\textsuperscript{130} As such it would be better to identify a non-membrane protein that can be used as a probe for LPA. An antibody for LPA has been developed,\textsuperscript{131} which could be used for biosensing applications. This antibody has unfortunately been patented, and was unable to be sourced for use in experiments. As such its efficacy and use as a biosensing probe could not be evaluated.

Another possible probe is the protein gelsolin,\textsuperscript{132} which binds to LPA through a small chain of amino acids known as the PIP\textsubscript{2}-binding domain (Fig. 4).\textsuperscript{133} Gelsolin binds LPA with a high
affinity measured by its $K_d$ of 6 nM, which on par with the previously mentioned receptors.\textsuperscript{119,132} The PIP$_2$-binding domain however only binds LPA with a $K_d$ of 920 nM, suggesting that the interactions of LPA and gelsolin are heavily dependent on the rest of the protein. The protein itself is a large 6 domain protein, with a molecular mass over 80 kDa,\textsuperscript{132} which makes its use as a probe in biosensing applications that require measuring mass changes difficult as LPA only weighs 436 kDa. Of the six domains the protein essentially exists as two identical components comprised of domains 1-3 and domains 4-6, with these halves individually being able to bind to LPA.\textsuperscript{134} As such half of gelsolin, which shall be referred to as gelsolin 1-3, could alone be used as a probe in binding to LPA.

![Crystal structure of gelsolin (grey) bound to actin (blue), with the PIP-2 binding domain highlighted in red. Protein data bank number 2FGH.](image)

Figure 4 - Crystal structure of gelsolin (grey) bound to actin (blue), with the PIP-2 binding domain highlighted in red. Protein data bank number 2FGH.

Gelsolin is also an actin binding protein, which regulates cellular motility and morphology by binding actin monomers, severing actin filaments, and nucleating actin polymerization to form new filaments all of which results in cytoskeletal changes in cells (Fig. 4).\textsuperscript{135–137} Actin can bind to gelsolin at three different sites and the affinity of this binding depends heavily on which binding site is targeted, and what salts are present. It has been measured with a $K_d$ as low as $4.5 \times 10^{-12}$ M, and as high as 400 $\mu$M.\textsuperscript{138–140}

Although the large mass of gelsolin in comparison to LPA makes methods such as QCM and EMPAS difficult, the binding of gelsolin to actin can remove this problem. This is due to the
fact that LPA is a regulator of gelsolin and actin binding, and will cause a release of actin from
gelsolin when present in solution.\textsuperscript{141} As a result of this release the small molecule LPA will
cause a large mass change on the biosensor surface, negating the issue of a small change being
difficult to detect. As well the actin can be pre-tagged with a signaling molecule that can be
measured in solution following the release of actin.

1.8 Introduction to Surface Linkers and Fouling

As was mentioned before, in order to use gelsolin and actin to detect LPA in serum samples a
surface linker is needed. Although the focus of this research was to immobilize gelsolin onto a
biosensing surface, the linker developed in this thesis is more versatile than this, and as such the
chapter on surface linkers will also make mention of broader applications for this linker.

Such a linker should also present some ability for anti-fouling as any biosensing platforms are
prone to fouling when exposed to physiological fluids (\textit{e.g.}, blood serum, plasma). Non-
specifically adsorbed proteins can affect the response of the biosensor by, for example, creating a
substantial increase in the background signal and/or decreasing the signal contribution from the
analyte by electrostatically and/or sterically excluding the analyte from the sensing surface.\textsuperscript{107}
This has inspired extensive efforts in the development of anti-fouling layers designed to resist or
altogether prevent NSA. Several excellent reviews have been written on this topic detailing
various classes of surface coatings including: amino acids, peptides, and peptoids; poly(ethylene
glycol)-based coatings; zwitterionic self-assembled monolayers (SAMs); and carbohydrate
derivatives.\textsuperscript{107,142,143}

Many biosensors incorporate ligands that enable molecular recognition, such as antibodies and
small molecules (\textit{e.g.}, biotin). These are typically connected to the sensing substrate covalently
with a linker molecule. An effective biosensor that uses this approach and that must be exposed
directly to undiluted or semi-diluted physiological fluids requires the harmonization of the
linker/ligand ensemble and an anti-fouling coating. One option is to construct a mixed monolayer
containing both the linker/ligand ensemble and an anti-fouling component. Another potential
solution is to design a linker whose structure contains motifs found in known anti-fouling surface
coatings and that can be functionalized with a targeting ligand. In chapter 3 the design and
preparation of 3-(3-(trichlorosilyl)propoxy)propanoyl chloride (\textbf{MEG-Cl, Fig. 5}), a linker that
forms SAMs on hydroxylated surfaces and that can be functionalized with a targeting group of
one’s choice through an acyl chloride head group, will be described.

![Chemical structure of 3-(Trichlorosilyl)propoxy)propanoyl chloride (MEG-Cl).](image)

**Figure 5 - Structure of 3-(3-(Trichlorosilyl)propoxy)propanoyl chloride (MEG-Cl).**

This molecule incorporates certain structural features found in MEG-OH (Fig. 6), a linker with
excellent anti-fouling (and anti-thrombogenic) properties that has been described in more detail
elsewhere.\(^{109,144,145}\) The anti-fouling capability of MEG-OH has been attributed to the ether
group that is thought to enable the formation of an interfacial layer of water molecules, which we
have observed previously using neutron reflectometry.\(^{146}\) The length of the linker makes it
spatially compatible with common spacers (linker diluents), such as trichloro(hexyl)silane, and
permits for the formation of an ultrathin surface coating.

![Chemical structure of MEG-OH.](image)

**Figure 6 - MEG-OH. The R group may represent a H atom, an atom in the underlying
substrate (e.g., Si in the case of silica), or a Si atom in an adjacent MEG-OH unit.**

MEG-Cl inherits the ether group from MEG-OH and has a similar chain length. Moreover, it
sacrifices its trichlorosilane group to form organosiloxane networks on hydroxylated
surfaces.\(^{147,148}\) Such networks have good thermal stability and may be formed on any surface
that can be hydroxylated by such simple methods as, for example, plasma electrolytic
oxidation.\(^{149,150}\) Biosensing surfaces that can be modified with these networks include quartz
discs used in electromagnetic piezoelectric acoustic sensors,\(^{110}\) as well as aluminum nitride-based
sensors.\(^{151}\)

The acyl chloride group can participate in a diverse range of reactions as a result of its sensitivity
to nucleophilic attack. In particular, treating the linker with an alcohol to form an ester,\(^{152}\) or with
an amine (under basic conditions) to form an amide (i.e., the Schotten–Baumann reaction),\(^{153,154}\)
are two possible and very general strategies for extending the monolayer. The choice of acyl
chloride is additionally motivated by the need to avoid autocyclization of MEG-Cl through the trichlorosilane group. This autocyclization would be a result of the reaction of the trichlorosilane group with the carboxylic acid, polymerizing the linker before it could be bound to a surface.\textsuperscript{155}

Chapter 3 investigates the usefulness of this linker for binding gelsolin will be demonstrated extending it with $N\alpha,N\alpha$-bis(carboxymethyl)-L-lysine, which will then be employed for binding the polyhistidine-tagged protein form of gelsolin.\textsuperscript{156}

\section*{1.9 Conclusions and Objectives}

Ovarian cancer is a common disease with no reliable blood based screening method. As such many women go undiagnosed until later stages of the disease when it is less treatable. Lysophosphatidic acid as a probe for ovarian cancer provides a high specificity and selectivity for the disease, and as such would be useful in screening for ovarian cancer. As such a reliable low cost test for this molecule that works rapidly in serum needs to be developed. Development of a test based on the complex of gelsolin and actin, which has been previously shown to be sensitive to LPA, is the focus of this thesis. The various component for the test need to first be developed, which includes production of the proteins gelsolin and actin, and development of a surface linker which can bind gelsolin down onto a biosensing surface. The test will be evaluated on acoustic wave based instruments known as quartz crystal microbalances. It will also be developed using a fluorescent tagged version of actin and detection of displaced actin by UV-visible absorbance spectroscopy and fluorescence spectroscopy.
Chapter 2

2 Expression and Characterization of Gelsolin and Actin

2.1 Abstract

Protein plasmids for the proteins gelsolin and gelsolin 1-3 were sequenced and found to contain the desired proteins in the correct location. The proteins gelsolin and gelsolin 1-3 were successfully produced using the Rosetta bl21 line of E. coli cells. These proteins were analyzed by SDS-PAGE and found to have the desired molecular mass, and were isolated in high purity. Further the protein actin was successfully modified with the dyes NHS-Rhodamine, and Fluorescein. The dye modified actin was found to bind to gelsolin using non-denaturing PAGE analysis. Further the complex between gelsolin and actin was disrupted by LPA in a concentration dependent manner, as analyzed using non-denaturing PAGE.

2.2 Contributions

Plasmids containing Gelsolin or Gelsolin 1-3 were provided by Professor Robert Robinson of the University of Singapore. DNA sequencing was performed by the labs at Sick Kids hospital.

2.3 Methods of Protein Expression and Modification

DH5α cells, bl21 cells, lysogeny broth (LB broth), SOC media, and agar plates were purchased from MedStore at the University of Toronto. NHS-rhodamine was purchased from Fisher Scientific. All other chemicals were purchased from Sigma Aldrich.

2.3.1 Growth of Gelsolin Plasmids

pSY5 plasmids containing the Gelsolin or Gelsolin 1-3 gene with a histidine tag in water (10 μL) were introduced to DH5α cells in LB broth (20 μL) and placed on ice for 20 minutes. The cells were then placed in a water bath at 42 °C for 90 seconds to heat shock the plasmids into the cells. The cells were then placed on ice for 2 minutes followed by the addition of SOC media (80 μL). The solution was incubated for 1 hour at 37 °C, followed by plating on LB agar ampicillin 100 plates. A single cell spot was then grown overnight in LB buffer at 37 °C (37.5 g/L LB broth, 10 μg/L ampicillin, 10 mL).
2.3.2 Isolation and Analysis of Gelsolin Plasmids

Cells were purified by Promega Wizard Plus SV miniprep DNA Purification System. LB buffer containing grown cells was pelleted by centrifugation (4,500 rpm, 20 min), decanted, and the resulting pellet of cells resuspended in cell resuspension solution (250 μL). To this was added cell lysis solution (250 μL) and mixed by inversion. Protease solution (10 μL) was added to the mixture, mixed by inversion, and incubated for 5 minutes. Neutralization solution (750 μL) was added followed by centrifugation (14,500 rpm, 10 min). The lysate was decanted into the provided spin columns and centrifuged (14,500 rpm, 1 min). The flow through was discarded and wash solution was added followed by centrifugation (750 μL at 14,500 rpm for 1 min, then 250 μL at 14,500 rpm for 2 minutes). The column was transferred to a clean centrifuge tube followed by addition of nuclease free water (100 μL) and centrifuged (14,500 rpm, 1 min). Water containing the purified plasmid (10 μL) was sent to Sick Kids hospital for DNA sequencing at T7 and T7term primers, and the remaining solution was stored at -20 °C.

2.3.3 Expression of Gelsolin and Gelsolin 1-3

Purified plasmids from the previous section were transferred in bl21 Rosetta cells for protein expression using the above procedure. Cells stocks were stored at -20 °C in 30% glycerol. Cells solutions from freezer stock (200 μL) were thawed and grown in LB buffer (37.5 g/L LB broth, 10 μg/L ampicillin, 30 mL) at 37 °C overnight. This solution was then diluted into LB buffer (37.5 g/L LB broth, 10 μg/L ampicillin, 1.5 L) and grown at 37 °C. Protein production was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) during Log phase II (OD600 0.4–0.8) overnight. Cells were pelleted by centrifugation at 4800 rpm for 20 min. Cells were re-suspended in lysis buffer (500 mM NaCl, 20 mM imidazole, 50 mM Tris pH 7.8, 0.1% Triton™ X, 5% glycerol, 1 mg/mL lysozyme, 1 protease inhibitor tablet, 4 mL) and sonicated for 30 min. DNAase I (1 μL) was added to the suspension and the suspension was rocked gently for 30 min. Cell debris was pelleted by centrifugation at 14 500 rpm for 50 min. Gelsolin was purified from the crude solution by use of a Ni-NTA column followed by dialysis into storage solution (20 mM Tris pH 8.0, 0.5 mM EDTA). Protein mass and purity were determined by SDS-PAGE (12% acrylamide, 120 V for 30 mins) and concentration by absorbance at 280 nm.
2.3.4 Modification of Actin

Actin from rabbit muscle was purchased from Alfa Aeser or Sigma Aldrich. The Actin (0.3-0.6 mg) was added to acetone (100 μL) and sonicated until broken up into a cloudy precipitate. Buffer A (2 mM Tris-Cl pH 8, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl₂ 200 μL) was added to the solution and sonicated for 15 minutes. To this was added acetone (200 μL) followed by sonication for 5 minutes. Finally Buffer A (400 μL) was added to the solution and sonicated until no large solids could be seen. To the solution, now containing 1:2 acetone:Buffer A, was added either NHS-rhodamine in DMF (10 mg/mL, 10 μL), or NHS-rhodamine and fluorescein disodium salt (10 mg/mL, 10 μL). The solution was then shaken under light-free conditions for 90 minutes, followed by dialysis into buffer B (2 mM Tris-Cl pH 8, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl₂, 3.5 mW cutoff). Protein concentration was determined by absorbance at 280 and 552 nM.

2.3.5 Non-denaturing Acrylamide Gel Analysis of Proteins

A separating gel (25 mM tris pH 7.4, 194 mM glycine, 0.2 mM ATP, 0.1% triton-X, 7.5% acrylamide, 0.2% N,N'-methylenebisacrylamide, 0.03% EDTA, 0.1% ammonium persulfate, 10 mL) was prepared and polymerized with 10% APS (100 μL APS), and TEMED (10 μL) for 20 minutes. A stacking gel (6% acrylamide, tris pH 6.8, 5 mL) was prepared, added to the separating gel, and polymerized with 10% APS (100 μL APS), and TEMED (10 μL) for 1 hour. The gel was pre-run in electrode buffer (25 mM tris pH 7.4, 194 mM glycine, 0.2 mM ATP, 0.5 mM CaCl₂) for 30 minutes at 120 V. Solutions containing gelsolin and actin mixtures were added to the gel with non-denaturing loading dye (225 mM tris pH 6.8, 45% glycerol, 10 nM bromophenol blue, 1/5 sample volume), and the gel was run at 120 V for 40 minutes. Proteins were visualized with Coomassie blue. Additionally the actin-gelsolin complex was exposed to LPA between 1 and 50 μM LPA in PBS buffer for 2 minutes before being run on another gel using the same method as above.

2.4 DNA Sequence Analysis of Gelsolin Plasmids

Sequencing of the gelsolin plasmid at the T7 primer resulted in the following sequence:

GMAGGTTAAAT TTCTCTAGA ATATTTTTGT TTAATTCTAA GAAGGAGATA TACCATGGCA GAAGAACACC ACCACCACCA CCACCACCCAC CTGGAGTTC TGTTCCAGGG GCCCCGGGCGG CCGATGGTGG TGGAACACCC
The bolded section above was found using the National Center for Biotechnology Information (NCBI) BLAST database to match to human gelsolin with 97% sequence identity. It should be noted that any mistakes found between the above sequence and gelsolin were found later in the sequence, which due to limitations in the sequencing method used is an area of the sequence more prone to errors.

Translation of the above sequence into an amino acid sequence using the ExPASy Bioinformatics Resource Portal yielded the following amino acid sequence:

```
MAEEHHHHHH HLEVLFQGP GRPMVVEHPE FLKAGKEPGL QIWRVEKFDL VPVPTNLGYD FFTGDAYVL KTVQLRNGNL QYDLHYWLGN ECSQDESGAA A1FTVQLDDY LNGRAVQHRE VQGFESATFL GYFKSGLKYK KGGVASEGFKH VVPNEVTVQR LFQVKGRRVV RATEVPVSWE SPNNGDCFIL DLGNIIHQWC GSNSNRYERL KATQVSKGIR DNERSGRGRV HVSEEGTEPE AMLQVLGPKF A1PAGTEDTA KEDAANRKLK KLYKVSNGAG TMSVSLVADE NPFAQGALKS EDCFILDHGK MEIFVWKKASS NTERRGGLQ L
```

The highlighted sequences show first the histidine tag, which was left on the protein for use as a reversible surface anchor on the biosensor. The second highlighted section, which follows a protease C cleavage site, matches favourably to gelsolin as determined using the NCBI BLAST database. Any errors found between the above sequence and previously reported gelsolin
Sequencing of the gelsolin plasmid from the T7 terminal primer results in the following sequence:

AGGATGAATT CCGTTAGGC AGCCAGCTCA GCCATGGCCC TGTCCAAGGG GTCCACAGAC CAGTAATCAT
CATCCCAAGCC AAGGAACCA GACCAAAAGG AGGGAGGCTC AAGACCTTGC TTTACCAGGG TGATGGGGCT
CCGCCGATCC CGATTTGAGTC GTTCCGCTTC GATGTACAGC TTAGCAAGAG TCAAGGCTTC TGTCTTTTCT
TCTTCTTGGAG AATCCTTCCC AACCCAGACA AAGACCTGTT CCCAGGTGTC CAGAAGCATG ACCTTCATCC
TGCAAGAGGT TTCCCTGATG AGCTCACCAG GAACCTCTTC GATCACAAAA CGTGTAATCG TGGGCCCGCA
GCCGCAGGTC CGATTGGCTG GGTCCGTCTC GATGTACCGC TTAGCAGAAG TCAAGGCTTC TGTCTTTTCT
TGCAAGAGGG GTTTTCCAGAA CAAAGGCATG GTTGGAGTTC AGTGCACGAC TCCCTACAA CAGGGCATTG
GCCGCCGCTTGG CTCCAGCGCT GTTGGCGCGG ACCTGGAAGA GGCGGGTCGT GTCTGGCCGC
CTCTGCGGGA GTTGCGCCGC TTTGAAGAGT GCACAAACAC AGGCTCATGA GGTGCGCGCA
CTCTTCTGGCT TGGAACACAC GGGCTGCGAC AGGGTATACG CACCGCTCCT CATCCAGCTG AGCAGTCA
GTCAGATGTC AGCGACCTCA TTCTGGGCTG ACTGGGCCAT CTCGCTGTGA TAGATTATCT CTCCTACAA
GCMCTCCTGC ACGCTCGCTG CTCTGGGCTG ACTGGGCCAT CTCGCTGTGA TAGATTATCT CTCCTACAA
ACGGGCACCTT GTGGAACCTC GATATCCTCA ATCTGCTCTT GCCTCAGTGC CATGTCGTGC AACGCTGGCG
GTCATGCAGT GAGTGTGCGT TGGGCGGCGA GCCCGGCCGTC CACGTGGGCA WTCCTGCCGA TAGACAGGCA
AGGCATCTGC TGCGTGTGCC AGTCTGAAC TCTGCACCGGA TYAGCCTTAGG AAGGACGAAA CCTGAA

The bolded section of the above sequence when reversed was found to match the terminal end of human gelsolin with 97% identity using the NCBI BLAST database, ending at base pair 2370 of the human gelsolin gene. This provides strong evidence that the entire protein of gelsolin is contained in the provided plasmid.

The plasmid containing gelsolin 1-3 was also sequenced at the T7 primer and resulted in the following sequence:

GGGGAAAATT ACCTCTAGAA TAATTGGTTT TAACTTTTAAG AAGGAGATAT ACCATGGCGA AAGAACACCA
CCACCCCTCC CACCACCCAC TTGGAAGTCT GCTCCAGGGG CCCGGGCGGC CGATGGGTGT GGAACACCCC
GAGTTTCTCCA AGGCGAGGA AAGGCGCTGC CTGCAGATCT GGCCTGTGGA GAAGCTCGAT CTGGTCGGCG
TGCCCCACCA CTTTTATGGA GACCTCTTCA CGGGGACGC AGTACATGAC TCGAACAGAC TGACAGCTGAG
Once again the bolded section was found to match human gelsolin by analysis with the NCBI BLAST database. Translating the above sequence using ExPASy results in the following amino acid sequence:

MAEEHHHHHH HHLEVLFLQGP GRPMVVEHPE FLKAGKEPGL QIWRVEKFDL VPVPNTNLYGD FFTGDAYVIL KTVQLRNGNL QYDLHYWLGN ECSQDESGAA AIFTVQLDDY LNGRAVQHRE VQGFESATFL GYFKSGLKYK KGVSAGFKH VVPNEVVVQR LFQVKGRRVV RATEVPVSWE SFNNGDCFIL DLGNQIHQWC GSNSNRYERL KATVQSVKIR DNRSSGRARV HVSEEETPE AMLQVLGPKP ALPAGTEDTA KEDAAARKLA KLYKVSNGAG TMSVSLVADE NPFARGP

As before the histidine tag and sequence found to match human gelsolin have been highlighted. The strong sequence match to gelsolin suggests the gelsolin gene is also present in this plasmid.

To determine if the gene present is full length gelsolin or the desired 1-3 portion of the protein sequencing at the T7 terminal primer was also performed on this plasmid resulting in the following sequence:

CSGCTGCATT CCGTTACTTG AGACTGCTTG AACAGTGGGG TCTCACCAGCC CTCAGAGGAC ACCGAGACCT GAGTCTGTCA GGTTAGTCTG GCCAGTGGAG ATCTCTCCCTC TCTGGCGGTGT CATTGGAGAC CTTCTCTAGC CCAGCAGGGG TCTTCTCCAG
Once again using the NCBI BLAST database the above bolded sequence in reverse was matched to human gelsolin. However, unlike the primer containing full length gelsolin, this sequence was matched ending at the center of the protein at base pair 1246. As a result it can be concluded that the gene contained in the plasmid codes for gelsolin 1-3 as opposed to full length gelsolin. The above sequences also match well with previously reported sequences.\textsuperscript{134}

### 2.5 Gel Electrophoresis of Gelsolin Proteins

The below acrylamide gel represents a typical production of gelsolin protein. The protein can clearly be seen in the crude lysate (Fig. 7 Lane 2, 1/5 dilution) as a large blob at 82 kDa, which matches the expected mass of gelsolin. The flow-through from the column which will show any proteins that did not bind the Ni-NTA column (Fig. 7, Lane 3, 1/5 dilution) showed a far smaller band at 82 kDa, suggesting that most of the gelsolin present in the cell lysate has been successfully bound by the column. Column washes with his-binding buffer (Fig. 7, Lanes 4-6) show a small band at 82 kDa, and only very faint other bands. This suggests that very little other protein remains on the column after washing. The pre-eluate, which is the estimated last mL of his-binding buffer before the elution buffer has flowed through the column (Fig. 7, Lane 7), shows some minor impurities, and a fairly strong band of gelsolin. Finally, the column eluate which was pulled off using his-binding buffer with imidazole (Fig 7, Lane 8) shows a very large blob at 82 kDa, suggesting gelsolin has been isolated in high concentration. There are some minor spots visible in the lane around gelsolin, however these are very small in comparison to the gelsolin spot suggesting gelsolin was also isolated in high purity.
Figure 7 - SDS PAGE analysis of gelsolin protein synthesis with lanes 1) BenchMark Protein Ladder, 2) crude lysate of cells, 3) column excess after incubation with Ni-NTA gel, 4) first wash with 15 mL his binding buffer, 5) second wash with 15 mL his binding buffer, 6) third wash with 15 mL his binding buffer, 7) pre-eluate, 8) isolated protein after dialysis.
UV-Visible absorption at 280 nm was used to quantify the produced protein using an estimated extinction coefficient of 113,750 M\(^{-1}\)cm\(^{-1}\), which was calculated using the ExPASy ProtParam tools. Gelsolin concentrations between 20 and 80 μM were typically achieved. This high variability is likely due to many factors such as cell optical density at the point of induction with IPTG, incubation time and temperature with IPTG, or how many times the Ni-NTA resin had been previously used. Despite this variability it is clear that the protein gelsolin was successfully produced with high purity.

Analysis of the gelsolin 1-3 protein by SDS PAGE yielded similar results to gelsolin (Fig. 8). The only band visible in the column eluate is one between 37 and 49 kDa. Given the expected mass of gelsolin 1-3 is approximately 41 kDa this band most likely corresponds to gelsolin 1-3. The band is however much narrower than that observed for gelsolin suggesting the protein has been isolated in lower concentration. This can be confirmed by UV visible analysis where an estimated extinction coefficient of 47,180 M\(^{-1}\)cm\(^{-1}\) at 280 nm was used based on calculations using ExPASy ProtParam. Concentrations of gelsolin 1-3 typically end up between 5 and 40 μM, about half what is typically observed for gelsolin. It is unknown why gelsolin 1-3 expresses in lower concentration, but the protein was successfully produced as evidenced by SDS PAGE analysis. Although mass spectrometry and circular dichroism spectroscopy could be used to further identify the protein, the DNA sequence and mass seen by PAGE analysis is sufficient evidence for the production.
Figure 8 - SDS PAGE analysis of gelsolin 1-3 protein synthesis with lanes 1) BenchMark Protein Ladder, 2) crude lysate of cells, 3) column excess after incubation with Ni-NTA gel, 4) first wash with 15 mL his binding buffer, 5) second wash with 15 mL his binding buffer, 6) pre-eluate, 7) isolated protein before dialysis, 8) isolated protein after dialysis.
2.6 UV-Visible Analysis of Dyed Actin

Following modification of actin with fluorescent dyes, UV-Visible spectrometric analysis was performed to determine the concentration of actin and dye in the solution.

When actin was dyed with NHS-Rhodamine the resulting spectrum featured two primary peaks (Fig. 9). The first peak at 290 nm corresponds to actin, and can be used to quantify the actin present using its molar extinction coefficient, which is approximately $26,400 \text{ M}^{-1}\text{cm}^{-1}$\textsuperscript{157}. The second major peak is seen at 552 nm, which corresponds to NHS-Rhodamine, and can be used to quantify the dye using it's molar extinction coefficient of $80,000 \text{ M}^{-1}\text{cm}^{-1}$\textsuperscript{158}.

Several different batches of actin were prepared and dyed with NHS-Rhodamine, and the concentration varied between 5 and 15 μM. Variation is likely due to differing amounts of actin dissolving into solution in each run. However as the absorbance peak at 290 nm is not found in NHS-Rhodamine, and no other proteins were introduced into the solution it can be concluded that actin was successfully dissolved in solution.

Quantification of the rhodamine dye via the peak at 552 nm resulted in a concentration similar to that of actin, suggesting a 1:1 relationship between NHS-Rhodamine and actin following modification. The structure of actin was solved by Robinson et. al.,\textsuperscript{159} and reveals that actin has several lysines near its surface (Figure 10).
Figure 9 - UV-Vis spectrum of actin dyed with NHS-Rhodamine.
Despite the presence of many lysines near the surface of actin we only get approximately one rhodamine molecule binding to each protein. As can be seen in the structure of actin most of these lysine residues face inward in the protein and are likely interacting non-covalently with other amino acids making them difficult to react with. As a result it is likely that only one or two of these surface lysine residues is reactive to NHS-Rhodamine, which would explain the 1:1 ratio between the molecules.

Reacting actin with not only NHS-Rhodamine, but also fluorescein disodium salt results in a very different UV-Visible spectrum after dialysis than the one obtained for just NHS-Rhodamine (Fig 11). The most notable difference is the large peak at 490 nm, characteristic of fluorescein. Since this spectrum was taken after dialysis the existence of this peak in the spectrum is strong evidence that fluorescein disodium salt has successfully modified actin.
Figure 11 - UV-Vis spectrum of actin dyed with NHS-Rhodamine and fluorescein disodium salt.
Unfortunately due to the ability for fluorescein to absorb at a wavelength of 290 nm, direct quantification of actin dyed with fluorescein is difficult, and instead the quantity of rhodamine must be used as it is bound to actin in a 1 to 1 ratio. Using a molar absorptivity of 76,900 M$^{-1}$cm$^{-1}$, it was found that there was between 3 and 15 times as high of a concentration of fluorescein in the protein sample as rhodamine. This high variability is most likely due to the non-specific nature of fluorescein salt interactions with actin, or due to the amount of time spent dialyzing the protein. Care should be taken when preparing actin with these two dyes to ensure that the ratio of dyes bound to the protein is kept consistent. This includes carefully massing the actin and fluorescein, as well as consistent reaction times and dialysis.

2.7 Actin Gelsolin Binding

To determine if dyed actin and the expressed gelsolin are still capable of binding together non-denaturing gels were prepared and performed on mixtures of actin and gelsolin. These types of gels have been used previously to analyze actin binding.

The non-denaturing gel (Fig. 12) presents with a considerable amount of streaking. Unfortunately due to the limited equipment available to run gels available a small gel run for a relatively short amount of time had to be used. Since there are no denaturing agents present the proteins move with drastically variable travel times due to packing differences. As a result of this streaking the actin itself was not visible on the gels. This is most likely due band widening as compounds progress down the gel. Since actin is much smaller than gelsolin, its band becomes too diluted by this streaking to be visible by Coomassie stain.

Despite the large amount of streaking some conclusions can still be made from the gel. Firstly gelsolin is visible as a streak that concentrates to 82 kDa as visible in the first lane. The binding of gelsolin and actin is thus visible as the disappearance of this band as the concentration if actin is increased, as well as the appearance of a new band above 120 kDa (the mass of the gelsolin-actin protein complex). It can be seen that at a 1:1 ratio of gelsolin to actin that there is no longer a band visible at 82 kDa (Fig. 12, Lane 7), suggesting complete or near complete binding of gelsolin to actin. As such actin modified with NHS-Rhodamine is still capable of binding to gelsolin, and thus being used as a molecular probe.
Figure 12 - A non-denaturing gel where mixtures of actin and gelsolin were run.
In order to be useful for the detection of LPA the dyed-actin and gelsolin complex must still be broken apart by LPA, and this must occur in a concentration dependent manner. Another gel was prepared to investigate this (Fig. 13).

As with the previous gel there is considerable streaking and band widening occurring throughout the gel, however unlike before two distinct bands at approximately 120 kDa and greater than 180 kDa are visible in the actin and gelsolin mixture (Fig. 13, Lane 2). This suggests that there is a mixture of discrete actin and gelsolin compounds with either 1 or 3 actin molecules bound to the gelsolin. As LPA is added to the mixture two new bands begin to appear, one at 82 kDa, and one between 120 and 180 kDa. Further, as higher concentrations of LPA are reached the band between 120 and 180 kDa begins to fade and there is a strengthening of the band at 120 kDa. This suggests the loss of actin molecules from the gelsolin actin compounds, resulting in free gelsolin, and gelsolin bound to 1 or 2 actins. This also strongly suggests that the loss of actin from gelsolin occurs in a concentration dependent manner, and is visible on a gel at a concentration of 5 μM suggesting that low concentrations of LPA are able to disrupt this complex in an easily detectible manner.

These gels show that not only is gelsolin capable of binding to dye-modified actin, but this complex is sensitive to LPA concentration upon exposure for a very short period of time as the proteins were exposed to LPA for only 2 minutes prior to being run on the gel. This gives strong evidence for the usefulness of the actin-gelsolin complex in the biosensing of LPA. Additional methods such as isothermal colorimetry or surface Plasmon resonance could be used to further verify and quantify these interactions, however these methods were not performed.
Figure 13 - A non-denaturing gel where each lane contains a 1:1 mixture of gelsolin and actin that has been incubated with a varying concentration of LPA.
2.8 Conclusions of Protein Production and Modification

DNA sequencing of the plasmids containing gelsolin showed that each plasmid contained the correct variant of gelsolin between the T7 and T7term primers. Growth of the protein in bl21 Rosetta cells was successful, though showed highly variable yield which is most likely due to cell phase and temperature at the time of induction. The proteins were produced in high purity without the need for column purification following dialysis as evidenced by SDS-PAGE analysis. Modification of actin with NHS-Rhodamine and fluorescein also proved successful as shown by UV-Visible spectrometry. Binding of the modified actin to gelsolin was found to occur readily and 1:1 binding was observed between the proteins. Finally LPA was found to disrupt this binding in a concentration dependent manner, suggesting the actin and gelsolin protein pair could be made into a biosensor for LPA.
Chapter 3

3 Synthesis of the Surface Linker MEG-Cl

The content of this chapter has been peer reviewed and published, and is reproduced here with additional insights. De La Franier, B.; Jankowski, A.; Thompson, M. Functionalizable Self-Assembled Trichlorosilyl-Based Monolayer for Application in Biosensor Technology. Appl. Surf. Sci. 2017, 414, 435–441. A modified introduction from this paper can be found in section 1.8.

3.1 Abstract

This chapter describes the design and synthesis of 3-(3-(trichlorosilyl)propoxy)propanoyl chloride (MEG-Cl), a compound capable of forming functionalizable monolayers on hydroxylated surfaces. The compound was synthesized in high purity, as suggested by nuclear magnetic resonance analysis, and in moderate overall yield. Contact angle measurement and X-ray photoelectron spectroscopy confirm the binding of MEG-Cl to an amorphous glass substrate and the further modification of the monolayer with a nickel (II)-binding ligand for the purpose of binding polyhistidine-tagged proteins. The compound will be useful in biosensing applications due to its ability to be easily modified with any number of nucleophilic functional groups subsequent to substrate monolayer formation.

3.2 Contributions

The synthesis of TBAP, APA, and APC was developed with the aid of Alex Jankowski. The synthesis of MEG-Cl was developed with the aid of Christophe Blaszykowski. XPS data was collected by Peter Brodersen of SI-Ontario.

3.3 Methods for Surface Linker Synthesis

Unless otherwise specified all compounds used in the following synthesis were purchased from Sigma Aldrich.
3.3.1 Synthesis of Tert-butyl-3-(2-allyloxy)propanoate

\[ \text{Figure 14 - Two-step synthesis of Tert-butyl-3-(2-allyloxy)propanoate (TBAP).} \]

A solution of N-benzyltrimethylammonium hydroxide (2.6 mL, 40% w/w in methanol) was concentrated \textit{in vacuo} in a flame-dried flask, then allowed to cool to room temperature. The residue was diluted in allyl alcohol (5.0 mL, 73.5 mmol) and the solution was stirred for 15 min at room temperature. Next, tert-butyl acrylate (9.6 mL, 65.5 mmol) was introduced and the mixture was stirred and held at 58 °C for 72 h. The product, tert-butyl 3-(2-allyloxy)propanoate (TBAP), was purified by flash chromatography with high-purity grade silica gel (60-Å pore size, 200-400 mesh) and hexanes/ethyl acetate (3/1 v/v) as the mobile phase.

3.3.2 Synthesis of 3-(2-allyloxy)propanoic acid

\[ \text{Figure 15 - Conversion of TBAP to 3-(2-allyloxy)propanoic acid (APA) via ester hydrolysis.} \]

In dichloromethane (DCM, 18 mL) were dissolved TBAP (1.75 g, 9.40 mmol) and trifluoroacetic acid (TFA, 4.6 mL, 60.1 mmol). The mixture was stirred at room temperature for 1 h. It was then repeatedly concentrated \textit{in vacuo} and enriched with DCM until no more TFA was present, yielding 3-(2-allyloxy)propanoic acid (APA) as the product.

3.3.3 Original Synthesis of 3-(2-allyloxy)propanoyl chloride

\[ \text{Figure 16 - Original conversion of APA to 3-(2-allyloxy)propanoyl chloride (APC).} \]

A flask was charged with APA (2.49 g, 19.1 mmol), then toluene (60 mL), and then oxalyl dichloride (10 mL, 116.5 mmol). The mixture was stirred at 40 °C overnight, after which it was
concentrated *in vacuo*. The resulting oil containing 3-(2-allyloxy)propanoyl chloride (APC) was optionally purified by vacuum distillation at 35–40 °C.

### 3.3.4 Updated Synthesis of 3-(2-allyloxy)propanoyl chloride

![Figure 17 - Updated conversion of APA to APC.](image)

A flask was charged with APA (2.49 g, 19.1 mmol), then DCM (20 mL), then *N*,*N*-dimethylformamide (312 μL, 4.03 mmol), and then oxalyl dichloride (4.8 mL, 55.9 mmol). The mixture was stirred at 0 °C for 2 h, after which it was concentrated *in vacuo*. The resulting solid residue was discarded while the liquid containing 3-(2-allyloxy)propanoyl chloride (APC) was optionally purified by vacuum distillation at 35–40 °C.

### 3.3.5 Synthesis of 3-(3-trichlorosilyl)propoxy)propanoyl chloride

![Figure 18 - Hydrosilylation of APC into 3-(3-trichlorosilyl)propoxy)propanoyl chloride (MEG-Cl) under inert atmosphere.](image)

To the product obtained in the previous step (1.2 g, 8.1 mmol) were added trichlorosilane (1.7 mL, 17 mmol) and hexachloroplatinic acid hexahydrate (42 mg, 0.081 mmol) under inert (N₂) and anhydrous (P₂O₅) atmosphere. The mixture was stirred at room temperature overnight. The product, 3-(3-(trichlorosilyl)propoxy)propanoyl chloride (MEG-Cl), was obtained via vacuum distillation at 75 °C.

### 3.4 Product Yields

The qualities of the products and the yields of the corresponding reactions are given in Table 1.

<table>
<thead>
<tr>
<th>compound</th>
<th>characteristics</th>
<th>reaction yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Description</td>
<td>Yield</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>TBAP</td>
<td>clear, light-yellow oil</td>
<td>80%</td>
</tr>
<tr>
<td>APA</td>
<td>clear, light-yellow oil</td>
<td>97%</td>
</tr>
<tr>
<td>APC</td>
<td>clear, colourless oil</td>
<td>60%</td>
</tr>
<tr>
<td>MEG-CI</td>
<td>clear, colourless oil</td>
<td>41%</td>
</tr>
</tbody>
</table>

The product yields above are the yields obtained following purification, and as such are lower than the actual amount of product synthesized. Firstly, TBAP is synthesized at nearly 100% yield, however the \(N\)-benzyltrimethylammonium hydroxide used to catalyze the reaction is present in the final product mixture, and must be removed by column chromatography, effectively lowering the yield.

As for the synthesis of APC and MEG-CI, much of the product is lost during distillation. Since the reactions are typically performed on a 1-2 g scale, there is only a small volume of product present. Both products also have high viscosity, causing them to stick to the distillation glassware. As such a small, but significant amount of each product gets stuck to the distillation column, and does not drip back down into the heating flask in order to be collected into the product flask. If synthesized on a larger scale the collected yield of both of these products should improve dramatically as this loss of product within the distillation apparatus will be minimized.

### 3.5 Notes on Product Synthesis

For the synthesis of TBAP impurities will often present as a darker orange colour, allowing their removal by silica filtration to be easily visualized. The product itself is a very pale yellow oil, so filtration should be performed to remove any dark orange from the product. A volume of silica gel equivalent to half the volume of TBAP product should be used to ensure that no impurities pass through the silica. Following elution with hexanes:ethyl acetate a solid precipitate will sometimes form in the TBAP product, which is possibly a salt of benzyltrimethylammonium, however the nature of this precipitate has not been investigated. This precipitate can be removed by use of a syringe filter of any appropriate size for the volume of TBAP produced.
Conversion of the TBAP to APA occurs rapidly and in high yield, however the removal of TFA can be quite difficult. Although TFA is highly volatile and has a low boiling point, a large amount of it is found to remain in the APA product following vacuum evaporation. This is possibly due to hydrogen bonding interactions between the newly formed APA and TFA preventing the simple evaporation of TFA. For APA to be successfully converted into APC in the subsequent reaction the majority of the TFA needs to be removed. In order to preserve yield of APA co-evaporation with additional volumes of DCM is recommended, but this frequently leaves a small amount of TFA in the product. Vacuum distillation can also be performed to remove the remaining TFA, however this will significantly reduce the overall yield.

As noted in the experimental sections 3.3.3 and 3.3.4 the conversion of APA to APC can be done via multiple methods. The updated method utilizing DMF as a catalyst is faster, less energy intensive, and allows the use of less oxalyl chloride. However it should be noted that the solid DMF often traps much of the product, reducing yield. The product is an orange oil at this stage of the conversion and is easily visible in the DMF. Yield can be maximized in this reaction by adding a small amount of DCM to the solidified DMF and sonicating the mixture to break up the solid into a fine suspension. The DMF will remain solid, but any trapped product will dissolve into the DCM, allowing the solid DMF to be filtered off followed by additional vacuum evaporation of DCM from the product. The filtered DMF will be a white powder following extraction of all of the product, allowing judgment to be made on how much DCM is necessary to extract all of the product. Distillation is of the APC product is recommended as well since sometimes not all of the DMF solidifies, and remaining DMF can often be seen in NMR analysis of non-distilled product.

The alternative synthesis of APC is more time consuming and requires elevated temperatures for the reaction to proceed, but it does not leave any undesired compounds in the final product. Removal of the toluene and excess oxalyl chloride can be easily done in vacuo leaving behind a highly pure product with rarely a need for distillation to purify it.

The final step of the synthesis must be done under inert reaction conditions as any moisture present with react with the trichlorosilane and final product destroying it. The distillation apparatus used in this reaction must also be pre-silanized with a non-reactive silane such as hexyltrichlorosilane (HTS) before use in the distillation; otherwise the product can be seen to
solidify in the distillation apparatus greatly reducing yield. This distillation is very difficult due to the very similar boiling points of the product, starting material, and any cross-linked bi-product, all of which can be seen in NMR spectra taken of the reaction before distillation. The product itself is a clear colorless oil, while impurities often take on a dark black colour, so careful monitoring of the distillation to ensure that no coloured impurities are taken into the distilled product is necessary. Removal of any impurities is very important as cross-linked bi-product seems to accelerate the degradation of the linker, causing the solution to rapidly darken, while pure linker will remain colourless for months provided it is properly stored under inert conditions of an oxygen and water free nitrogen atmosphere at -10 °C.

3.6 Spectral Analysis of Linker

3.6.1 Spectral Data of tert-butyl-3-(2-allyloxy)propanoate

![Figure 19 - Labeled structure of TBAP for spectral assignment.](image)

The first step in the synthesis of MEG-Cl involves the production of the tert-butyl protected form of 3-(allyloxy)propanoic acid (TBAP). Although 3-(allyloxy)propanoic acid is commercially available, its high cost makes it expensive to obtain, so a synthetic route to the acid is preferred. No previously reported spectra for this compound could be found, so FTIR and NMR analysis of TBAP was performed with reference to predicted spectra for the compound.
Figure 20 - Neat FTIR spectrum of tert-butyl 3-(2-allyloxy)propanoate.
Figure 21 - Hydrogen-1 NMR spectrum of tert-buty 3-(2-allyloxy)propanoate in chloroform-$d$ with 0.05% w/v tetramethylsilane at 500 MHz.
Figure 22 - Carbon-13 NMR spectrum of tert-butyl 3-(2-allyloxy)propanoate in chloroform-$d$ with 0.05% w/v tetramethylsilane at 125 MHz.
FTIR (neat): $\nu = 2978$ (m, sp$^2$ C–H) 2932 (m, sp$^2$ C–H), 2873 (m, sp$^2$ C–H), 1736 (s, C=O), 1648 (w, C=C) cm$^{-1}$.

FTIR data for TBAP shows key peaks at 1736 cm$^{-1}$ corresponding to the carbonyl present, and 1648 cm$^{-1}$ corresponding to the alkene (Fig. 20). The moderately high wavenumber shift for the carbonyl suggests the presence of a large attached group, suggesting tert-butyl is present on the molecule.

$^1$H–NMR (500 MHz, chloroform-$d$ + 0.05% w/v TMS): $\delta =$ 5.89 (1H, m, H5), 5.26 (1H, dm, $J =$ 17.5 Hz, H2), 5.16 (1H, dm, $J =$ 10.5 Hz, H1), 3.98 (2H, bs, H6), 3.66 (2H, bs, H7), 2.49 (2H, bs, H8), 1.44 (9H, s, H11) ppm.

Proton NMR data shows seven peaks outside of the chloroform solvent, which is the expected number based on the number of unique protons present in TBAP’s chemical structure (Fig. 19). Firstly, the most important peaks for this compound are those on atoms 6 and 7 (Fig. 21) which are adjacent to the newly formed ether linkage. These can be seen at 3.98 and 3.66 Hz respectively. Additionally, there are three peaks between 5 and 6 ppm, which match what is expected for the terminal alkene group. Finally there are two peaks below 3 ppm, one at 2.49 ppm which corresponds to a proton adjacent the ester group, and one at 1.44 ppm, which matches the expected tert-butyl protecting group.

$^{13}$C–NMR (125 MHz, chloroform-$d$ + 0.05% w/v TMS): $\delta =$ 171.0 (C9), 134.8 (C4), 117.1 (C3), 80.7 (C10), 72.1 (C6), 66.0 (C7), 36.5 (C8), 28.2 (C11) ppm.

For the carbon NMR spectra the expected number of peaks, 8, is again observed (Fig. 22). As with the proton spectra the most important peaks are adjacent the ether linkage, and these peaks can be seen at 72.1 and 66.1 ppm exactly where they would be expected. All other peaks match what would be expected as predicted by NMR software MESTre Nova. This gives strong evidence that the desired product was formed.
3.6.2 Spectral Data of 3-(2-allyloxy)propanoic acid

![Labeled structure of APA for spectral assignment.](image)

FTIR (neat): $\nu = 3000$ (s, br, O–H), 2925 (s, sp$^2$ C–H), 1717 (s, br, C=O), 1649 (w, C=C) cm$^{-1}$.

The collected FTIR data for APA (Fig. 24) closely matches literature spectra found in the Spectral Database for Organic Compounds.$^{164}$ Important spectral changes from TBAP can be observed as the shift of the carbonyl peak from 1736 to 1717 cm$^{-1}$, indicating the transformation of a ketone to a carboxylic acid. The appearance of a broad absorption at 3000 cm$^{-1}$ also supports this notion, suggesting the successful conversion of TBAP to APA. Unfortunately some TFA impurity could be seen on this spectrum at 1760 cm$^{-1}$. As was mentioned in the notes, removal of all of the TFA from APA after its synthesis is very difficult, and generally some of it remains in the product. However a small amount of TFA seems to have little negative impact on subsequent reactions, so in general it can be ignored so long as the amount of it in the product is minimized.
Figure 24 - Neat FTIR spectrum of 3-(2-allyloxy)propanoic acid with TFA impurity.
Figure 25 - Hydrogen-1 NMR spectrum of 3-(2-allyloxy)propanoic acid in chloroform-$d$
with 0.05% w/v tetramethylsilane at 500 MHz.
Figure 26 - Carbon-13 NMR spectrum of 3-(2-allyloxy)propanoic acid in chloroform-$d$ with 0.05% w/v tetramethylsilane at 125 MHz.
\(^1\)H–NMR (500 MHz, chloroform-\(d\) + 0.05% w/v TMS): \(\delta\) 5.90 (1H, ddt, \(J = 17.5\) Hz, 10.5 Hz, 5.5 Hz, H3), 5.29 (1H, dq, \(J = 17.5\) Hz, 2.0 Hz, H2), 5.20 (1H, dq, \(J = 10.5\) Hz, 1.0 Hz, H1), 4.02 (2H, dt, \(J = 6.0\) Hz, 1.5 Hz, H6), 3.72 (2H, t, \(J = 6.5\) Hz, H7), 2.65 (2H, t, \(J = 6.5\) Hz, H8) ppm.

\(^{13}\)C–NMR (125 MHz, chloroform-\(d\) + 0.05% w/v TMS): \(\delta\) = 176.5 (C9), 134.4 (C4), 117.6 (C3), 72.3 (C6), 65.2 (C7), 34.9 (C8) ppm.

Although this compound has both been previously published and is for sale, no previously reported NMR spectra could be found for it. As such the NMR data was once again analyzed with reference to predicted spectra. Most importantly were the spectral changes from TBAP which are consistent with the expected transformation. In particular, the absence of the \(^1\)H and \(^{13}\)C signals at 1.44 and 28.2 ppm, respectively, indicates the loss of the tert-butyl group following the conversion of the ester into a carboxylic acid (Figs. 25 and 26). The signals due to H7 and H8 are shifted slightly downfield because of the stronger electron-withdrawing ability of the acid group compared to that of the ester. The other signals are largely unchanged, suggesting that no undesired transformations occurred in the remainder of the molecule. Slight starting materials are visible in the above spectra however these could be removed via distillation of the product. TFA was not visible on these spectra as the NMR spectra were run on a more highly purified sample of the product.

3.6.3 Spectral Data of 3(-2-allyloxy)propanoyl chloride

![Figure 27 - Labeled structure of APC for spectral assignment.](image)
Figure 28 - Neat FTIR spectrum of 3-(2-allyloxy)propanoyl chloride.
Figure 29 - Hydrogen-1 NMR spectrum of 3-(2-allyloxy)propanoyl chloride in chloroform-
$\text{d}$ with 0.05\% w/v tetramethylsilane at 500 MHz.
Figure 30 - Carbon-13 NMR spectrum of 3-(2-allyloxy)propanoyl chloride in chloroform-$d$

with 0.05% w/v tetramethylsilane at 125 MHz.
FTIR (neat): ν = 2875 (m, sp² C–H), 1796 (s, C=O), 1647 (w, C=C) cm⁻¹.

The conversion of APA to APC involves only a small change in chemical structure, namely the replacement of the carboxylic acid’s hydroxide with a chloride. This change is visible in the FTIR data as a loss of the broad peak at 3000 cm⁻¹, as well as the shift of the carbonyl peak from 1717 to 1796 cm⁻¹ (Fig. 28). This strongly suggests the successful conversion of the carboxylic acid of APA into the acid chloride of APC.

¹H-NMR (500 MHz, chloroform-d + 0.05% w/v TMS): δ = 5.88 (1H, ddt, J = 17.5 Hz, 10.5 Hz, 5.5 Hz, H5), 5.28 (2H, ddt, J = 17 Hz, 1.5 Hz, 1.5 Hz, H2), 5.20 (2H, ddt, J = 10 Hz, 1.5 Hz, 1.5 Hz, H1), 4.00 (dt, 2H, J = 6 Hz, 1.5 Hz, H6), 3.74 (t, 2H, J = 6 Hz, H7), 3.12 (t, 2H, J = 6 Hz, H8) ppm.

¹³C-NMR (125 MHz, chloroform-d + 0.05% w/v TMS): δ = 171.8 (C9), 134.0 (C4), 117.6 (C3), 72.2 (C6), 64.6 (C7), 47.3 (C8) ppm.

From the NMR data the conversion of APA into APC is supported by the shifts of the H8 signal from 2.65 to 3.12 ppm, C9 from 176.5 to 171.8 ppm, and C8 from 34.9 to 47.3 ppm (Figs. 29 and 30). This corresponds to the increase in electron-withdrawing ability on replacing a carboxylic acid with an acid chloride, again lending strong evidence to the production of the desired acid chloride. It should be noted that both methods for making the acid chloride produce identical spectra, showing that each is capable of producing the product.

3.6.4 Spectral Data of 3-(3-(trichlorosylil)prooxy)propanoyl chloride

Figure 31 - Labeled structure of MEG-Cl for spectral assignment.
Figure 32 - Neat FTIR spectrum of 3-(3-(trichlorosilyl)propoxy)propanoyl chloride.
Figure 33 - Hydrogen-1 NMR spectrum of 3-(3-(trichlorosilyl)propoxy)propanoyl chloride in chloroform-$d$ with 0.05% w/v tetramethylsilane at 500 MHz.
Figure 34 - Carbon-13 NMR spectrum of 3-(3-(trichlorosilyl)propoxy)propanoyl chloride in chloroform-$d$ with 0.05% w/v tetramethylsilane at 125 MHz.
Figure 35 - $^1$H–$^{29}$Si NMR spectrum of 3-(3-(trichlorosilyl)propoxy)propanoyl chloride in chloroform-$d$ with 0.05% w/v tetramethylsilane at 500 MHz ($^1$H) and 100 MHz ($^{29}$Si).
FTIR (neat): $\nu = 2899$ (w, sp$^2$ C–H), 1795 (s, C=O) cm$^{-1}$.

The final step of the synthesis involves conversion of the alkene group of APC to a trichlorosilane. As the product is a novel compound spectra were assigned with respect to predicted spectra. This conversion is evident in the FTIR spectrum as a loss of the alkene signal at 1647 cm$^{-1}$, which corresponds to its replacement with another functionality (Fig. 32). This is most likely to be the desired trichlorosilane group, however as this is a novel molecule further spectral data is required to show this as no literature spectra for this molecule exist.

$^1$H–NMR (500 MHz, chloroform-$d + 0.05\%$ w/v TMS): $\delta$ 3.73 (2H, t, $J = 6$ Hz, H4$'$), 3.50 (2H, t, $J = 6$ Hz, H3$'$), 3.11 (2H, t, $J = 6$ Hz, H5$'$), 1.83 (2H, m, $J = 4$ Hz, H2$'$), 1.47 (2H, m, $J = 4$ Hz, H1$'$) ppm.

In the NMR data the conversion is supported by the loss of $^1$H signals at 5.88 and 5.28 ppm, as well as the appearance of $^1$H signals at 1.83 and 1.47 ppm, suggesting complete hydrosilylation of the alkene to the product, MEG-Cl (Fig. 33). These shifts match the shifts expected using software prediction, lending strong evidence to the production of the product.

$^{13}$C-NMR (125 MHz, chloroform-$d + 0.05\%$ w/v TMS): $\delta$ 171.9 (C6$'$), 71.6 (C3$'$), 65.5 (C4$'$), 47.4 (C5$'$), 22.7 (C2$'$), 21.0 (C1$'$).

Similar conclusions to the above proton NMR data can be drawn from the replacement of the $^{13}$C signals at 134.0 and 117.6 ppm with those at 22.7 and 21.0 ppm, respectively (Fig. 34). As with the proton NMR this shows the complete reaction of the alkene functionality, and its hopeful replacement with a trichlorosilane functional group.

$^{29}$Si-NMR (100 MHz, chloroform-$d + 0.05\%$ w/v TMS): $\delta = 12.12$ (SiCl$_3$).

In order to prove that the alkene functionality was replaced with the desired trichlorosilane group a 2D $^{29}$Si–$^1$H NMR was also taken of the final product which revealed a single $^{29}$Si peak at 12.14 ppm that is strongly coupled to H1$'$ and H2$'$ with slight coupling to H3$'$, strongly suggesting the terminal addition of trichlorosilane (Fig. 35). As no other peaks are visible in the spectra it can also be assumed that there is no cross-linked bi-product in the sample, which would give rise to peaks below 0 ppm.
3.6.5 Importance of APC Purity in MEG-Cl Synthesis

Although the spectra shown for APC show a highly pure product, this purity is rarely achieved without distillation of the product. Below is a typical spectrum that is obtained from the updated synthesis of APC using DMF as the catalyst, following removal of solid DMF by filtration. Although the majority of the sample in this spectrum is APC, there are noticeable impurities, namely the presence of unreacted APA which can be seen at 2.5 ppm (Fig. 36). In order to test the importance of APC purity on the synthesis of MEG-Cl, the above sample of APC was used in the conversion to MEG-Cl. On addition of the platinum catalyst to this mixture a hard solid formed around the catalyst, trapping most of it. The reaction did not proceed well, with most of the APC going unreacted, and much of the MEG-Cl cross-linking into a black solid. As such distillation of APC, to ensure its high purity before reaction to form MEG-Cl, is necessary for the success of the final reaction. However, small impurities in APA do not seem to have much of an effect on the synthesis of APC so distillation of APA is unnecessary unless major impurities are seen.
Figure 36 - Hydrogen-1 NMR spectrum of 3-(2-allyloxy)propanoyl chloride in chloroform-$d$ with 0.05% w/v tetramethylsilane at 500 MHz with no purification performed.
3.7 Surface Analysis of MEG-Cl Experimental

Unless otherwise indicated all compounds used in the following experimental section were purchased from Sigma Aldrich.

3.7.1 Cleaning and Surface Modification of Glass Discs

Glass coverslips/discs (Ted Pella, Inc., Redding, CA, USA), 12 mm in diameter and 0.13–0.16 mm in thickness, were first thoroughly washed with sodium dodecyl sulfate (SDS) solution (1% w/w in deionized water) followed by sonication in this solution for 30 min. The discs were next thoroughly washed with deionized water, then rinsed with acetone. The discs were dried under a stream of dinitrogen gas (Praxair), then plasma-oxidized for 10 min. They were then transferred to a humidity chamber, maintained at 80% relative humidity with a saturated aqueous solution of Mg(NO₃)₂ • 6 H₂O, and set aside overnight.

![Chemical structure](image)

**Figure 37** - Modification of glass discs to add MEG-Cl and Ni-NTA consecutively. This is a simplified representation that shows only one surface-bound molecule. The asterisks may represent an O atom in a siloxane group (and therefore a connection to another monomer), a surface-bound O atom, or a terminal silanol group.

Neat MEG-Cl (1 µL, synthesized as above) was diluted with anhydrous toluene (1 mL) under inert (N₂) and anhydrous (P₂O₅) atmosphere in a glovebox. The solution was added to glass vials (pre-silanized with trichloro(octadecyl)silane) containing a cleaned glass disc. The vials were capped and sealed with Parafilm™ M, removed from the glovebox, and placed on a spinning plate for 1.5 h. The discs were then rinsed with anhydrous toluene and sonicated in deionized
water for 5 min. The discs were rinsed again with deionized water. A solution of ab-NTA and nickel(II) chloride (2 mg/mL ab-NTA, and 2 mg/mL nickel(II) chloride in deionized water, 1 mL) was added to each vial along with pyridine (0.5 mL) and placed on a spinning plate overnight.

The discs, now modified with Ni-NTA, were rinsed with deionized water. Gelsolin solution (0.1 mg/mL in deionized water, 1 mL) was added to each vial containing a glass disc and placed on a spinning plate for 1 h. Each disc was then rinsed with deionized water.

3.7.2 Contact Angle Goniometry (CAG)

Static contact angle measurements were performed for the clean and bare glass discs, each step of chemical surface modification, and immobilization of gelsolin. Surfaces were analyzed with the KSV CAM 101 contact angle goniometer (KSV Instruments Ltd., Helsinki, Finland) using Type I water (18.20 MΩ cm) as the test liquid. Contact angle values were generated by the software provided with the instrument. Three discs were prepared for each step of the modification. Each side of each disc was tested twice.

3.7.3 X-ray Photoelectron Spectroscopy (XPS)

Angle-resolved XPS analysis was performed with a Theta Probe Angle-Resolved X-ray Photoelectron Spectrometer System (Thermo Fisher Scientific Inc., Waltham, MA, USA) located at Surface Interface Ontario (University of Toronto, Toronto, ON, Canada) for glass discs set aside after cleaning, each step of chemical surface modification, and immobilization of gelsolin. The samples were analyzed with monochromated Al Kα X-rays (elliptical spots of 400 μm along the long axis) with take-off angles of 90° relative to the surface. Peak fitting and data analysis were performed using the Avantage software provided with the instrument.
3.8 Contact Angle Results

![Graph showing contact angle results](image)

**Figure 38 - Surface contact angles of 18.20-MΩ cm water on cleaned glass discs, after each step of chemical surface modification of the discs, and after deposition of gelsolin.**

The shallow contact angle of $3 \pm 1^\circ$ ([Figs. 38, 39a]) observed for plasma-oxidized glass discs indicates a highly hydrophilic surface, which is evidence for a large concentration of silanol groups on the surface—a requirement for effective surface silanization. The modification of the surface with MEG-Cl increases the contact angle to $24 \pm 5^\circ$ ([Figs. 38, 39b]). This is consistent with the introduction of a more hydrophobic head group (the acyl chloride). The contact angle further increases to $31 \pm 3^\circ$ ([Figs. 38, 39c]) after extension of the surface coating with ab-NTA and binding of Ni(II) to the ligand. Although ab-NTA contains hydrophilic moieties, the elongation of the surface coating may mask the underlying glass substrate from the test liquid, thereby increasing the liquid’s surface tension and giving rise to a larger observed contact angle. Finally, the binding of gelsolin by the targeting group also increases the contact angle, this time to $38 \pm 5^\circ$ ([Figs. 38, 39d]). Overall, a progressive increase in the contact angle with each step of surface modification is observed, which emphasizes the functionalizability of the MEG-Cl-derived SAM.
Figure 39 - Contact angle measurements of a plasma-oxidized glass disc (a), a glass disc modified with MEG-Cl (b), a glass disc with Ni-NTA-extended MEG-Cl (c), and a glass disc with the same coating as in Figure 1c but enriched with gelsolin (d).
### 3.9 XPS Results

Table 2 - Relative atomic percentages of selected elements on the surfaces of glass discs after the various surface modification steps.

<table>
<thead>
<tr>
<th>relative atomic percentage</th>
<th>substrate</th>
<th>C1s</th>
<th>Cl2p</th>
<th>N1s</th>
<th>O1s</th>
<th>Si2p</th>
<th>Ni2p</th>
</tr>
</thead>
<tbody>
<tr>
<td>uncoated glass</td>
<td></td>
<td>14.3</td>
<td>0.7</td>
<td>0.3</td>
<td>58.1</td>
<td>26.5</td>
<td>—</td>
</tr>
<tr>
<td>MEG-Cl-coated glass</td>
<td></td>
<td>43.0</td>
<td>0.3</td>
<td>0.2</td>
<td>40.8</td>
<td>15.7</td>
<td>—</td>
</tr>
<tr>
<td>after Ni-NTA extension</td>
<td></td>
<td>40.3</td>
<td>0.3</td>
<td>0.6</td>
<td>42.5</td>
<td>16.0</td>
<td>0.4</td>
</tr>
<tr>
<td>after gelsolin binding</td>
<td></td>
<td>54.5</td>
<td>—</td>
<td>9.5</td>
<td>28.3</td>
<td>7.4</td>
<td>0.3</td>
</tr>
</tbody>
</table>

![Figure 40 - Visualization of the data presented in Table 3.2.](image-url)
Figure 41: Carbon 1s X-ray photoelectron spectra for the modified glass discs.

- uncoated glass  - after MEG-Cl coating  - after extension with Ni-NTA  - after gelsolin addition
The uncoated glass presents some degree of adventitious carbon contamination (283.1 eV), potassium (378, 294.8, 292.0 eV), chlorine (193.3 eV), and nitrogen (400.9 eV, possibly due to adsorbed dinitrogen) but consists mostly of oxygen (533.0 eV) and silicon (154, 103.8 eV) (Table 2, Fig. 40). These two elements are present at a Si2p:O1s ratio of approximately 1:2, which corresponds to the formula of silicon dioxide.

The modification of the glass substrate with MEG-Cl adlayer results in several changes. The C1s signal increases significantly because the adlayer is predominantly composed of carbon. There is also a noticeable change in carbon oxidation state with the disappearance of the peak seen at 283.1 and the appearance of lower binding energy peaks (282.9, 284.2, and 287.1 eV) (Fig. 41). These new peaks correspond to C-C, C-O, and C=O bonds, respectively, which are all present in MEG-Cl. The potassium peaks all disappear, suggesting that either the element has been washed off the surface or hidden by the organic adlayer. The O1s and Si2p signals fall since the adlayer partially obscures the underlying substrate. It is worth noting that the Si2p:O1s ratio is no longer 1:2. This is because (1) modification of the glass involves plasma-oxidation, which hydroxylates the surface of the substrate, and (2) the adlayer contains more oxygen than silicon. Thus, the Si2p signal decreases more relative to the O1s signal.

The decrease in the Cl2p signal may reflect a loss of chlorine due to X-ray bombardment of the surface. Additionally, chlorine is introduced to the surface at a smaller proportion than the other elements, so the relative atomic percent may decrease even though chlorine is being added.

The introduction of ab-NTA and binding of Ni(II) gives the expected increases in N1s, O1s, and Ni2p (856.5 eV) signals. A decrease in C1s signal is observed but this is likely due to the relatively lower atomic percentage of carbon in Ni-NTA bound to MEG-Cl as compared to just MEG-Cl. The oxidation state of C1s was not observed to change as the Ni-NTA headgroup contains carboxylic acid groups which would present similar peaks to those observed for MEG-Cl.

Finally, the binding of protein brings about an increase in the C1s and N1s signals, as is expected, and a substantial decrease in the O1s and Si2p signals. The oxidation state of C1s changes from what was observed with MEG-Cl and Ni-NTA but remains similar, most likely due to the large presence of C-C and C=C bonds found within the protein. Again, the Si2p signal decreases more strongly relative to the O1s signal since the underlying substrate (consisting of
silicon and oxygen) is being further obscured but the protein contains a fair amount of oxygen. The persistence of the Ni2p signal suggests that the poly-histidine tag functions as intended. We can also conclude that, at this point, most chlorine has been removed due to the absence of any signal.

Survey scans provide more information about the surface and potential substrate contaminants (Fig. 42). All samples present substrate peaks at 153–154 eV due to Si2s and 24–26 eV due to O2s. Each sample also gives a possible Sn3d signal at 497–498 eV, which decreases with each subsequent surface modification. This could represent a tin oxide impurity in the underlying substrate. The peaks at 1072 and 1071 eV for bare and MEG-Cl-coated glass, respectively, suggest that a small amount of sodium is present on the surface, likely bound to the glass substrate. Multiple signals in the survey scan for the bare glass also imply some degree of zinc contamination: 1022 (2p), 89 (3p), and 11 eV (3d). As with tin, these signals become progressively smaller relative to other signals in the scan, suggesting that zinc is a substrate contaminant. Finally, a small Ca2p signal appears at 347 eV when gelsolin is added. This may represent gelsolin-bound calcium, which regulates the protein’s actin-severing activity, further evidence of successful protein binding. 165
Figure 42 – XPS survey scans of a) bare glass after plasma cleaning, b) MEG-Cl coated glass, c) Ni-NTA addition to MEG-Cl on glass, and d) gelsolin immobilization on Ni-NTA and MEG-Cl coated glass.
3.10 Surface Linker Conclusions

3-(3-(Trichlorosilyl)propoxy)propanoyl chloride has been successfully synthesized in high purity and moderate yield. Surface contact angle measurements and X-ray photoelectron spectra support the successful two-step modification of the hydroxylated surfaces of glass discs to contain protein-binding Ni-NTA. Since the product’s trichlorosilyl group may react with hydroxyl groups in the presence of water to form a surface monolayer, this surface chemistry may be extended to any hydroxylated surface. Further applications of this chemistry will be explored in the biosensing context. Moreover, the ability of the resulting surface coatings to resist non-specific protein adsorption is also the target of further research.
Chapter 4

4 Transverse Shear Mode Device Studies

4.1 Abstract

Transverse shear mode devices (TSM), also known as quartz crystal microbalances are of interest in the sensing of biomarkers for diseases in various biological fluids, but suffer from the issue of non-specific adsorption of compounds other than those of interest to the surface affecting the output. Anti-fouling layers are typically used to reduce the issue of non-specific adsorption in these devices. For the detection of ovarian cancer the biomarker lysophosphatidic acid is of interest, and may be detectable in serum using a TSM. The aim of this study was to determine the level of non-specific adsorption on TSM discs from serum samples with added LPA in the presence of a MEG-OH anti-fouling layer. It was found that although the concentration of serum analyzed had little effect on the amount of fouling observed, there was a large amount of fouling with high standard deviation observed for all cases, rendering the TSM incapable of detection in serum samples even with a MEG-OH anti-fouling layer.

4.2 Contributions

The computer interface for the TSM device was developed by Gordon Hayward of the University of Guelph. The surface anti-fouling molecule MEG-OH was synthesized by Christophe Blaszykowski.

4.3 Development of a 9 MHz TSM Device

4.3.1 Hardware Development

The original transverse shear mode device used in the Thompson lab was based off of a Hewlett Packard 4395A Spectrum Analyzer, which was used to drive the quartz crystal and measure its frequency and resistance. This device was used for the initial experiments done on TSM devices, predominantly the early fouling experiments performed in PBS buffer described below. However the device broke down early in the project and a new TSM device had to be constructed to replace it, a device that could also operate at 9 MHz, which was the frequency of the original TSM.
The replacement TSM device, which was used for all subsequent TSM experiments, was based off of a PLO-10i oscillator (Fig. 43), which has an operational frequency of 5.1 to 10 MHz. Commercial QCM devices, such as the SRS QCM200, and the Biolin Scientific QCM-D, typically operate at 5 MHz, limiting which discs can be used when performing experiments, and making any new experiments not comparable to experiments previously performed in the Thompson lab due to the difference in natural frequency. The PLO-10i oscillator was chosen with the help of Gordon Hayward of the University of Guelph as it allows for the original 9 MHz quartz discs to be used which other devices would not. It also allows for a wider range of discs to be chosen due to the wide range of frequencies it can measure. The reason for the use of higher frequency 9 MHz crystals in the Thompson lab is because they are more sensitive to surface changes than the standard 5 MHz crystals.

In order to communicate with the oscillator a computer interface was needed. This interface was constructed based on designs by Gordon Hayward. It is based on a Propeller Quickstart programmable computer, which has a USB port to allow it to interface with a modern computer. An analogue to digital converter was also needed so that the Propeller Quickstart could send the signal from the oscillator to the computer via the USB interface (Fig. 44).
Figure 44 - Analogue to Digital converter circuit where the P numbers refer to pins on the Propeller Quickstart. Circuit designed by Gordon Hayward.
4.3.2 TSM Software

The software used to visualize and collect data coming from the PLO-10i oscillator was programmed in two parts. The first was coded by Gordon Hayward, and modified by myself, which is the programming for the Propeller Quickstart. This program takes the data coming from the PLO-10i, and converts it into readable numbers. This part of the software was written in Assembly, and the code can be found in Appendix A.

This code allows for the device frequency to be measured to 1 Hz once per second. Unfortunately the Propeller Software is limited to only outputting the measured frequency as a number, and cannot graphically display the frequency or save the data. To overcome this limitation a second piece of software was developed by myself that takes the data from the Propeller Quickstart and both displays and saves this data (Fig. 45).

![Software interface for the TSM controller.](image)

This software was coded in the visual basic language using Visual Studio 2012 Express. The code can be found in Appendix B.
4.4 TSM Experimental

Quartz crystal discs were purchased from LapTech Precision Inc. and are the xt2905 model of disc, which has an operational frequency of 9 MHz. 2-(2-mercaptoethoxy)ethan-1-ol was synthesized by Christophe Blaszykowski in large quantity prior to the start of this thesis work. PBS buffer, sodium dodecyl sulfate, and goat serum were all purchased from Sigma Aldrich. Ethanol was purchased from MedStore at the University of Toronto. Lysophosphatidic acid was purchased from Enzo Life Sciences. Nitrogen was purchased as a pressurized gas from Praxair. Deionized water was collected from the Analest facility at the University of Toronto with a resistance of at least 18.0 MΩ.

4.4.1 TSM Disc Cleaning

TSM discs were placed in small glass vials, which had been rinsed 2 times with deionized water (diH₂O) and once with 1% SDS in diH₂O (SDS). Each disc was then rinsed 2 times with SDS, and then placed on a rotator in SDS and rotated for 15 minutes. The discs were then given a thorough rinsing in diH₂O, then rinsed 3 times in acetone. After that they were rinsed 3 times with methanol, followed by 5 minutes of rotation in methanol. They were then rinsed thoroughly with methanol, and dried under a stream of nitrogen. They were then plasma cleaned under atmosphere for 5 minutes, followed by storage in glass vials.

4.4.2 TSM Disc Modification

Clean TSM discs were soaked in 5 µM 2-(2-mercaptoethoxy)ethan-1-ol (MEG-OH) in anhydrous absolute ethanol, and placed on a rotator for 30 minutes. The discs were then rinsed with absolute ethanol, followed by methanol and dried under a stream of nitrogen. Discs were then stored in glass vials.

4.4.3 TSM Operation

A TSM disc was placed in a custom flow-through chamber connected to an Inficon PLO-10i oscillator. A Harvard Apparatus 11plus syringe pump was used to pull 0.01 M PBS buffer at pH 7.4 (PBS) through the chamber and over the disc at 100 µL/min. Once the disc was saturated in PBS solution, the PLO-10i oscillator was calibrated using the calibration rotors on the front of the device. Analogue signal from the oscillator was converted into a USB digital signal by way
of a custom A/D circuit and Parallax Quickstart board (interface designed by Gordon Hayward), and the data was read by custom software programmed in Visual Basic. Once the frequency of the disc had stabilized in PBS the test solution (such as LPA in PBS, or dilute goat serum) was passed over the disc for 5 minutes, before returning the disc to PBS. The disc was kept under PBS flow for 30 minutes after the test solution was stopped, followed by switching the solution to diH₂O for final washing of the disc and chamber for 10 minutes.

### 4.4.4 Data Analysis

Data was analyzed by taking the average frequency of the TSM discs in PBS for 10 minutes prior to injection of sample. The frequency of the disc when in sample was averaged for 1.5 minutes 5 minutes after injection. Finally, the return frequency was averaged from 20-30 minutes after return to PBS solution for washing. Calculations and graphing were done in Microsoft Excel. At least 3 runs were averaged for each solution and the errors shown are the standard deviations from the multiple runs.

### 4.5 Results of TSM Fouling Studies

#### 4.5.1 Frequency Results
Figure 46 - Frequency data for individual TSM runs of 100% PBS buffer containing a) 0 μM LPA, b) 25 μM LPA, c) 50 μM LPA, and d) 100 μM LPA. Colours indicate individual experiments.
Figure 47 - Frequency data for individual TSM runs of PBS buffer with 10% serum containing a) 0 μM LPA, b) 25 μM LPA, c) 50 μM LPA, and d) 100 μM LPA. Colours indicate individual experiments.
Figure 48 - Frequency data for individual TSM runs of PBS buffer with 20% serum containing a) 0 μM LPA, b) 25 μM LPA, c) 50 μM LPA, and d) 100 μM LPA. Colours indicate individual experiments.
Figure 49 - Frequency data for individual TSM runs of PBS buffer with 50% serum containing a) 0 μM LPA, b) 25 μM LPA, c) 50 μM LPA, and d) 100 μM LPA. Colours indicate individual experiments.
Figure 50 - A graph representing the frequency drop of the TSM signal on addition of PBS and serum samples. From light blue to dark blue indicates the concentration of LPA with 0 μM being the lightest blue, 25 μM slightly darker, 50 μM darker still, and 100 μM the darkest blue.
In the raw data a ringing can be seen in the signal (Fig. 46a), which can be attributed to the instrument outputting a value between the two frequencies, which is rounded up or down dependent on the frequency counter. This 1 Hz ring can be ignored as it is simply the instruments built in noise.

The first piece of information that can be obtained from a TSM run is the lowest frequency of the disc after sample injection, which will be referred to as the frequency drop (Fig. 50). From the data it can be seen that the frequency drop is lowest when there is no serum present, and is larger when there is serum present. However there is little change in the frequency drop between 20% and 50% serum, which suggests that the amount of material deposited onto the QCM disc during injection is the same between these two conditions.

Addition of LPA in to the injection solution causes an increase the frequency drop in all conditions above those without LPA, which is as little as 30 Hz for pure PBS and as high as 130 Hz for 50% serum (Fig. 50). Though there appears to be an increasing trend between LPA concentration and frequency drop in pure PBS due to the increase of 30, 40 and 70 Hz respectively for 25, 50, and 100 µM LPA, high error in runs with 50 µM LPA make this trend unreliable. As well no trends of increasing frequency drop in relation to LPA concentration are visible in serum, predominantly due to high error within each condition. At 10% serum there was no significant difference in frequency drop between the different concentrations of LPA present, though all three were greater than the condition where no LPA was added.

When the serum concentration is increased to 20% there is no significant effect of LPA on frequency drop with all conditions exhibiting frequency drops close together and within error of each other (Fig. 50). The only time when frequency drop changes greatly based on LPA concentration is at 50% serum, where there is an increase for both 25 µM and 50 µM LPA, with 100 µM LPA being within error of 50 µM. This suggests that at 50% serum LPA is having an effect on the deposition of materials onto gold, which saturates between 25 µM and 50 µM LPA; an effect which is not present at lower concentrations of serum.
Figure 51 - A graph representing the change in frequency of the TSM from the baseline in PBS buffer to the final wash. From light blue to dark blue indicates the concentration of LPA with 0 μM being the lightest blue, 25 μM slightly darker, 50 μM darker still, and 100 μM the darkest blue.
Another important piece of information that can be ascertained from the TSM data is the change in frequency from before injection to after wash-off with PBS, which will be referred to as the overall frequency change (Fig. 51). This change is indicative of the amount and characteristic of new matter on the TSM disk from the injection that has not washed off, or the amount of fouling material present. A larger change in overall frequency suggests a greater amount of surface fouling from the injected sample.

From the samples with no additional LPA it can be seen that all three serum concentrations exhibit a similar amount of overall frequency change, or fouling, suggesting when it comes to fouling increasing serum concentration, and thus the concentration of the analyte of interest, from 10 to 50% has little impact on the amount of fouling, and thus higher concentrations of serum are desirable versus lower concentrations in TSM experiments. This could be due to there being a maximum amount to which a disc can be fouled, and that 10% serum is enough to cause this.

The addition of LPA to any samples increases the overall fouling, suggesting that LPA itself deposits on the surface and some of it remains unwashed by the PBS wash-off. The concentration of LPA above 25 µM seems to have no effect on the overall frequency change, with all concentrations exhibiting similar frequency changes within error of each other. This is similar to what was observed with the drop in frequency, suggesting a saturation of fouling above 25 µM LPA. Also, the error between runs with identical conditions is quite high for the overall frequency change, suggesting a large variation in fouling between runs.
Figure 52 - A graph representing the percent recovery of frequency of the TSM signal between the initial frequency in PBS buffer and the frequency following the final wash. From light blue to dark blue indicates the concentration of LPA with 0 μM being the lightest blue, 25 μM slightly darker, 50 μM darker still, and 100 μM the darkest blue.
Due to the difference in scale of frequency drop on solution change, and the overall frequency change, a percent frequency recovery, which was calculated by dividing the overall frequency change by the frequency drop on solution change, is a potential way to compare runs with different serum concentrations (Fig. 52). The higher the percentage recovery of the frequency, the less fouling there is based on the amount of sample initially deposited on the surface.

This calculation helps to remove some of the error between runs, though there are exceptions to this case such as those runs when no serum is present. At 50% serum there is little difference in percentage recovery between different concentrations of added LPA, suggesting consistent levels of fouling versus the amount of material deposited. At lower concentrations of serum there is a clear drop in the percentage recovery after the addition of LPA to the sample, suggesting that the LPA contributes strongly to the surface fouling in these cases. The additional surface fouling that is present at lower concentrations of serum is potentially attributed to micelle formation in serum which would likely reduce fouling, and may require higher concentrations of serum to be facilitated.\textsuperscript{170} More study would have to be done to analyze the ability of LPA to form micelles in different concentrations of serum in order to determine if this is the case.

As can be seen from the data overall, there is a large amount of surface fouling on gold even in the presence of an anti-fouling monolayer, and there is a large variation between identical runs in the amount of fouling. This fouling was observed for all conditions, even those with just LPA in PBS, showing that even LPA causes noticeable fouling on its own. As a result of this, it is unlikely that a useful signal could be achieved in serum samples, regardless of the quality of molecular probe, since this change in frequency would be difficult, if not impossible to separate from the change as a result of surface fouling. As a result it cannot be recommended to use a TSM device in serum for the detection of biomarkers, as such detection could not be made accurate or quantifiable. However, if one does wish to analyze serum with a TSM device, the above study has shown that all concentrations of serum from 10% to 50% provide similar levels of surface fouling, and thus higher concentrations of serum can be used in the analysis. As well a better anti-fouling layer that reduces the amount of fouling and error between runs would need to be developed.

4.5.2 Resistance Results
Figure 53 - Resistance data for individual TSM runs of 100% PBS buffer containing a) 0 μM LPA, b) 25 μM LPA, c) 50 μM LPA, and d) 100 μM LPA. Colours indicate individual experiments.
Figure 54 - Resistance data for individual TSM runs of PBS buffer with 10% serum containing a) 0 μM LPA, b) 25 μM LPA, c) 50 μM LPA, and d) 100 μM LPA. Colours indicate individual experiments.
Figure 55 - Resistance data for individual TSM runs of PBS buffer with 20% serum containing a) 0 μM LPA, b) 25 μM LPA, c) 50 μM LPA, and d) 100 μM LPA. Colours indicate individual experiments.
Figure 56 - Resistance data for individual TSM runs of PBS buffer with 50% serum containing a) 0 μM LPA, b) 25 μM LPA, c) 50 μM LPA, and d) 100 μM LPA. Colours indicate individual experiments.
Before discussing the resistance change results for the TSM experiments, it should be noted that the PLO-10i does not itself output a resistance value for the crystal. Instead the PLO-10i outputs the crystal's overall capacitance as a voltage, which must then be converted to a resistance value. This is done by dividing 100 by the capacitance and subtracting 20 (the system's internal resistance), which gives a value for the crystal's resistance. Since the resistance is calculated by division with a typically small voltage value large jumps can be seen in the resistance data, limiting the precision of the data. Despite this overall trends in resistance can be observed, and may be useful in measuring crystal surface changes.

Typically with a TSM resistance acts in the opposite way to frequency, where a decrease in crystal frequency is observed with injection of serum, the resistance is seen to increase (Fig. 57). This increase in resistance is barely observed with pure PBS solution. The resistance can be seen to increase more with the injection of serum solutions, where 50% serum causes the largest increase in resistance on injection.

The addition of LPA to the injected solutions seems to have little effect on the resistance increase in the case of pure PBS and 10% serum. However at higher concentrations of serum LPA concentrations seems to cause a large variation in the observed resistance rise. However in many of these tested conditions a large error can be seen in these values. This is primarily due to the starting resistance of the system.

Unlike the crystal frequency, which tends to be very similar disc to disc in PBS buffer, the resistance shows far greater starting variation. It was found to be as low as 80 Ω, and as large as 360 Ω. This is most notable in the runs performed in 50% serum with 50 μM LPA, where two of the experimental runs had a baseline resistance at approximately 90 Ω, and the other two runs at approximately 360 Ω. The rise in resistance is directly correlated to the starting resistance, with a lower starting resistance resulting in a lower overall rise in resistance. As such it is important to factor the starting resistance of the system into any resistance measurements.
Figure 57 - A graph representing the increase in resistance of the TSM signal on addition of PBS and serum samples. From light blue to dark blue indicates the concentration of LPA with 0 μM being the lightest blue, 25 μM slightly darker, 50 μM darker still, and 100 μM the darkest blue.
Figure 58 - A graph representing the overall change in resistance of the TSM between the initial resistance in PBS buffer and the resistance following the final wash. From light blue to dark blue indicates the concentration of LPA with 0 μM being the lightest blue, 25 μM slightly darker, 50 μM darker still, and 100 μM the darkest blue.
A similar trend to the rise in resistance can be observed with the overall resistance change (Fig. 58). There is little overall change to the resistance of the crystal at low concentrations of serum with each LPA condition being within 0.2 Ω of each other with no statistical difference between them, and a large overall change can be observed with high concentrations of serum as much as 5 Ω in the case of the 20% serum sample. As with the resistance rise, LPA seems to have little effect on the overall change in resistance at low concentrations of serum with each LPA condition being statistically the same, but causes large variation in experiments performed at higher concentrations. The highest variation is seen in the condition of 20% serum with 50 μM LPA, which had a change in resistance of 4.7 ± 3.8 Ω, an error nearly as large as the measurement itself. Taken on its own the overall resistance change is not useful in analyzing the system due to the high variability that can be observed above.

In order to account for error resulting from variation in the starting crystal resistance the percent recovery of crystal's resistance can be analyzed (Fig. 59). Although there is very large error in each of the experimental conditions performed in pure PBS buffer this can be easily explained by the very small change observed in resistance upon injection of PBS. As a result a small 1 Ω change, which is within the error of the system, results in a large observed change in percent recovery.

However at higher concentrations of serum, which have correspondingly higher changes in both resistance rise and overall resistance, there is far less variation in the resistance recovery. This is especially true in the case of experiments performed in 50% serum, where all concentrations of LPA resulted in a recovery of approximately 80%, with very low between run error observed. Each of the LPA conditions in 50% serum are statistically the same, between 82 and 84% with an standard deviation of 4%. This is in sharp contrast to the high between run error observed in the resistance rise and overall resistance change, showing that the percent recovery is an excellent tool to correct for starting resistance variability.

Although there is little effect caused by LPA on the percent resistance recovery of the crystal, making it the most useful measurement for analyzing the system, the low precision of the resistance measurements will greatly limit the sensitivity of any test relying on it to measure changes in the system.
Figure 59 - A graph representing the percentage recovery of resistance of the TSM between the initial resistance in PBS buffer and the resistance following the final wash. From light blue to dark blue indicates the concentration of LPA with 0 μM being the lightest blue, 25 μM slightly darker, 50 μM darker still, and 100 μM the darkest blue.
4.6 Conclusions of TSM Use in Serum

Transverse shear mode devices have shown promise as potential bio-detectors due to their relative low cost and ease of use. The frequency change from an equilibrium state in PBS buffer to the injection of a sample, and to the overall frequency change after wash-off in solution, is indicative of the amount of new material on the surface. This paper has shown that even with an anti-fouling SAM, and no molecular probe, the injection of the biomarker LPA with or without serum consistently fouled the surface. As a result it cannot be recommended to use a TSM device when analyzing biological samples until a more effective anti-fouling SAM has been developed. However in all concentrations of serum analyzed (10-50%) there is a similar level of fouling, suggesting that higher concentrations of serum can be studied with TSM devices in the future. Further study into the micellular formation of LPA in serum should be studied in order to determine the cause of LPA’s effects on surface fouling in serum.
Chapter 5

5 Colorimetric Detection of LPA

A patent has been filed on the technology developed in this chapter, along with the methods of its production; patent application number PCT/CA2016/050545 by Brian De La Franier and Michael Thompson.\textsuperscript{171}

5.1 Abstract

Development of a low cost test for quantifying LPA in human serum, with the desire of screening for ovarian cancer, was explored using colorimetric analysis. The backbone of this test relies on the complex of the proteins gelsolin and actin, and its sensitivity to being disrupted by LPA in a concentration dependent manner. To develop the test first actin is dyed with fluorescently active dyes, and then introduced to gelsolin to form a complex. This complex is bound via the gelsolin to a solid surface which can be exposed to human serum. LPA in the serum will cause a release of the dyed actin, which is then measured via colorimetric analysis. It was found that by using a mixed surface of MEG-Cl and HTS on solid silica gel was optimal for protein complex binding. It was also found that LPA in serum caused a measurable signal via fluorescence spectroscopy, which was concentration dependent; though more work needs to be done optimizing this test to lower the limit of detection.

5.2 Contributions

The surface linker PFP-TTTA was synthesized by Christophe Blaszykowski. Human serum was provided by Alex Romaschin of St. Michael’s Hospital.

5.3 General Idea

Due to the prevalence, ease, and relative low cost of UV-visible and fluorescence spectroscopic analysis a colorimetric approach to using actin and gelsolin to detect LPA is a desirable route to explore. By attaching gelsolin to a solid surface, dyed actin can be bound to that surface in a way that is susceptible to release by LPA. Due to the previously established concentration dependent nature of actin release by LPA, the amount of dyed actin released from the solid into a liquid sample should be directly correlated to the concentration of LPA present. By measuring
the amount of dye, and thus the concentration of actin present, the sample concentration of LPA could be determined (Fig. 60).

5.4 UV-Visible analysis of LPA

In order to determine if UV-Visible analysis would be suitable for a gelsolin-actin based sensor for LPA, analysis of LPA in PBS buffer as well as in human serum by UV-visible spectroscopy was performed from 400-700 nm.

In PBS buffer there was a clear upward trend in absorbance between 400 and 700 nm with an increasing concentration of LPA (Fig 61). This trend is quite small overall, but must be accounted for in any testing performed for LPA. LPA itself does not contain any functional groups that are known to absorb visible light, so the absorbance observed must be due to another property of LPA such as the formation of micelles.

Unlike when LPA was dissolved in PBS there is no clear trend observed in the absorbance scans of LPA in serum (Fig 62). Buffer A (2 mM Tris-Cl pH 8, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl$_2$) was chosen as the blank solution instead of serum to highlight the absorbance patterns of serum. The differences in scans are very small and can likely be explained as a result of instrumental error. It's possible that the lack of upward trend that was observed in PBS buffer is due to the difference in critical micelle concentration of LPA in serum versus other buffers. Since LPA does not appear to have any noticeable effect on uv-visible absorbance of serum it is possible that this technique can be used to measure dyed actin in serum in relation to a test for LPA.
Figure 61 - Scans of PBS buffer containing the specified concentration of LPA with PBS buffer used as the blank.
Figure 62 - Scans of serum containing the specified concentration of LPA with buffer A used as the blank.
5.5 Fluorescence analysis of dyed actin and LPA

In order to determine if dyed actin is suitable for detection by fluorescence measurement, fluorescence measurements of dyed actin were taken, with and without LPA present in solution (Fig 63). When rhodamine dyed actin was introduced to serum first (Fig 63, orange trace) a linear relationship between the fluorescence signal ($\lambda_{ex} = 552$ nm, $\lambda_{em} = 572$ nm) and actin concentration was observed. Although the above plot does not look linear this is due to the mixed nature of the x-axis where LPA and actin concentrations were used on different scales. The relationship resulted in a limit of detection of 3.5 nM for the actin concentration. It should be noted that the fluorescence spectrophotometer used in these experiments is an older model which was not optimized. Better fluorescence spectrophotometers could likely sense a much lower concentration of rhodamine dyed actin.

More importantly when LPA was added to the serum now containing 75 nM actin there was no observed change in fluorescence signal at any concentration of LPA up to 25 μM. This suggests that LPA itself would not interfere in a fluorescence test reliant on accurate quantification of rhodamine dyed actin.

A second set of fluorescence measurements in serum was taken, only this time LPA was first added to serum followed by addition of rhodamine dyed actin (Fig 63, blue trace). Addition of LPA to the serum did not result in any measurable fluorescence signal up to a concentration of 25 μM. Addition of actin into the serum now containing 25 μM LPA was found to cause a linearly increasing fluorescence signal with actin concentration, which was a close match to the relationship found when actin was added to the serum first. As LPA at biologically relevant concentrations has no effect on the ability of actin-rhodamine to fluoresce in serum, dyed actin can be used to test for LPA in serum samples.
Figure 63 - Fluorescence analysis of rhodamine dyed actin in serum where in orange actin was first added to serum at the specified concentrations followed by the addition of LPA to the final serum solution, and in blue LPA was added to serum at the specified concentrations followed by the addition of actin to the final solution.
5.6 Plastic testing strips

One possible avenue for LPA testing in serum is to create a disposable cuvette that has been modified with gelsolin and dyed-actin. Serum could be introduced to the cuvette and the UV-visible or fluorescence signals for the dyed actin can be measured over time. Any LPA in the serum should cause the release of actin from the walls of the cuvette into the serum, causing the absorbance and fluorescence to change. Monitoring the rate and magnitude of this change could then be correlated back to the concentration of LPA in the serum sample.

In order to determine if this is feasible plastic discs made of PET were modified with MEG-Cl, followed by reaction with ab-NTA and NiCl$_2$ to create a surface that could bind to the His-tag of gelsolin. This was done using the same procedure as glass modification outlined in chapter 3, only substituting toluene with heptanes. Gelsolin and actin-rhodamine were added to the surface and incubated for 1 hour (~1 μM concentration of each in buffer A). The stripes were then rinsed thoroughly with deionized water to create a strip that should be sensitive to LPA in solution.

Initially the modified discs were incubated in a solution of buffer A or a solution of buffer A containing 25 μM LPA for 30 minutes. UV-visible absorbance was measured at 552 nm and for buffer A exposed to the discs was found to be 0.000 ± 0.001 absorbance units (n = 3). This suggests that there was no non-specific adsorption of actin-rhodamine to the surface. The solution with LPA was found to produce a signal of 0.044 ± 0.002 absorbance units (n = 3) after correction for the absorbance of LPA in buffer. This signal was quite small, but could still be measured suggesting that the use of an actin-gelsolin complex to detect LPA is plausible.

In order to determine the incubation time necessary between samples and the plastic discs time trials were performed where modified PET strips were placed in a cuvette such that they did not block the beam of the UV-visible spectrometer. Buffer A samples containing LPA were then added and the absorbance at 552 nm was recorded every 10 seconds. Samples included either 1, 10, or 25 μM LPA and experiments were performed in triplicate.
Figure 64 - UV-visible absorbance at 552 nm for buffer A samples containing LPA exposed to modified PET plastic strips.
There was a noticeable increase in absorbance versus time for the first 4 minutes of exposure to the plastic strips for each concentration of LPA tested (Fig. 64). Unfortunately there was a very high difference observed between experimental runs with the same concentration of LPA, both in the rate and magnitude of signal increase. As well there was visible overlap in this error between samples containing different concentrations of LPA. Although there is a noticeable signal for LPA exposed to the strips, the high error makes these strips less than ideal for use in clinical analysis. The very low signal is likely a result of the limited amount of protein that can pack onto a flat surface, so a higher surface area solution is desired.

5.7 Column Based Fluorescence Detection

Due to Ni-NTA being able to bind the gelsolin made in this lab a high surface area alternative to the plastic strips used previously could be store bought Ni-NTA agarose gel used in protein purification. In order to determine the plausibility of using this resin to detect LPA fluorescence analysis was used instead of UV-visible analysis to reduce the effect of LPA itself on the measurements taken. Initial testing was performed as a flow-through column, with the fluorescence signal of the sample being taken (λ<sub>ex</sub> = 552 nm, λ<sub>em</sub> = 572 nm) followed by passing the sample through a column containing 0.5 mL of the agarose gel covered in the gelsolin and rhodamine-actin complex, and then taking the fluorescence signal again.

To prepare the gel a protein mixture of gelsolin and actin-rhodamine was added to store bought gel and incubated for 1 hour (~1 μM concentration of each in buffer A). The gel was mixed with either buffer A containing 700 μM imidazole as a positive control, or 500 μM LPA. The fluorescence signal observed for the sample containing imidazole was found to be 3.26 ± 0.12 fluorescence units, while the sample containing LPA was found to create a signal of 0.25 ± 0.22 fluorescence units. Not only did the LPA sample create virtually no signal even with a very high concentration of LPA, but the positive control also created only a very small signal. This suggests that store bought Ni-NTA agarose gel is unsuitable for binding the actin-gelsolin complex, and a different solid phase needed to be developed.
5.8 Initial Development of Solid Support based on Silica Gel

5.8.1 Methods for Initial Development

All compounds used in this chapter were purchased from Sigma Aldrich, or made as described in previous chapters.

To try and improve upon the limited amount of protein captured by commercial Ni-NTA agarose gel, a new solid phase with a high surface area had to be determined. One possibility is silica gel, commonly used in column chromatography. Several steps would be needed to modify commercially available silica gel with a Ni-NTA linker attached to bind the gelsolin-actin complex.

The first step is plasma cleaning the silica gel in air. This serves to not only remove unwanted organics from the silica, but also to ensure the surface presence of hydroxyl groups which a trichlorosilane linker can be bound to. Plasma cleaning was initially done on 2 mL of silica gel (150 Å pore size, 200-425 mesh) in a shallow open-top container with a kimwipe used to prevent the silica from being pulled out of the container under vacuum while still allowing plasma to reach the silica gel. The gel was plasma cleaned for 2 minutes before being removed from the vacuum.

The second step is to saturate the surface with a thin film of water. This film of water is necessary for the formation of a silane adlayer on the surface by allowing for crosslinking between trichlorosilane molecules after they have bound to the surface. Bulk water in the solvent would not be suitable for this since it would present the dissolved trichlorosilane to crosslink before settling on the surface of the silica. The thin film is achieved by placing the now hydroxylated silica gel in a humidity chamber at 80% humidity overnight.

![Figure 65 - Structure of perfluorophenyl 12-(trichlorosilyl)dodecanoate (PFP-TTTA).](image-url)
The third step is the addition of a trichlorosilane adlayer. Initial experiments used an amine reactive linker perfluorophenyl 12-(trichlorosilyl)dodecanoate (PFP-TTTA, Fig. 65) due to issues purifying MEG-Cl at the time. PFP-TTTA is also reactive towards amine groups, forming an amide linkage, allowing it to function similarly to MEG-Cl. This linker was initially deposited on the surface with trichloro(hexyl)silane as a spacer in a 1:1 ratio in anhydrous toluene. The reaction was rotated for 90 minutes, followed by removal of solvent using a cotton filter in a syringe column. This was followed by washing the gel with 20 mL of toluene followed by 5 mL of deionized water by passing the solvent through the gel.

The fourth step is to extend the linker with a Ni-NTA head group. This was initially done by reacting the now modified silica gel with ab-NTA in the presence of 10% triethylamine in deionized water overnight (5 mg/mL ab-NTA, 5 mg/mL NiCl₂, 5 mL). The gel was then cleaned with 15 mL deionized water in the same way as above.

Actin and gelsolin (0.1 mg of each) were pre-incubated together for 1 hour in buffer A before being mixed with the modified silica gel (1 mL buffer A per mL of silica gel, with 1 mg/mL NiCl₂). The gel was rocked for 1 hour to ensure adequate mixing. Columns were prepared by plugging a 1 mL syringe with cotton, and transferring 0.5 mL of wet silica gel into each. The liquid containing excess protein was pushed through the column and collected for analysis, followed by the gel being washed with 10 mL buffer A.

The following sample solutions were prepared and had their fluorescence measured ($\lambda_{ex} = 552$ nm, $\lambda_{em} = 572$ nm): buffer A as a negative control, buffer A with 700 μM imidazole as a positive control, and buffer A with 25 μM LPA as a test. Then the sample was added to a column test, and pushed through the gel in a flow-through set-up (Fig. 66). The fluorescence was measured again for each, with the difference in fluorescence before and after exposure to the column being the signal.
5.8.2 Fluorescence Results of Initial Silica Gel Solid Support

The negative control of buffer A produced a fluorescence signal of 6.1 ± 0.6 fluorescence units. This signal suggests that some of the protein has non-specifically adsorbed onto the surface suggesting inadequate washing of the silica gel before testing. The positive control with imidazole produced a signal of 36.7 ± 0.6 fluorescence units. This signal is significantly higher than the one observed for just buffer A, suggesting that there is a reasonable amount of gelsolin-actin complex specifically bound to the ni-NTA. This is also much higher than the positive control signal observed for the store bought agarose gel, suggesting this surface is better at binding to the gelsolin-actin complex.

When preparing the silica gel columns the protein solution added to the silica gel was filtered off and retained. Fluorescence analysis of this column excess produced a signal of 118 ± 2 fluorescence units. As well the first 5 mL of water used to wash the silica gel was also retained and produced a signal of 74 ± 1 fluorescence units. These high signals show that most of the protein that has been added to the silica gel has not bound and washes off the column, suggesting there is still room for surface improvement.

Finally the signal produced by buffer A with 25 μM LPA produced a fluorescence signal of 6.7 ± 0.6 fluorescence units. Though this appears to be higher than the blank signal, there is overlap in the signal errors resulting in a p-value of only 0.25 and no significant difference between the two. As well it falls far from the signal observed with imidazole. Improvements need to be
made to the surface modification in order to attach more protein to the surface, and to make it more accessible to binding with LPA.

5.9 Modification of Silica Gel Preparation

Competition studies were done with multiple batches of silica gel being prepared with one of the steps in the procedure outlined above being changed to see what effect it would have. For this and all following sections the error is standard deviation (n=5), unless otherwise stated.

5.9.1 Linker to Diluent Ratio

The ratio of PFP-TTTA to HTS was varied between pure PFP-TTTA to half as much PFP-TTTA to HTS, with the following signals observed:

<table>
<thead>
<tr>
<th>Linker ratio (PFP-TTTA-HTS)</th>
<th>Buffer A Fluorescence</th>
<th>LPA Fluorescence</th>
<th>LPA Signal - Buffer A Signal</th>
<th>Imidazole Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:0</td>
<td>0.17 ± 0.06</td>
<td>0.32 ± 0.06</td>
<td>0.15 ± 0.06</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>2:1</td>
<td>0.5 ± 0.2</td>
<td>0.9 ± 0.3</td>
<td>0.4 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>1:1</td>
<td>0.23 ± 0.06</td>
<td>0.27 ± 0.07</td>
<td>0.04 ± 0.07</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>1:2</td>
<td>0.40 ± 0.05</td>
<td>0.61 ± 0.04</td>
<td>0.21 ± 0.05</td>
<td>4.3 ± 0.4</td>
</tr>
</tbody>
</table>

Although none of the signals were as high as the initial conditions, direct comparison of linker ratios is possible since all the ratios were done with otherwise identical procedures. As can be seen from the above data a ratio of 1:2 linker:diluent ratio produced the highest signal for the imidazole control. Although the signal produced for LPA after subtracting out the negative control of buffer A was slightly lower than the signal observed for a 2:1 ratio, the error was much lower making this ratio more reproducible and thus better for a test. The reason for the 1:2 linker:diluent ratio being superior to pure linker or a 1:1 ratio could be due to protein packing on the surface. Having slightly less linker, and thus Ni-NTA, on the surface could allow for better spacing between proteins preserving their accessibility and function.

5.9.2 Silica Gel Pore Size

Another possible change in the solid support was the pore size of the silica gel used. To see which pore size is preferable silica with a 60 Å pore size was compared to 150 Å pore size under otherwise identical preparations using a 1:2 PFP-TTTA-HTS linker ratio.
Table 4 - Fluorescence results for varying silica gel pore size done in buffer A.

<table>
<thead>
<tr>
<th>Pore Size</th>
<th>Buffer A Fluorescence</th>
<th>LPA Fluorescence</th>
<th>LPA Signal - Buffer A Signal</th>
<th>Imidazole Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 Å</td>
<td>4.2 ± 0.5</td>
<td>3.0 ± 0.5</td>
<td>-1.2 ± 0.5</td>
<td>9.8 ± 0.6</td>
</tr>
<tr>
<td>150 Å</td>
<td>0.6 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>3.8 ± 0.9</td>
</tr>
</tbody>
</table>

From the fluorescence results of a column test it can be seen that silica gel with a pore size of 150 Å is preferable to a pore size of 60 Å. This is partly due to a large reduction in non-specific adsorption of the protein complex, evidenced by the much larger signal of 4.2 fluorescence units for the negative control with 60 Å, where under the same conditions a signal of only 0.6 was achieved for 150 Å. Although a larger signal was also seen for the LPA test conditions, this signal is completely swamped by the negative control in the case of silica gel with 60 Å pores. Although the imidazole signal is higher in the case of 60 Å silica gel this signal is most likely due to non-specifically adsorbed proteins as evidenced by the negative control signals.

The increase in non-specific adsorption for 60 Å silica gel could be in part due to the large size of the full gelsolin-actin complex. The complex has a length of 146 Å across its longest side when one actin is bound, making it unable to enter the pores of the 60 Å silica gel. The smaller pores could cause the complex to break apart, allowing the smaller actin, which has a width of 55 Å, to enter the pores and remain on the silica gel in a non-specific manner. As such the larger pores of 150 Å silica gel would allow the entire complex to enter the pores and bind the protein complex specifically, reducing the negative signal in the absence of LPA. Admittedly this is purely speculative and no surface analysis of the silica gel has been performed to determine the cause of the non-specific adsorption.

5.9.3 Plasma cleaning time

The length of time the silica gel is initially plasma cleaned was also investigated to determine the necessity of plasma cleaning the silica and how long this should be done for. Columns were prepared with a 1:2 PFP-TTTA:HTS ratio and 150 Å silica gel.
Table 5 - Fluorescence results for varying silica gel plasma cleaning time done in buffer A.

<table>
<thead>
<tr>
<th>Plasma cleaning time</th>
<th>Buffer A Fluorescence</th>
<th>LPA Fluorescence</th>
<th>LPA Signal - Buffer A Signal</th>
<th>Imidazole Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 minute</td>
<td>1.2 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>-0.4 ± 0.5</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>10 minutes</td>
<td>0.7 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
</tbody>
</table>

The results show that a longer plasma cleaning time is preferable due to not only a higher test signal, but also a reduction in non-specific adsorption of the protein complex, as shown by the higher buffer A signal and lower LPA signal observed for 1 minute of plasma cleaning versus 10 minutes. This is likely due to insufficient hydroxylation of the surface of the silica gel when plasma cleaned for only 1 minute. The lack of hydroxylation would limit the ability of the linker and diluent to bind and form an adlayer, which would also leave patches of bare silica gel exposed. This could increase the amount of non-specific adsorption of the proteins, which causes any signal from LPA to be masked by the negative control. Thus a plasma cleaning time of at least 10 minutes should be used in the production of these tests.

5.9.4 Linker nature

Once new batches of MEG-Cl could be synthesized in high purity tests using MEG-Cl as the linker were compared to those using PFP-TTTA. The other conditions used were the best conditions previously established, with the spacer being HTS.

Table 6 - Fluorescence results for varying linker structure done in buffer A.

<table>
<thead>
<tr>
<th>Linker</th>
<th>Buffer A Fluorescence</th>
<th>LPA Fluorescence</th>
<th>LPA Signal - Buffer A Signal</th>
<th>Imidazole Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFP-TTTA</td>
<td>0.3 ± 0.2</td>
<td>0.0 ± 0.2</td>
<td>-0.3 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>MEG-Cl</td>
<td>1.2 ± 0.2</td>
<td>2.2 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>2.3 ± 0.5</td>
</tr>
</tbody>
</table>

MEG-Cl as a linker outperformed PFP-TTTA in the signal achieved from buffer A containing LPA by a fairly large margin, with a signal of 1.0 ± 0.3 versus -0.3 ± 0.2 which has a p-value of 0.01 (df = 3). This could be due to the structure of MEG-Cl, with the interior ether which provides an area for water to bind into the layer,\textsuperscript{114,146} as well as the more reactive nature of
MEG-Cl towards ab-NTA allowing for better coverage of Ni-NTA on the surface of the silica gel.

With MEG-Cl as the linker we see a lower signal for the positive imidazole control than we do when PFP is the linker. Although this would suggest that we should see improved performance in the presence of LPA, the opposite is true. The LPA signal observed when MEG-Cl is used as the linker is almost as high as the positive control, suggesting that under the tested conditions that 25 μM LPA can saturate the test. Unfortunately this signal is still quite small, so further improvements to the silica surface need to be made.

5.9.5 Gelsolin versus Gelsolin 1-3

As was discussed previously the first 3 domains of gelsolin, referred to as gelsolin 1-3, are also capable of binding actin and releasing it in the presence of LPA. As such another area of interest in improving the surface binding of the protein complex is the difference between gelsolin and gelsolin 1-3 as the anchor protein. The difference in binding and LPA response between gelsolin and gelsolin 1-3 on the surface was thus investigated. This was done using PFP as the linker, and HTS as the spacer in a 1 to 2 ratio.

Table 7 - Fluorescence results for varying gelsolin construct done in buffer A.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Buffer A Fluorescence</th>
<th>LPA Fluorescence</th>
<th>LPA Signal - Buffer A Signal</th>
<th>Imidazole Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelsolin</td>
<td>0.6 ± 0.3</td>
<td>0.2 ± 0.2</td>
<td>-0.5 ± 0.3</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Gelsolin 1-3</td>
<td>0.6 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.3 ± 0.2</td>
<td>2.7 ± 0.2</td>
</tr>
</tbody>
</table>

As can be seen both proteins exhibit the same amount of non-specific adsorption as evidenced by the same fluorescence being measured for their negative buffer A controls. However a larger signal was observed for both LPA and the positive imidazole control when gelsolin 1-3 was the bound protein than when full length gelsolin was used. This could be due to the smaller size of gelsolin 1-3, allowing it to better pack onto the surface of the silica.

5.9.6 Spacer Nature

Another area of interest in the surface modification was the nature of the spacer. As MEG-Cl was determined to be a better linker than PFP-TTTA, possibly due to the presence of its anti-fouling ether group, a spacer with the same ether was chosen. This spacer is known as MEG-OH...
and has the same structure as MEG-Cl with an alcohol group instead of an acid chloride. Since the alcohol group would be reactive to the trichlorosilane, MEG-OH is prepared with a TFA protecting group that is removed by incubation in ethanol for 1 hour following surface deposition of the adlayer.

Table 8 - Fluorescence results for varying spacer structure done in buffer A.

<table>
<thead>
<tr>
<th>Linker</th>
<th>Buffer A Fluorescence</th>
<th>LPA Fluorescence</th>
<th>LPA Signal - Buffer A Signal</th>
<th>Imidazole Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTS</td>
<td>2.1 ± 0.4</td>
<td>2.3 ± 0.3</td>
<td>0.2 ± 0.3</td>
<td>13.8 ± 0.7</td>
</tr>
<tr>
<td>MEG-OH</td>
<td>2.4 ± 0.3</td>
<td>2.7 ± 0.3</td>
<td>0.3 ± 0.3</td>
<td>4.0 ± 0.3</td>
</tr>
</tbody>
</table>

As can be seen HTS as a spacer showed slightly lower non-specific adsorption of proteins than MEG-OH, which is surprising as MEG-OH should act as an anti-fouling group. The mix in structures, combining the ether of MEG-Cl with the alkyl of HTS, could provide better anti-fouling properties on the surface; however this was not otherwise investigated.

It can also be seen that MEG-OH produced a slightly larger signal for LPA, though the LPA results were within error of each other, but a much lower signal for the positive imidazole control. This suggests that the mixed adlayer of MEG-Cl and HTS allows for a much larger amount of protein to specifically bind to the surface; however there does not seem to be a direct link between the amount of protein specifically present and the signal obtained from LPA. Further investigation as to why this is the case should be explored, but the accessibility of the proteins to LPA as well as the surface's own binding to LPA could be the cause of this discrepancy.

The smaller positive control signal could also be due to destruction of the acid chloride group of MEG-Cl during deprotection of the TFA group, which would prevent reaction with ab-NTA to complete the linker formation. As such HTS provides an advantage over MEG-OH in that it does not require additional steps in the preparation of the solid phase, increasing efficiency and reducing the likelihood of surface destruction.

5.9.7 Conclusions of Early Solid Phase Modifications

From the direct comparison experiments performed in Buffer A, detailed in this section, there are some conclusions that can be drawn regarding the optimal solid phase in the detection of LPA.
Firstly the linker should be added to the surface with a spacer in order to obtain a better signal. The nature of this spacer should be a straight chain alkyl group, while the linker performs best when it is comprised of an ether group in the chain and an acid chloride head group. Additionally any pores in the surface need to be large enough to fit the protein complex, which itself is very large, otherwise the amount of protein present in the column is significantly limited. Along the same lines, the smaller gelsolin 1-3 protein complex outperforms the larger full length gelsolin complex, which is possibly due to the ability to pack in greater density on the surface. Finally, it is important to ensure the surface is sufficiently hydroxylated to allow for surface adlayer deposition. Although these tests were all performed on silica gel, it is likely that the results extend to other types of surfaces, such as magnetic nanoparticles, and biosensor chips.

5.10 Analysis of Flow-through Methods

In the previous section the tests were performed by flowing 1 mL of a sample through the prepared silica gel column, and measuring the fluorescence of the flow through all at once (Fig. 66). The silica gel itself most likely traps some of the sample, which if collected may increase the fluorescence signal observed. It may also be that most of the measured actin flows through the column in the first portion of the sample, and that by using too much sample the fluorescence signal could be diluted. To determine the location of the protein in the eluate flow-through of the column should be investigated. As the fluorometer used in these experiments does not support flow-through of sample, instead low volume fractions of the sample were collected as it flowed through, each of which then had its fluorescence measured.

5.10.1 Passing Through LPA or Imidazole First

Two columns containing 0.5 mL silica gel prepared from the same batch using the best methods determined in the previous section were prepared. The following solutions were flowed through the columns and had their fluorescence measured:

<table>
<thead>
<tr>
<th>Flow Order</th>
<th>Column 1</th>
<th>Volume</th>
<th>Signal</th>
<th>Column 2</th>
<th>Volume</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer A</td>
<td>750 μL</td>
<td>1.5 ± 0.1</td>
<td>25 μM LPA</td>
<td>750 μL</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>Imidazole</td>
<td>750 μL</td>
<td>6.0 ± 0.5</td>
<td>Buffer A</td>
<td>750 μL</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>25 μM LPA</td>
<td>750 μL</td>
<td>12.4 ± 0.8</td>
<td>25 μM LPA</td>
<td>750 μL</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>Buffer A</td>
<td>750 μL</td>
<td>0.4 ± 0.3</td>
<td>Imidazole</td>
<td>750 μL</td>
<td>7.7 ± 0.4</td>
</tr>
</tbody>
</table>
As can be seen from the first column, when only 750 μL of sample is used there is a significant amount of fluorescence signal trapped on the column following the imidazole sample. This can be seen by the very high fluorescence found for the sample containing LPA passed through the column immediately following the sample containing imidazole, which had a fluorescence of 12.4 fluorescence units, which is more than twice the fluorescence seen from the imidazole sample. Although it's possible the signal originates as a result of LPA interactions with the gelsolin actin complex, this is unlikely due to the signal being much higher than ever previously observed as well as the small signal seen when sample containing LPA was first passed through the second column. Overall this suggests that when a sample volume of 750 μL is used, some of the released protein is still trapped on the column due to the proteins’ mobility through the column.

In order to test if there is indeed released actin still within the column when only 750 μL of sample is used another 2 columns were prepared in the same manner as the above columns. These columns each had 750 μL buffer A flowed through them, followed by 750 μL imidazole control which then had their fluorescence measured. One of the columns then had buffer A passed through it, while the other had buffer A with LPA passed through it, with the following results:

<table>
<thead>
<tr>
<th>Flow Order</th>
<th>Column 1</th>
<th>Volume</th>
<th>Signal</th>
<th>Column 2</th>
<th>Volume</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer A</td>
<td>750 μL</td>
<td>1.7 ± 0.4</td>
<td>Buffer A</td>
<td>750 μL</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>Imidazole</td>
<td>750 μL</td>
<td>4.0 ± 0.3</td>
<td>Imidazole</td>
<td>750 μL</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>Buffer A</td>
<td>750 μL</td>
<td>11.0 ± 0.3</td>
<td>25 μM LPA</td>
<td>750 μL</td>
<td>10.4 ± 0.7</td>
</tr>
</tbody>
</table>

In this case it can be seen that passing a sample containing imidazole through the column before passing through the sample containing LPA results in no observable signal increase by the LPA. Although it appears that the LPA signal at the end of column 2 is lower than the buffer A signal at the end of column 1, the two are statistically similar due to the error of each. However any slight difference can be explained by the higher imidazole signal from column 2 than column 1, resulting in less protein complex left on the column to end up in the LPA solution. This result was most likely caused by differences in flow rate and slight differences in column trapping volume. Unfortunately as a possible solution to increase the measured signal from LPA in buffer
samples flowing through solution containing imidazole either before or after the LPA results in no signal difference than if buffer A lacking LPA is used.

5.10.2 Location in Sample Volume of Fluorescence Signal

As with the above tests the lack of a flow through fluorometric set-up limits the ability to test where in the sample solution the labeled actin ends up, as a flow-through set-up would enable the generation of a fluorescence vs. volume plot which could be used to determine the exact trapping volume of the silica gel, and the mobility of the actin through it. However using smaller fractions of 500 μL and collecting multiple fractions of the same solution this can be tested.

<table>
<thead>
<tr>
<th>Flow Order</th>
<th>Column 1</th>
<th>Volume</th>
<th>Signal</th>
<th>Column 2</th>
<th>Volume</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer A</td>
<td>750 μL</td>
<td>1.1 ± 0.3</td>
<td>Buffer A</td>
<td>750 μL</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>Imidazole</td>
<td>500 μL</td>
<td>0.8 ± 0.3</td>
<td>25 μM LPA</td>
<td>500 μL</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>Imidazole</td>
<td>500 μL</td>
<td>7.4 ± 0.4</td>
<td>25 μM LPA</td>
<td>500 μL</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>Imidazole</td>
<td>500 μL</td>
<td>6.8 ± 0.3</td>
<td>25 μM LPA</td>
<td>500 μL</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>Imidazole</td>
<td>500 μL</td>
<td>5.3 ± 0.5</td>
<td>25 μM LPA</td>
<td>500 μL</td>
<td>0.2 ± 0.3</td>
</tr>
</tbody>
</table>

As with the previous experiments a negative control consisting of 750 μL of buffer A was passed through the column to determine the amount of non-specifically adsorbed proteins before passing through the desired samples.

Using buffer A containing imidazole as the positive control in the first column it can be seen that little to no protein comes off the column in the first 500 μL of sample. Further fractions of 500 μL show a largely increased fluorescence signal, with the second and third fractions displaying very similar signals, and only a small drop off in signal with the fourth fraction. This suggests that the majority of the protein comes off the column between the 500 μL and 1.5 mL of sample passed through the column. Although there is still some signal at between 1.5 and 2 mL of sample through the column it is smaller than the previous fractions so tests using the developed silica gel in a column based set-up should measure the fluorescence of this middle 1 mL of sample to prevent signal dilution, and column trapping of the measured actin. Further fractions were not performed due to the observed drop off in signal.

Unfortunately very little signal was seen in the second column where buffer A containing LPA was used instead of imidazole. This suggests that although LPA is capable of disassociating the
actin-gelsolin complex, and the presence of this complex on the silica gel suggested by the results of column 1, on this column LPA is incapable of releasing the actin in a large measureable amount. This could be due to the time of interaction between LPA and the gelsolin-actin complex, which was found earlier to saturate the UV-visible absorbance of rhodamine after 3 minutes (Fig. 64). It was hoped that despite this, the larger amount of protein available on the silica gel surface would be enough to compensate for the interaction time allowing for more rapid testing, however this does not seem to be the case. Instead experiments should incubate the silica gel with the sample for a few minutes before filtering out the silica gel and measuring the sample fluorescence.

Before moving onto incubating samples, a last flow-through test was performed to look into the idea that the best flow-through signal is measured between 0.5 and 1.5 mL, which was tested with Buffer A against Buffer A with 25 μM LPA:

<table>
<thead>
<tr>
<th>Flow Order</th>
<th>Column 1</th>
<th>Volume</th>
<th>Signal</th>
<th>Column 2</th>
<th>Volume</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer A</td>
<td>500 μL</td>
<td>1.1 ± 0.4</td>
<td>25 μM LPA</td>
<td>500 μL</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>Buffer A</td>
<td>750 μL</td>
<td>0.9 ± 0.2</td>
<td>25 μM LPA</td>
<td>750 μL</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>Imidazole</td>
<td>1 mL</td>
<td>6.3 ± 0.6</td>
<td>Imidazole</td>
<td>1 mL</td>
<td>7.4 ± 0.6</td>
</tr>
</tbody>
</table>

From the above the signal for buffer A the second pass of buffer A through the column results in an apparently lower signal, but the error results in the two not having any statistical difference. The first pass of LPA through the second column shows that a statistically similar signal to the first pass of the buffer A column. The LPA signal from the second pass seems to better maintain its signal, but due to the error present this value is not significantly different from that observed for the second pass of buffer A through its column. As with the previous samples when imidazole was passed through the column that had LPA sample passed through it a seemingly larger signal was observed than when it was passed through the buffer A column, but the resultantly higher errors makes these values not significantly different, so little can be concluded from these experiments. As such a route of incubating the sample with modified silica gel is still preferred, and will be investigated.
5.11 Removal of Toluene

Before moving on to test incubation of the samples with the prepared silica gel, there was an additional step that was added to the procedure. It was noticed in previous preparations of the silica gel that the gel itself retained an odor of toluene even after the addition of the protein complex. It is likely that this is a result of toluene that has remained trapped in the pores of the silica gel due to the hydrophobicity of the MEG-Cl surface (Chapter 3). This remaining toluene may have a negative impact on the reaction between MEG-Cl and ab-NTA, or on the addition of the proteins in the following step. As such methods are needed to remove this toluene.

The step that was added to remove the toluene is to sonicate the silica gel in deionized water for 5 minutes following the wash of the gel in toluene after addition of the surface linker. Although MEG-Cl may be sensitive to reaction with water replacing the acid chloride with a carboxylic acid, this method was still attempted as the acid chloride may be robust enough to remain intact through a short period of sonication.

![Figure 67](image_url)

Figure 67 - Visualization of the fluorescence data for silica gel columns prepared with an additional sonication step in water to remove excess toluene from the surface.

The results of adding in this sonication steps were immediately apparent with a much higher positive imidazole signal than had been seen previously (Fig. 67). As well the signal from 25 μM LPA was 2.1 fluorescence units higher than the buffer A negative control signal, which as
with the imidazole control is higher than had ever been seen previously. As such this step is important for ensuring a large amount of specifically bound protein to the surface, which is required to produce a significant signal for biologically relevant concentrations of LPA.

Another possible method for the removal of toluene is to instead place the silica gel in an oven at 180 °C in order to boil off the toluene, while leaving the acid chloride free from exposure to water until it’s reacted with ab-NTA.

![Bar chart showing fluorescence signal minus blank for Buffer A Blank, Imidazole Control, and Buffer A + 25 μM LPA.](image)

**Figure 68 - Visualization of the fluorescence data for silica gel columns prepared with an additional heating step to remove excess toluene from the surface.**

Heating the silica gel to remove excess toluene from the surface also allowed for a large amount of protein complex to bind to the surface as suggested by the large signal observed for the imidazole control (Fig. 68). This increase was nearly twice as large as what was observed when a water sonication step was used. However the larger amount of protein complex on the surface failed to translate into a larger signal in the presence of LPA, which was just 0.4 fluorescence units larger than the blank buffer A solution, a much lower value than what was observed in the previous experiments.

It should be noted that due to dwindling supplies of the proteins used in the previous experiment, new batches of both gelsolin 1-3 and actin-rhodamine were prepared for this experiment, which may have resulted in some of the observed difference. It is possible that the new batches of protein are less capable of being disrupted by LPA, which would explain the lower than expected LPA signal even with the larger amount of specifically adsorbed protein complex present.
Despite the proteins being prepared using the same procedure, an unknown variable such as cell integrity during protein production causing differences in protein expression is possible.

5.12 Moving Into Serum

Although the fluorescence results at this time of the solid phase modifications were quite small it was decided to move onto working in serum since for this test to be considered for use in clinics it must work in unprocessed serum.

To facilitate this serum was collected from St. Michael's Hospital by pooling together the serum from male patient's test vials where the age of the males was between 18 and 100. Only serum samples that were clear of any visible precipitate were chosen to be combined together, and as many as 50 samples would be combined at once to create a supply of serum for 2 weeks to 1 month of testing. Serum was stored at 4 °C to prevent the formation of precipitate when serum is repeatedly frozen and thawed. The reason for only including male serum samples was to reduce the amount of initial LPA that may be present in the sample, reducing the likelihood of interference in the negative control and a higher than calculated concentration of LPA in the testing samples.

5.12.1 Initial Difficulties in Serum

As a note, in the previous experiments in buffer A the modified silica gel was transferred by volume into 1 mL syringe tubes stopped with a wad of cotton while still in buffer A containing protein. The columns were then washed with deionized water by flowing it through the column. As such when the solution to be tested was added to the column and passed through it the silica gel was still saturated with water.

The first tests when working in serum were prepared in the same way. Unfortunately when serum was passed through columns prepared in this manner a noticeable white precipitate formed in the serum and passed into the fluorescence cuvette before testing. This precipitate made measuring the fluorescence unreliable as it interfered with the paths of excitation and emission light, likely changing the fluorescence results. The precipitate is likely composed of denatured proteins from the serum, however it was not analyzed to determine this.
With these first tests it was found that shaking the serum sample in the fluorescence cuvette after exposing it to the column caused the precipitate to visibly disappear. However, this would be an unreliable method for serum analysis, and as such a different method for preparing the silica gel columns was needed.

5.12.2 Serum Transfer Method

The first attempt to reduce the precipitate formed during testing was what shall be called the serum transfer method. This was done by passing the buffer A that the silica gel is still submerged in following protein addition through the washing column, followed by the addition of a 2:1 volume of serum:silica gel. This solution of serum and modified silica gel was then transferred to the 1 mL syringe tubes, and the excess serum was pushed through the gel to form the testing columns.

The reason for trying this serum transfer method is twofold. Firstly, it is possible that removing the protein complex from solution could result in a denaturation of the proteins effectively destroying the test. As such using serum to transfer the silica gel into the test columns would allow the protein complex to remain in a solution that would not cause precipitate to form on testing. Secondly, the sacrificial transfer serum should work to remove any excess buffer A and non-specifically adsorbed proteins, which should reduce the background signal present from the negative control.
Figure 69 - Fluorescence results of the serum transfer method of test preparation for use on serum.

This method allowed for the test to be performed with minimal precipitate formation in the serum after it was passed through the columns. As such the fluorescence data obtained was more reliable and had lower error than experiments where no serum transfer was performed. The positive control of serum containing imidazole produced a fairly high fluorescence signal, similar to the signal obtained in the final column based experiments in buffer A (Figs. 67 and 69). The signal obtained for serum containing 25 μM LPA was also higher than the negative control of pure serum by 1.6 fluorescence units, though the error results in a p value of only 0.1. Although still quite small with a somewhat low p value, as well as smaller than the signal obtained in the earlier buffer A tests, this result shows that the test has the potential to work in serum.

5.12.3 Vacuum Drying Method

Although it was believed that removing the protein complex from solution could cause problems with denaturation, having the tests made and shipped in serum would also create problems in test production and distribution. As such another method was also tested; which was to dry the prepared silica gel by vacuum filtration in a hirsch funnel. The silica gel would then be transferred into 1 mL syringe tubes as a dried powder. The dried silica gel was transferred by
mass to ensure a consistent amount was placed in each test, with 400 mg chosen as the mass since this is approximately 0.5 mL in volume making these tests comparable to previous efforts.

![Diagram showing fluorescence results for modified silica gel tested in serum following dried vacuum transfer.](image)

**Figure 70 - Fluorescence results for modified silica gel tested in serum following dried vacuum transfer.**

The vacuum transfer method resulted in a slightly lower amount of non-specific adsorption of the protein complex than when the serum transfer method was used, which is shown by the lower fluorescence signal of the blank serum control (Figs. 69 and 70). Additionally there seems to be a greater visible presence of specifically adsorbed proteins on the surface given the higher fluorescence signal obtained for the positive imidazole control than what was obtained from the serum transfer method.

Most importantly however is the signal obtained when LPA is used in the test. The signal obtained for serum containing 25 μM LPA was slightly lower than what was found for the serum transfer method, being 1 fluorescence unit greater than the serum control, which due to the error gives it a p value of only 0.25. With this method another test was performed using a very high concentration of LPA, which was 700 μM, to see what maximum signal could be obtained. A much higher signal of 6.4 fluorescence units above the serum control was obtained, suggesting that this test has quantifiable results, though a calibration curve has yet to be prepared.
5.12.4 Nickel Chloride During Protein Incubation

Another variable that was tested in the production of the modified silica gel for use in serum was the presence or absence of NiCl₂ in the buffer used during protein incubation with the Ni-NTA covered silica gel. The following experiments used 150 Å pore size silica gel with a 1:2 MEG-Cl:HTS layer. The proteins used were gelsolin 1-3 and rhodamine-actin pre-incubated for 1 hour. For one experiment NiCl₂ (2 mg/mL) was added to the protein solution after addition of the silica gel, while no NiCl₂ was added to the other experiment.

Table 13 – Fluorescence results for silica gel tested in serum with NiCl₂ added to the protein incubation buffer.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Blank Fluorescence</th>
<th>After Test Fluorescence</th>
<th>Test Signal - Blank Signal</th>
<th>Increase over serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>4.9 ± 0.4</td>
<td>10.3 ± 0.3</td>
<td>5.5 ± 0.5</td>
<td>0 ± 0.7</td>
</tr>
<tr>
<td>10 μM LPA</td>
<td>4.7 ± 0.5</td>
<td>10.4 ± 0.2</td>
<td>5.8 ± 0.5</td>
<td>0.3 ± 0.7</td>
</tr>
<tr>
<td>25 μM LPA</td>
<td>4.3 ± 0.2</td>
<td>9.7 ± 0.4</td>
<td>5.4 ± 0.4</td>
<td>0 ± 0.6</td>
</tr>
</tbody>
</table>

Table 14 - Fluorescence results for silica gel tested in serum without NiCl₂ added to the protein incubation buffer.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Blank Fluorescence</th>
<th>After Test Fluorescence</th>
<th>Test Signal - Blank Signal</th>
<th>Increase over serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>4.7 ± 0.3</td>
<td>8.3 ± 0.4</td>
<td>3.6 ± 0.5</td>
<td>0 ± 0.7</td>
</tr>
<tr>
<td>10 μM LPA</td>
<td>4.5 ± 0.3</td>
<td>10.9 ± 0.2</td>
<td>6.5 ± 0.4</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>25 μM LPA</td>
<td>4.5 ± 0.4</td>
<td>11.9 ± 0.7</td>
<td>7.4 ± 0.8</td>
<td>3.8 ± 0.9</td>
</tr>
</tbody>
</table>

These experiments showed a small but measureable signal for serum with 10 or 25 μM LPA versus serum with no LPA when NiCl₂ was absent from the protein incubation buffer. However when NiCl₂ was added to the incubation buffer no such signal was observed. The actual fluorescence measured for the LPA containing samples in the tests absent NiCl₂ was not much higher than when in the tests with NiCl₂, but the blank serum solution generated a higher fluorescence signal when NiCl₂ was present during protein incubation. This lends further evidence to the idea that a large background signal as a result of non-specific adsorption of the protein complex is deleterious to the performance of the test, and further work needs to be done to reduce this.
5.12.5 Signal Reduction Testing

At this point all testing has been performed by first binding the protein complex to a solid surface, followed by exposing the solid to buffer or serum and measuring an increase in fluorescence of the tested solution. Although this method is preferred as it is easy to see an increase in signal from a small baseline, the results of the opposite form of measurement where the protein complex is first added to the sample solution followed by exposure to a solid phase to trap gelsolin and any intact complex was also tested for.

As such a known amount of protein complex was added to serum samples with and without LPA and incubated for 5 minutes before measuring the solutions fluorescence. Dried silica gel prepared with 1:2 MEG-Cl:HTS followed by addition of Ni-NTA (400 mg) was then added to the samples and incubated for 1 hour. The silica gel was then filtered from the samples, and the samples' fluorescence was again measured. This was done with gelsolin 1-3 as well as full length gelsolin in the protein complex.

For this experiment LPA should break apart the protein complex resulting in gelsolin without actin binding to the silica gel, while the actin stays in the sample. As such the fluorescence measured should remain close to the initial measured fluorescence. If a sample does not contain LPA, the protein complex should remain intact causing the actin to bind to the silica gel and reducing the fluorescence measured after incubation.
Figure 71 - Fluorescence results for initial column trapping tests. Blue is fluorescence before incubation while orange is fluorescence after incubation.

For each of the experiments there was a drop in fluorescence signal from before to after incubation with Ni-NTA modified silica gel (Fig. 71). Although this drop was noticeable for each run, there was little difference in the amount of signal reduction for blank serum samples versus serum samples with 25 μM LPA. When gelsolin 1-3 was used in the protein complex the difference in amount of drop between no LPA and the presence of LPA was -1.5 fluorescence units, while the difference when gelsolin was used in the complex was 2.7 fluorescence units. Analysis of the errors gives a slightly greater statistical relevance for the gelsolin signal (p value 0.25 for gelsolin 1-3, and 0.1 for gelsolin), but neither of these values is particularly relevant.

In the case of this experiment a higher number means a better preservation of fluorescence, so the experiments using gelsolin as the primary protein performed as expected, while those done with gelsolin 1-3 performed in the opposite way. This is somewhat strange given that gelsolin 1-3 in general performs better than gelsolin in the previously tested column tests. The stronger result for gelsolin could be a result of accessibility, with gelsolin and its two actin binding sites being accessible to LPA in solution before being incubated with the silica gel, allowing LPA to act on the complex and generate a positive signal. This does not explain why a negative signal was seen with gelsolin 1-3, though this negative signal was of low statistical significance.
Moving forward a small calibration curve was attempted using this method of testing with gelsolin as the primary protein. Otherwise the methods were the same as the previous experiment.

![Figure 72 - Fluorescence results for signal reduction experiments attempting a calibration curve. Blue is fluorescence before incubation while orange is fluorescence after incubation.](image)

Again a drop in signal was observed for all tested conditions, but the continued fluorescence of the serum sample suggests not all of the protein is being bound by the silica gel and that too much protein was added to the samples before incubation (Fig. 72). Unfortunately for the samples containing LPA the only one that showed a lower drop in fluorescence versus the serum sample was the 25 μM sample with a 1.2 fluorescence unit lower drop than serum. Both the 10 μM and 50 μM samples showed greater drops than the serum sample, thus there was no observed trend between LPA concentration and the level of drop in sample fluorescence. This suggests that the positive signal observed for the 25 μM LPA sample could be a result of error. As such it is not recommended to perform signal drop experiments for quantifying LPA, and that binding the protein to the solid surface before adding it to the sample is the best way to utilize this test.

### 5.12.6 Test Incubation

As was suggested by earlier data, incubation of the silica gel with the serum samples is likely to increase the measured signal. Up until now this method had not been tried due to the increased
time and labour that it adds to the test. However if a greater signal can be obtained this additional time and labour would be worth it to have a test sensitive to low concentrations of LPA while still solving many of the issues of current LPA testing discussed in chapter 1.

The silica gel for these tests had a pore size of 150 Å and was prepared with a 1:2 MEG-Cl:HTS linker system, which was sonicated in deionized water for 5 minutes following linker addition. To this was reacted ab-NTA and NiCl$_2$ in 2:1 water:pyridine overnight (2 mg/mL). The protein complex of 1:3 gelsolin 1-3:Actin-rhodamine was incubated for one hour and added to the no Ni-NTA covered silica gel. After protein addition the resin was washed with deionized water by vacuum filtration (50 mL), and dried on the hirsch funnel. Each serum sample tested consisted of 1 mL of sample, to which was added 400 mg of dry modified silica gel, and the resulting solution was mixed by rotation for 20 minutes. The solid silica gel was filtered off, and the sample solutions had their fluorescence measured.

![Figure 73 - Fluorescence results for silica gel tests incubated in serum samples for 20 minutes.](image-url)

The incubation of silica gel in the serum samples for a period of 20 minutes proved successful in increasing the measured fluorescence signals for LPA containing samples (Fig. 73). A signal difference of 7.2 fluorescence units between the serum sample containing 25 μM and the serum sample with no added LPA was much larger than the previously obtained largest difference of 1.6 fluorescence units. As can be seen the samples with 50 μM or 100 μM LPA added to them
produced even greater signals than this. The 100 μM LPA sample show a slightly lower fluorescence signal than the 50 μM sample, which suggests a saturation of the test above a 50 μM LPA concentration. This could be due to micelle formation of LPA in the sample as LPA has been shown to have a critical micelle concentration of 50 μM under physiological conditions.\textsuperscript{170}

Considering the data points of 0, 25, and 50 μM LPA from the above data, and discounting the 100 μM sample due to the likelihood that micelle formation affected the observed fluorescence, a near linear relationship was found. Using the error in fluorescence of the 0 μM LPA sample a limit of detection of 6 μM might be possible for the test as currently developed, though further data points would be necessary to conclude this. Although higher than the required LoD of 1 μM to determine the cutoff for women with and without ovarian cancer, it is quite close to this value and further improvements to the test could push the limit of detection down to the required level.

Although these results were very exciting, the protein and linker solutions used to prepare the tests ran out from this experiment. New batches of proteins were prepared, as well as fresh linker, and more testes were made using the same procedure. Unfortunately tests prepared with these new batches of protein and linker weren't able to replicate the large signal seen above. This lead to each protein and MEG-Cl being prepared and tested again. After multiple batches of each ingredient being swapped out in the silica gel preparation and tested against LPA, the following result was the best match to the above experiment:
Although the large signal increase for 25 μM LPA sample is only about half as large as the previous experiment, the large signal observed for 50 μM LPA was successfully reproduced (Fig. 74). The trend also is unfortunately not linear, so a limit of detection cannot be accurately calculated, but the sample containing 10 μM LPA did produce a reasonably large signal compared to the blank sample, which was significantly different from it. The primary change between this set of tests, and those that did not create a sizeable signal for 50 μM LPA was the batches of proteins used.

These experiments show that the protein solutions used are of the utmost importance in determining the success of an LPA detecting tests based on gelsolin 1-3 in complex with actin. Unfortunately the exact reasons why these particular batches of protein were able to produce a measureable signal, while other batches made just before were not is not known. The methods used to produce the proteins were the same in each case, and any differences between them were as a result not known. Further experimentation into the exact nature of the protein batches, and further characterization of the proteins used to produce the test should be investigated.
5.12.7 PBS as Final Washing Buffer

The final wash of the silica gel to remove any non-specifically adsorbed proteins was done with deionized water in previous experiments. A different buffer that more closely resembles physiological conditions could prove to be better at removing these proteins and leaving only proteins that are specifically adsorbed to the Ni-NTA on the surface. This would reduce the signal originating from serum samples with no LPA, potentially lowering the limit of detection for the test. As such PBS buffer (pH 7.4) was used to wash the silica gel after incubation with the gelsolin 1-3 and actin-rhodamine protein complex.

Table 15 - Fluorescence results when PBS buffer was used to perform the final washing of the silica gel.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Blank Fluorescence</th>
<th>After Test Fluorescence</th>
<th>Test Signal - Blank Signal</th>
<th>Increase over serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>6.9 ± 0.2</td>
<td>49.2 ± 0.7</td>
<td>42.4 ± 0.8</td>
<td>0 ± 0.8</td>
</tr>
<tr>
<td>10 μM LPA</td>
<td>6.5 ± 0.4</td>
<td>46.5 ± 0.8</td>
<td>40.0 ± 0.9</td>
<td>-2.4 ± 0.9</td>
</tr>
<tr>
<td>25 μM LPA</td>
<td>6.4 ± 0.2</td>
<td>44 ± 1</td>
<td>37 ± 1</td>
<td>-5 ± 1</td>
</tr>
<tr>
<td>50 μM LPA</td>
<td>6.0 ± 0.4</td>
<td>57.1 ± 0.7</td>
<td>57.2 ± 0.8</td>
<td>8.8 ± 0.8</td>
</tr>
</tbody>
</table>

Although it was hoped that PBS would cause a decrease in non-specific adsorption of the proteins from the above it can be seen that PBS as a final washing solution is inferior to deionized water due to the increase in non-specific adsorption seen. The serum blank with no LPA had a 42.4 fluorescence unit increase after exposure to the silica gel washed with PBS buffer, compared to the 0-7 fluorescence unit increase seen in previous experiments where deionized water was used for the final washing solution.

Due to this high background signal resulting from protein fouling, the signals for LPA are swamped out, and in the cases of 10 and 25 μM LPA samples lower than what was observed for serum. Only sample containing 50 μM LPA produced an increased signal over the blank, but due to the lower signal observed for other concentrations of LPA, this signal cannot be taken as a strong result and instead must be considered due to chance. As such washing the silica gel with PBS or similar buffers following protein addition is not recommended and instead deionized water should be used. Why this is the case is currently unknown, however it could be due to greater solubility of the protein complex in deionized water versus PBS which would result in better removal of non-specifically adsorbed proteins into deionized water.
5.13 New Standard Procedure

From the previous experiments detailed throughout this chapter a new standard procedure for the preparation of a gelsolin-actin complex test could be developed as a baseline for further improvements.

First silica gel with a pore size of at least 150 Å is plasma cleaned under atmosphere for at least 10 minutes to ensure adequate surface hydroxylation. It is then placed in a humidity chamber of 75-80% humidity overnight to saturate the surface with water.

Under a dry nitrogen atmosphere the silica gel is submerged in anhydrous toluene (1.2x volume) in pre-silanized glassware, to which is added MEG:Cl (1 μL per mL toluene) and HTS (2 μL per mL toluene). The solution is then mixed by rotation for 90 minutes to ensure full surface coating by the linkers.

Following this the toluene solution containing excess linkers is filtered from the silica gel, and the silica gel is washed with toluene (~3x volume). The silica gel is then submerged in toluene and sonicated for at least 3 minutes. This toluene is filtered off and the silica gel is again rinsed with toluene (~2x volume).

The silica gel is then submerged in deionized water and sonicated for 5 minutes, followed by removal of this water by filtration. The gel is then rinsed with deionized water (~4x volume), and partially dried under a stream of air.

The silica gel is added to a solution made of ab-NTA and NiCl$_2$ (2-3 mg/mL) dissolved in a water:pyridine mixture (2:1 ratio, ~2x volume of silica gel). The mixture is then rotated overnight to react the ab-NTA with the surface acid chloride groups creating a Ni-NTA surface. The silica gel is then filtered from this solution, rinsed with deionized water (~4x volume), and partially dried under air flow.

A mixture of gelsolin 1-3 and fluorescently dyed actin is then pre-mixed and stored at 0 °C for 1 hour. This mixture is diluted with buffer B (buffer A without ATP) to increase its volume to match the silica gel volume, and is added to the silica gel, and mixed by rotation for 1 hour. The solution containing excess protein is removed from the silica gel by vacuum filtration, and the
now modified silica gel is washed with deionized water (~15x volume). The gel is allowed to dry under vacuum, at which point it is measured out into tests (400 mg each).

To each test is added 1 mL of serum sample, which is incubated under rotation for 30 minutes, though a shorter incubation time may be sufficient this longer time was used to ensure all experiments had the same incubation time. Following this the solid silica gel is filtered off, and colorimetric measurements of the serum is then performed using serum samples no exposed to the silica gel as the instrument blanks.

Future experiments would build off this procedure in the hopes of further improving the performance of this LPA test.

5.14 Fluorescein

Moving forward additional fluorophores were also investigated using the standard procedure above as a baseline. Ideally long wavelength fluorophores with high extinction coefficients would be tested, but these dyes are frequently very expensive. Instead the more common low cost dye fluorescein was looked at in order to keep the price of the test as low as possible, ideally having it function in the cheapest possible production.

5.14.1 Fluorescence Testing of Fluorescein Tests in Serum

Actin was dissolved according to the procedure outlined in chapter 4 as with all previous preparations of the protein. However, when dying the protein fluorescein disodium salt (10 molar equivalents) was added to the actin solution alongside NHS-rhodamine. This dye has an absorbance maximum of 490 nm, and an emission maximum of 513 nm under physiological conditions. This is an area of the spectrum where serum shows some native fluorescence, which was measured to be 150 to 155 fluorescence units depending on the serum sample when the above excitation and emission wavelengths were used.
Figure 75 - Fluorescence measurements of fluorescein in serum samples.

Despite the native fluorescence of serum at the measured wavelengths, a large difference in fluorescence signal was observed between the serum sample and the serum sample with LPA after exposure to the modified silica gel (Fig. 75). The sample containing 25 μM LPA had a measured fluorescence 29 units higher than the sample with no LPA following exposure to the test. The sample with 50 μM LPA showed an even greater signal increase of 34 fluorescence units, suggesting that this test remains able to quantify LPA. Unfortunately the sample containing 5 μM LPA resulted in no significant increase in fluorescence over the blank sample, suggesting that the test as is with fluorescein dye has a limit of detection higher than this value, limiting its ability to be used in ovarian cancer screening.

Part of the reason the signal for lower concentrations of LPA may be indistinguishable could be due to the large amount of non-specifically adsorbed protein interfering with the signal. This is suggested by the very high signal increase of 126 fluorescence units measured for the serum sample with no LPA between the initial fluorescence measurement and the measurement taken following exposure to the silica gel. As such another set of tests was prepared using the same method and proteins as the previous experiments, but doubling the amount of water used to wash the silica gel following protein addition (~30 times volume).
Figure 76 - Fluorescence measurements of fluorescein in serum samples with additional washing of silica gel before testing.

Oddly when the silica gel was washed with twice the volume of deionized water the fluorescence observed after exposure of the serum samples to the silica gel ($\lambda_{ex}$ 490 nm, $\lambda_{em}$ 513 nm) was lower than the fluorescence observed before exposure (Fig. 76). A drop in fluorescence like this had never been observed when analyzing rhodamine, which is a longer wavelength fluorophore, under any of the conditions that the silica gel was prepared.

This drop in observed fluorescence could be a result of the silica gel trapping native serum proteins that happen to fluoresce at the same wavelength as fluorescein, resulting in a reduced signal after exposure to the silica gel. As such a larger amount of fluorescein, even if it is non-specifically adsorbed to the silica gel surface is necessary to overcome this potential trapping action of the silica gel and produce an increase in fluorescence following testing.

Despite the decrease in fluorescence a trend of increasing fluorescence was still observed from 5 to 50 μM LPA containing serum samples, furthering the idea that this test is capable of quantifying LPA, as each value was significantly different from the others, and became less negative as the LPA concentration was increased. Oddly though the serum blank does not fit this trend, with a fluorescence decrease that ends up between the 25 and 50 μM samples’ fluorescences. It is unknown why this was the case, as the other values observed have an expected trend for the action of this test.
5.14.2 UV-Visible Analysis of Fluorescein Tests in Serum

Silica gel tests were prepared with the same method as that used for the first fluorescein fluorescence tests outlined in the previous chapter. For these tests the exposed serum was analyzed by UV-Visible analysis between 450 and 600 nm to see what effect the fluorescein and rhodamine dyed actin had on the absorbance spectra of the serum. The instrument was blanked for each test using the same serum sample before exposing it to the modified silica gel.

A trend can be seen where increasing concentrations of LPA in the serum causes an increase in UV-visible absorbance at wavelengths below 510 nm, with 25 and 50 μM samples flipping above 510 nm (Fig. 7). The samples containing 25 and 50 μM LPA giving very similar absorbance spectra, and the sample containing 5 μM LPA between those samples’ and the serum blank’s absorbance spectra. The absorbance as a result of rhodamine is small, but visible in all conditions as a bump between 530 and 560 nm, which shows dyed actin is being removed from the silica gel by LPA.

Oddly, for all of the samples the measured absorbance at 490 nm is quite low, helping to explain the odd fluorescence results obtained where fluorescence after exposure to the test was lower than before testing when excess protein was washed from the silica gel. Whatever compounds in native serum absorb light at 490 nm, and possibly contribute to serum’s native fluorescence, are being removed from the serum by the silica gel as suggested by the serum absorbance going below zero versus untested serum at this wavelength.
Figure 77 – UV-Visible absorbance spectra for serum exposed to silica gel modified with actin-rhodamine-fluorescein and gelsolin 1-3.
5.14.3 Conclusions of Fluorescein Tests

What these experiments showed was that shorter wavelength fluorophores be used in the dyeing of actin, and used in this type of test, a larger amount of non-specifically adsorbed protein to the surface is desired to produce positive results for detecting LPA. This is actually the opposite of what was observed at longer wavelengths with rhodamine as the dye, where a smaller amount of non-specifically adsorbed protein resulted in a better performing test. Despite the stronger fluorescence of actin dyed with fluorescein versus actin dyed with rhodamine, the longer wavelength dye is better for use in this test due to reduced interference of native serum proteins on the measured fluorescence at those wavelengths.

5.15 Notes on Gelsolin Production

Part of the reason for testing fluorescein in the previous section of this chapter was that during the final year of this PhD any tests produced using previously successful methods no longer responded to LPA in either buffer or serum samples, a problem alluded to in section 5.10.5. The reason for this was likely the degradation of the cells used to produce gelsolin and gelsolin 1-3 for these experiments. Unfortunately this possibility was only discovered after months of work had gone to waste. This was determined through a long period of testing new batches of linker, actin, the dyes used, the silica gel, and gelsolin, which are not detailed in the previous sections as they do not add to the results of the work performed. As such any future work in this lab should begin by creating new cell lines for the production of gelsolin and gelsolin 1-3.
Chapter 6

Conclusions and Future Work

A low cost and low labour detection and quantification method for LPA in serum has proven to be a challenging endeavor. The protein complex of actin and gelsolin was found to be sensitive to LPA concentration in buffer and serum samples, leading to a potential testing method for this molecule. Although a truly reproducible test with a low limit of detection was not achieved in this work, further study of this system should allow for such a test to be developed and commercialized.

Although a large amount of time and effort was devoted to the development and use of a transverse shear mode instrument (also known as a quartz crystal microbalance), it was ultimately found to be inadequate for use in serum samples. Even with the application of a strong anti-fouling layer, MEG-OH, a large amount of fouling was observed as a result of serum and LPA. This is possibly due to the degradation of the thiol adlayer in serum, which would allow for a large build-up of fouling material. Although TSM instruments based on standard quartz crystals with gold electrodes are useful in the lab for studying buffer solutions, extending these instruments into the clinical laboratory for use in messy biological fluids does not seem feasible at this point. As such the focus of this work was shifted to systems already in place in the clinical laboratory, namely colorimetric analysis.

In order to work in a colorimetric system a solid phase capable of binding to the gelsolin-actin complex needed to be developed. In order to do this a surface linker capable of binding to a variety of molecules needed to first be produced. The designed molecule was based on MEG-OH, an anti-fouling trichlorosilane molecule capable of forming SAMs resistant to degradation with any hydroxylated surface. Since gelsolin protein could be produced with a histidine tag, a surface composed of Ni-NTA would be ideal for binding the protein complex. NTA is commonly available as a lysine construct, giving it a reactive amine tail. As such the linker was developed to have an acid chloride group terminal to the trichlorosilane, which could not only be reacted with amine groups, but could be extended to other reactions and functionalities. The linker which was denoted as MEG-Cl was produced in high purity and moderate yield. Spectral analysis of the linker showed the expected structure was present. Surface analysis of the linker
binding to silicon oxide, and its subsequent reaction with Ni-NTA, showed that the desired layer was formed and could be successfully reacted with ab-NTA generating the desired Ni-NTA surface for gelsolin binding. Further analysis of the linker’s long term stability both in solution and on surfaces should be investigated.

Beyond this the binding of the linker to additional materials should be studied using such surface techniques as XPS and contact angle. Although it is expected that the linker can form adlayers on a variety of materials, this has not yet been investigated due to the time and cost involved in such studies. As well additional chemistries of the linker with other functional groups reactive to acid chlorides should be investigated. Although not explored in this work due to a different goal, MEG-Cl could prove useful in applications beyond protein immobilization. Finally the anti-fouling nature of the linker should be investigated. Although the linker inherits the structure of MEG-OH, which is very effective at preventing non-specific adsorption, this has not yet been tested with regards to MEG-Cl.

Moving beyond the linker was the development of the gelsolin-actin protein complex and verification of its sensitivity to LPA concentration. As the lab that this work was performed in was not set-up for protein production, this stage of the work took quite a bit of time. Firstly plasmids each containing the gene for gelsolin and gelsolin 1-3 had to be procured, as they could not be produced in this lab. Prof. Robert Robinson of the University of Singapore was kind enough to supply these plasmids, however this process still took several months, delaying the work that could be done with these proteins. Once in possession of the plasmid production of gelsolin and gelsolin 1-3 on a small scale was successfully performed.

Actin fortunately is available for purchase, though it is in its filamental form and as such needed to first be broken apart. Due to limitations in methods of purification of large proteins available in this lab, a method of breaking apart the filaments without introducing any large molecules was successfully developed, allowing actin to be dissolved in buffered solutions at reasonably high concentrations. Modification of this actin with the fluorescent dyes NHS-rhodamine and fluorescein disodium salt was also successful.

Binding studies between actin and gelsolin as analyzed using non-denaturing PAGE gels showed that not only was gelsolin able to bind dye modified actin, but that the resulting complex was sensitive to LPA concentration in solution. As such the ground work for beginning to develop a
test based on this complex was complete and the test could then be developed. It should be noted that after multiple batches of protein had been produced over the last few years, the cell line used to produce them appears to have degraded somewhat. This was only discovered recently, and as such a new cell line was not produced due to time constraints. Any future work should be done on a novel cell line, which should be regularly analyzed for cell stability and their resulting ability to produce gelsolin or gelsolin 1-3 protein. Further investigation of gelsolin and actin should also be performed, such as through circular dichroism spectroscopy to investigate the secondary structure of the proteins.

Development of a test for LPA based on a gelsolin-actin complex was reasonably successful, though hindered by degradation of the gelsolin producing cell line in the final months of this work. Despite this several conclusions can be made regarding this test:

Firstly, the developed MEG-Cl linker is better for the binding of the gelsolin-actin complex than the previously developed alternative linker PFP-TTTA. However only these two linkers were investigated, and work could be done in the future to investigate additional linker systems. It is recommended that trichlorosilane linkers be the focus of this work, as they are capable of forming adlayers with a large variety of surfaces, giving them a large amount of potential in multiple applications.

Secondly, the solid phase used to immobilize the gelsolin-actin complex needs to not only have a high surface area allowing a large amount of protein to bind, but this surface must be accessible to the large complex. Since gelsolin and actin are both large proteins, any material which relies on small pores to increase the surface area will be unable to bind the intact protein complex, and may in fact break apart the complex resulting in a large amount of non-specifically adsorbed actin on the surface. A surface with pores of 150 Å were found to allow this test to function and respond to LPA, but this is barely larger than the size of the gelsolin actin complex. Surfaces with even greater pore sizes should be investigated, though an inability to purchase silica gel with larger pores prevented this work from being done.

Thirdly, the removal of toluene from the surface following addition of the MEG-Cl adlayer to it is crucial to the function of this test. Contact angle measurements of glass surfaces through each stage of production showed an increasing hydrophobicity of the surface. Unfortunately this likely causes the silica gel to retain toluene throughout the production, evidenced by the
noticeable smell of toluene through test production, but this toluene may hinder reaction of the acid chloride with ab-NTA and eventually protein binding to the surface. Two methods of removing the toluene were explored; one where the solid phase was sonicated in water and the other where the solid phase was heated to 180 °C. Both of these methods allowed for a larger amount of protein to bind to the surface than when the surface was prepared without removing the toluene, but the method using water sonication allowed for better protein interaction with LPA. This may have been due to the gelsolin production issues discussed earlier, as these methods were tested using different batches of protein. Further study of this should be performed using gelsolin produced through new cell lines to ensure its proper structure and characteristics.

Although several more conclusions were made regarding production of this LPA test, the three mentioned above had the largest impact on the tests performance. Additional insights are outlined in chapter five. Continued work on this test should build upon the test production procedure outlined in section 5.11, which was found to provide a reasonably strong fluorescence signal for samples containing LPA.

Despite the advances in test production, a low enough limit of detection for this test to be used in ovarian cancer screening was never obtained. This could be in part due to the limitations of the instrument that was used for serum analysis, which had very high error and was not very sensitive to fluorescence. Better fluorescent dyes that function at higher wavelengths with greater extinction coefficients than rhodamine should be tested to see if a greater signal can be obtained than what was seen with rhodamine dye. As well a more sensitive fluorometer may be enough to see the smaller signal produced by the rhodamine dye.

Additionally alternate forms of detection can be explored such as chemiluminescence. It was a desire in this work to test this method, as it was noted that it is frequently used in clinical analysis, but degradation of the gelsolin cell line, and subsequent difficulty producing a functional gelsolin-actin complex prevented this work from being done. Despite this, the work done by fluorescence spectroscopy gives hope that this test will function even better under chemiluminescence, and lower limits of detection may be achieved once a new line of gelsolin producing cells can be made.
Another important area of the test to investigate is changing the solid phase from silica gel to magnetic nanoparticles, which are commonly used in clinical detection where silica gel is not. The primary reason for using silica gel instead of nanoparticles to begin with is one of cost and the amount required. The only fluorometer that the lab had convenient access to, allowing for multiple tests to be evaluated weekly, required a high volume of sample. As such a large volume of the solid phase test was needed for each experiment, something that would have been very expensive to do with nanoparticles. Since much of the tests production has been explored and optimized, work with magnetic nanoparticles in the future should go faster and require fewer experiments. It is expected that the test will easily transition since nanoparticles with hydroxylated surfaces can be obtained, allowing for their modification with MEG-Cl and eventually immobilization of the protein complex. This should be the next area explored, as it is the most relevant to eventual clinical deployment of the test.

Overall the test developed here is one that has the potential to be used in screening. It is low cost, rapid, requires few reagents and steps, works in serum, and has the potential to be automated. This test therefore solves many of the problems discussed in section 1.4 which currently hinder the adoption of other LPA tests in clinical screening.
References


(16) Cancer Institute, N. Cancer of the Ovary (Invasive): SEER Incidence and U.S. Death Rates, Age-Adjusted and Age-Specific Rates, by Race


http://sdb.db.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi.

(165) Sun, H. Q.; Yamamoto, M.; Mejillano, M.; Yin, H. L. Gelsolin, a Multifunctional Actin


Acid-Induced Amyloid Fibril Formation of β2-Microglobulin in Vitro under Physiological

(171) De La Franier, B.; Thompson, M. Fluorescent Biosensors and Methods for Detection of
Lysophosphatidic Acid for Ovarian Cancer Diagnosis, 2016, PCT/CA2016/050545.


(173) Bhatt, N.; Huang, P.-J. J.; Dave, N.; Liu, J. Dissociation and Degradation of Thiol-
Appendix A - TSM Propeller Code

{ Crystal - Propeller module that measures frequency and voltage analogous to open loop Nose05 and Isys05.

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September 11, 2013

Frequency Counter based on Parallax AN001 (Propeller Counters v1.0)
Serial input routines expunged to save space GLH 01-VI-13
Synchronization ready for circular hub buffer GLH 08-VIII-13
Analog input from 3202B SPI chip GLH 11-VIII-13

CON _clkmode = xtal1 + pll16x
_XinFREQ = 5_000_000

BUFFER_LENGTH = 32 'enough for full screen
BUFFER_MASK = BUFFER_LENGTH - 1 'circular buffer mask

CON
TX_PIN = 30
RX_PIN = 31

CON
PC_BAUD = 38_400 'Optimum Baud Rate for PC application

MODE = %0000
VAR long freq 'frequency variable
    long volt '12 bit voltage
    long time 'run time clock in 1/100 sec
    long output[2]

OBJ

com1 : "FullDuplexSerial"

PUB Go | x, idx

com1.start(RX_PIN, TX_PIN, MODE, PC_BAUD)

waitcnt(clkfreq + cnt) 'wait 1 second

cognew(@counter, @freq) 'start counter in third cog

cognew(@analog, @volt) 'start analog in fourth cog

cognew(@timer, @time) 'start clock in fifth cog

com1.rxflush

repeat
    x:=com1.rxtime(100)
    if x=="a"
        output[0] := freq
        output[1] := volt
        repeat idx from 0 to 7
            com1.tx(byte[@output+idx])
        x:=-1

DAT org

{ Assembly code for run time clock }
timer mov updt, cnt 'setup time delay
    add updt, ticks
:times waitcnt updt, ticks 'wait 100 msec
    add stamp, #1 'increment run time
    wrlong stamp, par 'put time in hub
    jmp #:times

stamp long 0 '0 = now
ticks long 8_000_000 '100 msec delay
updt res 1 'time for next clock update

DAT     org
{ Assembly code for frequency counter. Accumulates counts over 1 second to give Hz. }

counter mov ctra, temp 'establish mode and start counter
    mov frqa, #1 'increment for each edge seen
    mov cnt_, cnt 'setup time delay
    add cnt_, cntadd

:loop waitcnt cnt_, cntadd 'wait for next sample
    mov new, phsa 'record new count
    mov temp, new 'make second copy
    sub new, old 'get delta
    mov old, temp 'set next delta's base
    wrlong new, par 'move delta to hub for display
    jmp #:loop 'do it all again forever

temp long %01010 << 26 + 26 'mode + APIN / temporary
cntadd long 80_000_000 'wait 1 second, answer in Hz
cnt_ res 1 'next time
new res 1 'current edge counter
old res 1 'previous edge counter

DAT org

{ Assembly code for A/D converter. Averages over 1/60 sec so line noise vanishes }

analog or dira, shiftin wz 'establish output lines (z=0)
muxnz outa, enable 'deselect a/d
muxz outa, clock 'clock starts low

:read mov sample, #256 'average 256 readings
mov volts, #0 'clear voltage accumulator
mov nxt, cnt 'get the time now
add nxt, half 'get next time

:smpl muxz outa, enable 'select a/d and start conversion
mov clkcnt, #17 'conversion takes 17 cycles

:conv waitcnt nxt,half 'wait half cycle
test input, ina wc 'read the bit
rcl shiftin, #1 'rotate into shift reg
muxnz outa, clock 'clock up
waitcnt nxt, half 'wait half cycle
muxz outa, clock 'clock down
djnz clkcnt, #:conv

muxnz outa, enable 'deselect a/d - conversion done
waitcnt nxt, half 'wait another clock cycle
muxnz outa,clock 'clock up
and shiftin, mask12 'kill top stuff from shifting
add volts, shiftin 'add the reading to the average
waitcnt nxt, makeup 'fix 60 Hz roundoff
muxz outa, clock 'take clock back down
djnz sample, #:smp

shr volts, #7           'divide average by 256 samples
              'multiply by 2 for 5v scale
wrlong volts, par       'move voltage to hub for display
jmp #:read              'do it all again

shiftin long %1100 << 20     '3202B mask / shift register
enable long %1  << 23       'chip enable mask
clock long %1  << 22         'clock mask
input long %1  << 20         'input mask
mask12 long $FFF           '12 bit mask
half  long 144              'ticks in half a clock
makeup long 168             'add for 60Hz roundoff
nxt  res  1                 'next time
volts res  1                'average voltage
sample res  1               'number of conversions to go
clkcnt res  1               'number of cycles to go

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FOR ANY CLAIM, DAMAGES OR OTHER LIABILITY, WHETHER IN AN ACTION OF CONTRACT, TORT OR OTHERWISE, ARISING FROM, OUT OF OR IN CONNECTION WITH THE SOFTWARE OR THE USE OR OTHER DEALINGS IN THE SOFTWARE.}}
Appendix B - TSM Visual Basic Code

Imports System
Imports System.IO
Imports System.Text
Imports System.Threading
Imports System.Net
Imports System.Net.Sockets
Imports Microsoft.VisualBasic

'Imports System.Web.UI.DataVisualization.Charting

Public Class Form1
    Dim point As Integer
    Dim minutes As Single
    Dim output As String
    Dim tick As Integer
    Dim fs As FileStream
    Dim dt As New DataTable
    Dim maxTime As Single
    Dim path As String

    Dim frequency As Single
    Dim startFreq As Single
    Dim freqDiff As Single

    Dim inbuff(8) As Byte

    Dim voltage As Single
Dim resistance As Single
Dim startRes As Single
Dim resDiff As Single

Private Sub Form1_Load(sender As Object, e As EventArgs) Handles MyBase.Load
    'create datatable for charts to use
    dt.Columns.Add("Frequency")
    dt.Columns.Add("Resistance")
    dt.Columns.Add("Minute")

End Sub

Private Sub StartButton_Click(sender As Object, e As EventArgs) Handles StartButton.Click
    'get filepath and maximum time entered
    path = location.Text & filename.Text & ".txt"
    maxTime = maximum.Text

    'check to see if file already exists, if so prompt user to either overwrite and start run, or rename
    If My.Computer.FileSystem.FileExists(path) Then
        If MsgBox("A file with this name already exists, overwrite it?", MsgBoxStyle.YesNo, "Overwrite File?") = 6 Then
            StartRun()
        End If
    Else
        StartRun()
    End If

End Sub

Public Sub StartRun()
'start communication with parallax board
If Not SerialPort1.IsOpen Then
    Try
        SerialPort1.Open()
        StartButton.Text = "Running"
    Catch ex As Exception
        MsgBox("Port Unavailable!", MessageBoxButtons.OK + MessageBoxIcon.Warning, "Error")
    End Try
End If

'create text file for saving data from run
fs = File.Create(path)

'write header line into text file
Dim info As Byte() = New UTF8Encoding(True).GetBytes("Point, Minute, Frequency (Hz), Resistance (Ohm), Delta Frequency (Hz), Delta Resistance (Ohm)")
fs.Write(info, 0, info.Length)
fs.Close()

'prep chart that will display frequency versus time data from datatable
With FreqChart
    .DataSource = dt
    .Series(0).Name = "Frequency"
    .Series(0).YValueMembers = "Frequency"
    .Series(0).XValueMember = "Minute"
End With

'prep chart that will display resistance versus time data from datatable
With ResChart
    .DataSource = dt
    .Series(0).Name = "Resistance"
Series(0).YValueMembers = "Resistance"
.Series(0).XValueMember = "Minute"
End With

'generate first frequency and resistance values
SerialPort1.DiscardOutBuffer()
SerialPort1.Write("a")

Threading.Thread.Sleep(100) 'give time for the data to arrive at the port buffer
SerialPort1.Read(inbuff, 0, 8) 'read 8 bytes
frequency = BitConverter.ToUInt32(inbuff, 0)
voltage = BitConverter.ToUInt32(inbuff, 3) / 100000
If voltage <= 0 Then
    voltage = 0.0001
End If
resistance = (100 / voltage) - 20

startFreq = frequency
startRes = resistance

'write zero point of data into datatable
Dim dr As DataRow
dr = dt.NewRow()
dr("Frequency") = 0
dr("Resistance") = 0
dr("Minute") = 0
dt.Rows.Add(dr)

'update charts to display current data
FreqChart.DataBind()
ResChart.DataBind()
'establish zero point of time
point = 0
minutes = 0

'write zero point data into text file
output = vbCrLf + CStr(point) + "," + CStr(minutes) + "," + CStr(frequency) + "," + CStr(resistance) + ", 0, 0"
My.Computer.FileSystem.WriteAllText(path, output, True)

'show zero point data in labels to side of charts
PtDisplay.Text = point
MinDisplay.Text = minutes
FreqDisplay.Text = startFreq
ResDisplay.Text = startRes
voltDisplay.Text = voltage

disable start button and enable stop button
StartTime.Enabled = False
calButton.Enabled = False
StartTime.Text = "Running"
StopTime.Enabled = True

'set timer interval to data points per minute entered and start timer to collect and
display updating data
TimerRun.Interval = (60 / Val(DataPoints.Text)) * 1000
TimerRun.Start()
'contact propellor and send letter a
SerialPort1.DiscardOutBuffer()
SerialPort1.Write("a")

'wait for return signal from propellor and collect in variables
Threading.Thread.Sleep(100) 'give time for the data to arrive at the port buffer
SerialPort1.Read(inbuff, 0, 8) 'read 8 bytes
frequency = BitConverter.ToUInt32(inbuff, 0)
voltage = BitConverter.ToUInt32(inbuff, 3) / 100000
If voltage <= 0 Then
    voltage = 0.0001
End If
resistance = (100 / voltage) - 20

'calculate change in frequency and resistance from first data point
freqDiff = frequency - startFreq
resDiff = resistance - startRes

'update data point number and time
point += 1
minutes = (point * TimerCal.Interval) / 60000

'convert time from mins to minutes and seconds
mins = Math.Floor(minutes)
secs = (minutes - mins) * 60

'update labels with current data point
PtDisplay.Text = point
If secs < 10 Then
    MinDisplay.Text = mins & ":0" & secs
Else
    MinDisplay.Text = mins & ":" & secs

End If

'write current data point to text file
output = vbCrLf + CStr(point) + ", " + CStr(minutes) + ", " + CStr(frequency) + ", ", "+ CStr(resistance) + ", " + CStr(freqDiff) + ", " + CStr(resDiff)
My.Computer.FileSystem.WriteAllText(path, output, True)

FreqDisplay.Text = frequency
ResDisplay.Text = resistance
dFreqDisplay.Text = freqDiff
dResDisplay.Text = resDiff
voltDisplay.Text = voltage

'write new row of data into datatable
Dim dr As DataRow
dr = dt.NewRow()
    dr("Frequency") = freqDiff
    dr("Resistance") = resDiff
    dr("Minute") = minutes
    dt.Rows.Add(dr)

'update charts with new data points
FreqChart.DataBind()
ResChart.DataBind()

'stop run if time has reached the maximum time
If minutes >= maxTime Then
    dt.Clear()
    StartButton.Text = "Start Run"
    StartButton.Enabled = True
calButton.Enabled = True
    StopButton.Enabled = False
SerialPort1.Close()
TimerRun.Stop()
fs.Close()
End If

End Sub

Private Sub StopButton_Click(sender As Object, e As EventArgs) Handles StopButton.Click

'check to see if user wants to stop the run and if so stops the timer collecting data
If MsgBox("Are you sure you want to stop the run", MsgBoxStyle.YesNo, "Stop Run?") Then
  dt.Clear()
  StartButton.Text = "Start Run"
  StartButton.Enabled = True
  calButton.Enabled = True
  StopButton.Enabled = False
  SerialPort1.Close()
  TimerRun.Stop()
  fs.Close()
End If

End Sub


'ensures only numbers allowed in datapoints textbox
If Not IsNumeric(e.KeyChar) Then
  e.Handled = True
End If
End Sub


' ensures only numbers allowed in maximum time textbox
If Not IsNumeric(e.KeyChar) Then
    If Not e.KeyChar = Chr(8) Then
        e.Handled = True
    End If
End If

End Sub

Private Sub calButton_Click(sender As Object, e As EventArgs) Handles calButton.Click

If calButton.Text = "Calibrate" Then
    ' start communication with parallax board
    If Not SerialPort1.IsOpen Then
        Try
            SerialPort1.Open()
        Catch ex As Exception
            MsgBox("Port Unavailable!", MessageBoxButtons.OK + MessageBoxIcon.Warning, "Error")
        End Try
    End If
End If

' prep chart that will display frequency versus time data from datatable
With FreqChart
    .DataSource = dt
    .Series(0).Name = "Frequency"
    .Series(0).YValueMembers = "Frequency"
End With
.Series(0).XValueMember = "Minute"
End With

'prep chart that will display resistance versus time data from datatable
With ResChart
  .DataSource = dt
  .Series(0).Name = "Resistance"
  .Series(0).YValueMembers = "Resistance"
  .Series(0).XValueMember = "Minute"
End With

'generate first frequency and resistance values
SerialPort1.DiscardOutBuffer()
SerialPort1.Write("a")

Threading.Thread.Sleep(100) 'give time for the data to arrive at the port buffer
SerialPort1.Read(inbuff, 0, 8) 'read 8 bytes
frequency = BitConverter.ToUInt32(inbuff, 0)
voltage = BitConverter.ToUInt32(inbuff, 3) / 100000
If voltage <= 0 Then
  voltage = 0.0001
End If
resistance = (100 / voltage) - 20

startFreq = frequency
startRes = resistance

'write zero point of data into datatable
Dim dr As DataRow
dr = dt.NewRow()
dr("Frequency") = 0
dr("Resistance") = 0
dr("Minute") = 0 
dt.Rows.Add(dr)

'update charts to display current data
FreqChart.DataBind()
ResChart.DataBind()

'establish zero point of time
point = 0
minutes = 0

'show zero point data in labels to side of charts
PtDisplay.Text = point
MinDisplay.Text = minutes
FreqDisplay.Text = startFreq
ResDisplay.Text = startRes
voltDisplay.Text = voltage

'disable start button and enable stop button
StartButton.Enabled = False
calButton.Text = "Stop Calibration"

'set timer interval to data points per minute entered and start timer to collect and
display updating data
TimerCal.Interval = (60 / Val(DataPoints.Text)) * 1000
TimerCal.Start()
Else

dt.Clear()
calButton.Text = "Calibrate"
StartButton.Enabled = True
calButton.Enabled = True
StopButton.Enabled = False
SerialPort1.Close()

TimerCal.Stop()

End If

End Sub

Private Sub TimerCal_Tick(sender As Object, e As EventArgs) Handles TimerCal.Tick

Dim mins, secs As Integer
Dim freqPrev As Single
Dim checkCal As Single

freqPrev = frequency

SerialPort1.DiscardOutBuffer()

SerialPort1.Write("a")

Threading.Thread.Sleep(100) 'give time for the data to arrive at the port buffer

SerialPort1.Read(inbuff, 0, 8) 'read 8 bytes

frequency = BitConverter.ToUInt32(inbuff, 0)
voltage = BitConverter.ToUInt32(inbuff, 3) / 100000

If voltage <= 0 Then
    voltage = 0.0001
End If

resistance = (100 / voltage) - 20

checkCal = frequency - freqPrev

If checkCal < 0 Then
    checkCal = checkCal * -1
End If

If checkCal > calTol.Text Then
    StartButton.BackColor = Color.Red
Else
    StartButton.BackColor = Color.Green
End If

'calculate change in frequency and resistance from first data point
freqDiff = frequency - startFreq
resDiff = resistance - startRes

'update data point number and time
point += 1
minutes = (point * TimerCal.Interval) / 60000

'convert time from mins to minutes and seconds
mins = Math.Floor(minutes)
secs = (minutes - mins) * 60

'update labels with current data point
PtDisplay.Text = point
If secs < 10 Then
    MinDisplay.Text = mins & ":0" & secs
Else
    MinDisplay.Text = mins & ":" & secs
End If
FreqDisplay.Text = frequency
ResDisplay.Text = resistance
dFreqDisplay.Text = freqDiff
dResDisplay.Text = resDiff
voltDisplay.Text = voltage

'write new row of data into datatable
Dim dr As DataRow
dr = dt.NewRow()
dr("Frequency") = freqDiff
dr("Resistance") = resDiff
dr("Minute") = minutes
dt.Rows.Add(dr)

'update charts with new data points
FreqChart.DataBind()
ResChart.DataBind()

End Sub
End Class
Appendix C - Synthetic Schemes

Two-step synthesis of Tert-butyl-3-(2-allyloxy)propanoate (TBAP).

Conversion of TBAP to 3-(2-allyloxy)propanoic acid (APA) via ester hydrolysis.

Original conversion of APA to 3-(2-allyloxy)propanoyl chloride (APC).

Updated conversion of APA to APC.

Hydrosilylation of APC into 3-(3-trichlorosilyl)propoxy)propanoyl chloride (MEG-Cl) under inert atmosphere.
Modification of glass discs to add MEG-Cl and Ni-NTA consecutively. This is a simplified representation that shows only one surface-bound molecule. The asterisks may represent an O atom in a siloxane group (and therefore a connection to another monomer), a surface-bound O atom, or a terminal silanol group.