Developing a Protocol to Fractionate and Identify Cardiac Extracellular Matrix Proteins Crucial to Cardiomyocyte Proliferation Using Size-Exclusion Chromatography

An Honors Thesis for the Department of Chemical & Biological Engineering

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Abstract

Engineered heart tissue is one of the solutions currently being investigated as an option for regenerating and replacing cardiac tissue post-myocardial infarction (MI). The constructs that are needed for this treatment option vary depending on the source of the cells used, the culture conditions and the scaffold material used. Although a number of polymers, both natural and synthetic are being used, decellularized extracellular matrix (ECM) is becoming more important in these studies as it is a blueprint of how nature creates scaffolds. Its usefulness has been found to include promoting cardiomyocyte proliferation, among other advantages as a scaffold biomaterial. The important factors utilized by ECM in its function are not well understood. Therefore this study sought to examine whether the molecular weight of ECM peptides was an important factor in cardiomyocyte proliferation. Using size-exclusion chromatography to fractionate digested ECM, a protocol was designed to separate the peptides by their molecular sizes. The fractions were characterized by concentration and molecular weight, before being coated onto tissue culture plastic (TCP) and studied for how they influence proliferation of cardiomyocytes cultured on them. The results showed statistical significance in the proliferation of cardiomyocytes on the fractions as compared to standards of TCP and collagen I. Overall, there was no significant difference in the number of proliferating cells between the fractions; while a larger percentage of proliferating cardiomyocytes tended to be on the fractions than the standards. Based on these findings, it was concluded that despite there not being one specific peptide size responsible for proliferation, the peptides obtained in fractions 5 to 7 (with molecular weight sizes of 1 kDa to 6.2 kDa) showed a little more potential to promote proliferation than the other fractions.

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1.0 Introduction

1.1 Significance

The healthy adult heart weighs ~300 g and contracts ~100,000 times a day and 3 x 10^9 times in a lifetime, but during a heart attack, or myocardial infarction close to 25% of its left ventricle-beating cells may be lost in a matter of hours (Bouten et al., 2011; Laflamme and Murry, 2011). The American Heart Association estimates that every 34 seconds, one American suffers a coronary event, while one will dies of a stroke approximately every 4 minutes (Go et al., 2012). Both of these conditions fall under the umbrella term of cardiovascular diseases (CVDs), which has been the leading cause of death in the United States every year since 1900, excluding the 1918 influenza epidemic (Goldthwaite, 2007). The ability of the human heart to regenerate tissue after injury or disease has been an issue of debate for more than 150 years (Carvalho and de Carvalho, 2010).

In exploring ways to curb heart disease, it is generally agreed that heart regeneration, if present, is not as significant as in other tissues like skin and bone (Laflamme and Murry, 2011). Although cardiac transplantation has been the golden standard to solve end-stage heart disease, its efficacy is greatly limited by a shortage of donors and by the need for retransplantation or a lifelong commitment to immunosuppressant and other anti-side effect drugs as patients' bodies may reject the new organ (Kriett and Kaye, 1990; Lindenfeld et al., 2004). Other avenues that are being explored to repair and maintain heart function after events like heart attacks include pharmacologic solutions like beta-blockers, and electromechanical interventions like inserting assistive devices like pacemakers and stem-cell therapy (Goldthwaite, 2007; Segers and Lee, 2008). These options however, suffer from the inability to restore function to damaged tissue, eliciting immune responses and their effects have not yet proven to be clinically substantial (Goldthwaite, 2007; D'Alessandro and Micheler, 2010).

The introduction of engineered heart tissue to replace damaged tissue has come about as an alternative solution. Such replacements need to mimic native tissue as much as possible to overcome the challenge of activating an immune response. These constructs need to have additional abilities to contract synchronically with native tissue, have adequate blood supply networks and also respond to the same electrochemical signals (Zimmermann et al., 2004). One application of this idea has been the use of acellular constructs to deliver bioactive molecules to infarcted regions to help target cells to the region (Zhang et al., 2009). Another important application of this technique has been to use 3D scaffolds to deliver cells to damaged regions.

Tissue engineering techniques offer a unique way to improve cell retention by creating an environment that is not only similar in structure and function to native tissue, but one that is also biochemically and electromechanically similar to it as well (Kreutziger and Murry, 2011). In the creation of myocardial tissue, three important factors always have to be considered, namely, the source of the cells to be implanted, the scaffold to be used and the culture conditions (Ye and Black, 2011). Many different cells have been studied, including cardiomyocytes and embryonic stem cells, each with varying pros and cons (Zimmerman, 2001; Huang et al. 2010). The choice of scaffold is pertinent to achieving the goals of a scaffold that is biocompatible and biodegradable, as well as functioning mechanically like native myocardium (Ye and Black, 2011). Lastly, the culture conditions also need to be controlled to ensure the right signals and influences affect the tissue.

In the quest to engineer the ideal construct it has increasingly become obvious that a deeper understanding of the myocardium's own native environment, the extracellular matrix (ECM) is essential. The cardiac ECM forms the structure and framework in which cardiomyocytes live and function. It is a protein rich structure that not only caters to the needs of its resident cells, but also changes and reacts to various stimuli. It's importance in tissue

generation and regeneration is undisputed as whole decellularized ECM has been successfully used as to create bioartificial organs like the heart and liver (Ott et al, 2008; Uygun et al., 2010) The ECM is "nature's ideal biologic scaffold" and is being explored as a natural biomaterial for engineered heart tissue (Badylak, 2007; Benders et al., 2013). Although the individual protein components that make up the ECM are known, little is known about how they function together (Pelouch et al., 1994; Valentin et al., 2009). The creation of an ECM-like scaffold has so far proved impossible as a comprehensive knowledge of the ECM's functions has not been achieved yet (Sreejit and Verma, 2013; Benders et al., 2013).

The fundamental goal of tissue engineering is to be able to isolate specific cells from a patient, culture them on a biomimetic scaffold and then implant the scaffold into the affected area in the patient's body to allow for tissue regeneration (Vacanti and Langer, 1999). Although many cells are currently being studied for EHT, cardiomyocytes have been identified as the ideal cell type because of their natural functional characteristics, but they are difficult to acquire and proliferate (Leor et al., 2005). However, cardiomyocytes are generally considered to be fully differentiated shortly after birth (Zak, 1974) and there is no source of them that is clinically substantial. Fortunately, ECM has been shown to promote cardiomyocyte proliferation and there exists a need to increase cardiomyocyte proliferation for their use in EHT, but since ECM function is still not fully understood, *there exists a need to identify specific peptides or sets of peptides found in the ECM that promote cardiomyocyte proliferation which can be used both in increasing cardiomyocyte populations and also in improving ECM-based cardiac constructs.*

1.2 Goals and Specific Aims

The overall objective of this project was to use size-exclusion chromatography to identify a specific peptide or set of peptides from cardiac ECM that most significantly promotes

cardiomyocyte proliferation. To achieve this objective, the project was broken down into these three specific aims:

The first aim of this project was develop a procedure to fractionate digested ECM by applying size-exclusion chromatography. Size exclusion chromatography is one of the work horses of industry when it comes to separating proteins by molecular weight. It allows for fragments of differing size to elute at different time points through a resin-filled column. Important factors influencing separation include diffusion and bulk flow. We hypothesized that this procedure would fractionate digested ECM fragments by their various sizes, with large peptides eluting first and smaller peptides eluting last. To test this hypothesis, different resins with different fractionation ranges were used.

The second aim of the project was to characterize the peptides by concentration and molecular weight. Since ECM is made up of a variety of proteins, we needed to have a reliable method of determining concentration of each fraction, and for this we used the Bicinchoninic Acid (BCA) assay. Proteins of known molecular weights from a kit were run through the column and their elution profiles analyzed to create a selectivity curve which could be used to determine weights of unknown fragments. Alternatively, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Imperial Staining with a PAGE rainbow molecular weight marker were also used to determine molecular weights. We hypothesized that there would be one or two particular molecular weight ranges with peptides that significantly influence cardiomyocyte proliferation as defined in the third aim.

The third aim of the project was to study the effects of each ECM fraction on cardiomyocyte proliferation. To study this, we coated tissue culture plastic (TCP) with the ECM fractions and cultured neonatal cardiomyocytes on them. Immunohistochemistry was then applied to visualize and quantify the differences between fractions versus standards of: stock

ECM, TCP, Collagen I and Poly-L-Lysine (with and without serum). We hypothesized that one or two fractions would show significantly more cardiomyocyte proliferation when compared with the other fractions and based on the successful fulfillment of the second aim, we would be able to pinpoint a particular molecular weight range.

1.3 Long Term Goal

In the long run, the purpose of this research is to identify a specific peptide or group of peptides that may be used in enhancing cardiomyocyte proliferation for EHT constructs. Identifying these crucial proteins would also go a long way in improving the design of currently used 3D models that use ECM as a scaffold. Additionally, other features of these peptides like charge and hydrophobicity may be explored in future studies to further characterize these peptides.

2.0 Background

2.1 Prevalence and Impact of Cardiovascular Diseases

Cardiovascular or coronary heart disease refers to a set of disorders relating to heart function and blood flow in arteries. Conditions leading to CVDs include diabetes, plaque build-up in arteries (atherosclerosis) and the heart's inability to supply enough oxygenated blood to the body (heart failure). Although the rate of deaths attributed to CVD in the United States has decreased in the years leading up to 2009 (Go et al., 2012), CVDs are still the number one cause of death throughout the world, approximately 30% of all deaths worldwide in 2008 and are estimated to remain the leading cause of death in the next two decades (World Health Organization). In America alone, it is estimated that for the period from 1999 to 2009, 1 in every 3 deaths, or 1 death every 40 seconds was attributed to CVD (Go et al., 2012). Although most CVDs can be avoided by maintaining a healthy lifestyle which includes regular physical activity, a healthy diet and avoiding the use of tobacco, in the United States, CVDs annually account for $312.6 billion of health expenses or 15% of all health expenditures in 2009, which also makes CVD the largest health expenditure, even surpassing all cancer related costs (Go et al., 2012).

Current projections estimate that by the year 2030, 40.8% of the American population will have a CVD-related illness (Go et al., 2012) and 39 states may have obesity rates of over 50% (Trust for America's Health). The cost of CVD-related illnesses, both direct and indirect is also estimated to be more than $1 trillion in 2030 (Go et al., 2012). Thus, it is an undeniable fact that steps need to be taken to address this global pandemic.

2.2 Cardiovascular Disease Pathways

Commonly mentioned CVDs include stroke, atherosclerosis, heart failure and heart attack. During a stroke, arteries in or around the brain either rupture or become clogged causing a portion of the brain to suffer from cerebral anoxia or hypoxia, complete or partial oxygen

deprivation or cerebral ischemia, insufficient blood flow to the brain and the result may be irreversible brain damage. Atherosclerosis involves the buildup of fat and cholesterol along arterial walls leading up to blockages and heart attack or stroke. Heart failure or congestive cardiac failure occurs when the heart is unable to pump enough blood to meet the body's demand for oxygenated blood. In such cases, the heart first compensates by enlarging or pumping faster and the body narrows the blood vessels to build up pressure, but symptoms soon take root in the form of fatigue while performing basic activities like climbing stairs.

Heart attack, or myocardial infarction (MI) occurs when a cumulative amount of fat, cholesterol and other substances build up in coronary arteries, which when they rupture, cause thrombosis (clotting) and the eventual blockage of the artery and cell death in cardiac tissue that relied on the particular artery for oxygenated blood. The human body has some mechanisms to mitigate the effects of MI which include negative remodeling of the damaged tissue and pathological hypertrophy or enlarging of the left ventricle, but neither of these can completely compensate for the damage suffered. The heart eventually becomes susceptible to other fatal maladies like congestive heart failure.

2.3 Cardiovascular Disease Treatment

Myocardium is known to be "one of the least regenerative organs in the body," and with MI able to wipe out 25% of the heart's cardiomyocytes in a matter of hours, the battle for ways to repair myocardium has been raging on for years (Laflamme and Murry, 2011). Given that the average left ventricle of a heart weighs ca. 200 g and that it's made up of approximately 4 billion cardiomyocytes, any treatment of MI would need to find a way to replace at least 1 billion of these cells and ensure that they can contract in harmony with the rest of the myocardium (Murry et al. 2006). For a while, heart transplants served as the only definitive form of treatment but the shortage of donors has greatly inspired the study of alternative solutions (Stock and Vacanti,

2001). Treatments currently under study include stem cell therapy where adult bone marrow-derived stem cells and pluripotent stem cells are injected intravenously, or into coronary arteries or the myocardium (Segers and Lee, 2008) and into damaged areas in the hopes they differentiate into cardiomyocytes. These attempts have yielded some positive results with left ventricular function improving although there are doubts about whether such improvements are permanent or transient (Chen et al., 2004; Collins et al., 2007) and although promising, stem cell therapy has not yet proven to be clinically effective (Adler and Maddox, 2007).

Another avenue of treatment being explored involves the engineering of cardiac tissue which is then implanted into an MI patient's heart to replace damaged tissue. Such constructs need to be similar to native tissue in form, function, vascularity, contractility and electrophysiological performance, but such ideal models are still yet to be created (Zimmermann et al., 2004). Engineered heart tissue (EHT) models can be constructed with cardiomyocytes but due to the inability of these cells to proliferate, other cell lines are being explored, particularly embryonic and adult stem cells which possess great potential for proliferation (Zimmermann et al., 2004). Tissue engineering has benefits over cell therapy in that there are no immunological responses or viral infection troubles as autologous cells are used in construction (Stock and Vacanti, 2001). Since the extracellular matrix plays an important role in the functioning of myocardium, both healthy and diseased, it is also vital in the creation of ideal biomimetic tissue (Kim et al., 2012)

2.4 Tissue Engineering

The field of myocardial tissue engineering offers a lot of promise in the quest to curb the impact of CVDs as such constructs may be used in many applications including the culturing of healthy cells for cell-based therapy and in studies of cell differentiation, organ development and how cells interact with their environment (Chen et al., 2008). The field seeks to imitate nature's

design of the heart by incorporating the 3 essential components viz. cells, extracellular matrix and signaling systems (Chen et al., 2008). Scaffolds serve a number of uses in EHT models as they act as physical supports and also aid cell-cell interactions during tissue formation. The ideal scaffold would be one that is mechanically similar to the native tissue onto which the cells are being transplanted, provides the necessary structural and signaling environment (chemical and biological), does not elicit an immune response and is easily degraded by the body after a set period of time at a rate similar to the formation of new tissue. Different regimens may be utilized in the design of EHTs. These methods include in vitro engineering, which involves creating grafts in a bioreactor and injecting the biomaterials or implanting the scaffolds for in vivo growth, and in vivo engineering, where the scaffolds and cell are implanted at the site of injury to promote regeneration or replacement of damaged tissue. In vitro studies have been found to be useful especially in disease model studies, while in vivo constructs provide more control of the shape, size and development of the replacement tissue, although this means contending with the possibility of eliciting an immune response. Within both fields of EHT, synthetic and natural biomaterials have been studied extensively with regards to their usefulness in creating biomimetic scaffolds, with natural biomaterials being favored mainly due to the reduced risk of immunogenicity.

Synthetic scaffolds have been made from materials like polycaprolactone (PCL) (Eschenhagen et al., 2002), polyurethane (Fujimoto et al., 2007, Fromstein et al., 2008) and poly(glycerol-sebacate) (PGS) (Radisic et al., 2008, Kenar et al., 2010). The advantages offered by synthetic scaffolds are that they can be modified in the laboratory by mixing various polymers and altering scaffold preparation techniques to meet desired mechanical and structural needs. For example, hydrogels have mechanical features similar to native ECM, high water content and offer gel properties to scaffolds which are controllable and possible to reproduce (Wang et al.,

2008). In addition to this, the scaffold's porosity, pore size and degradation time can be controlled, which makes them very attractive options (Rabkin and Schoen, 2002; Kenar et al., 2010; Mirensky and Breuer, 2008).

Unfortunately, synthetic biomaterials are not without problems. The trouble they present is their inability to grow or regenerate tissue also adds to their increased likelihood of sparking an immune response or an aneurysm (Miresnky and Breuer, 2008). In a classical example of this, a teflon patch was seen to induce a fibrotic response and calcification in the pericardium of a patient who had had a pericadectomy 25 years earlier, in addition to obstructive tissue ingrowth , which was only resolved after the patch was removed (Endo et al., 2001). To add to this, synthetic patches are now linked to infective endocarditis and thrombus formation (Shrivastava and Radhakrishnan, 1989; Di Eusanio and Schepens, 2002).

Natural biomaterials, on the other hand, are both biocompatible and biodegradable (Yang et al., 2001). These scaffolds are made with biomaterials that are derived from or similar to native ECM, such as collagen, fibrin and gelatin, or from natural polymers like alginate (Xiang et al., 2006; Yoneno et al., 2005; Leor et al., 2000; Malafaya et al., 2007). Decellularized ECM is also being explored as a natural scaffold (Robinson et al., 2005; Ott et al., 2008; Benders et al., 2013). These biomimetic polymers are either modified existing materials or are synthesized anew (Leor et al., 2000). They offer a wide range of advantages as compared to their synthetic counterparts. For example, fibrin gels are biopolymers that can be manufactured from the patients' own blood, thus reducing risk of immune actions, and its degradation may be controlled (Jockenhoevel et al., 2001). Additionally, natural biomaterials provide a natural framework for cells to adhere, proliferate and differentiate and they possess the ability to coordinate cell migration, growth and organization during tissue regeneration and stabilizing transplanted cells (Chen et al., 2008; Malafaya et al., 2007). Despite the promise held by such biomaterials, they

too are also with some challenges that still need to be addressed. For example, both collagen and gelatin mesh scaffolds have been shown to offer no improvement of ventricular function and are also prone to the challenge of poor vascularization, which is a common problem for 3D tissue constructs (Chen et al., 2008). Other drawbacks to these biomimetic scaffolds are their batch-to-batch variability and weak mechanical properties (Malafaya et al., 2007; Bouten et al., 2011).

Thus, there exists a need to develop more suitable scaffolds and patches which not only meet the basic requirements of biocompatibility, biodegradability, specific mechanical properties and a lack of immunogenicity, but also closely resembles native ECM is its other roles in cellular growth.

2.5 The Extracellular Matrix

In physiological terms, the myocardium is made up of about 30% cardiomyocytes and 70% non-myocytes, while from a volumetric standpoint, cardiomyocytes constitute 75% of cardiac muscle and fibroblasts account for about a third of the total cell count (Zimmermann et al., 2004; Bouten et al., 2011). The extracellular matrix forms the framework and structure in which the cells live and operate in. Malafaya et al. describe it as being a "soft, tough, and elastomeric proteinaceous network that provides mechanical stability and structural integrity to tissues and organs." Myocardial ECM, mainly deposited by fibroblasts, is primarily composed of collagen subtypes, elastin, fibronectin, laminin, proteoglycans and glycoproteins (Leor et al., 2005; Bouten et al., 2011). The collagen (mainly collagen I) and elastin are responsible for providing tensile strength and resilience during the cardiac cycle, while the proteoglycans offer resistance to compression (Badylak et al., 2009; Bouten et al., 2011). Proteoglycans like decorin and heparin sulfate proteoglycans facilitate cell-ECM interactions while glycosaminoglycans like dermatan sulfate and hyaluronic acid are in charge of immobilizing growth factors (Seif-Naraghi and Christman, 2013). Other functions of the ECM include playing host to signaling factors

responsible for angiogenesis, cell migration, proliferation and orientation; in addition it also functions as a "conduit for blood vessels, nerves and lymphatics and for the diffusion of nutrients from the blood to the surrounding cells," (Badylak, 2002; Sreejit and Verma, 2013).

The extracellular matrix has been defined as "nature's ideal biologic scaffold material" and is known to not be static but always in a state of dynamic equilibrium with its surrounding environment (Badylak, 2007; Sreejit and Verma, 2013; Martins-Green and Bissel, 1995). As discussed earlier, it undergoes remodeling following myocardial infarction, but this is just one of the many instances that ECM composition may change. The ECM is composed of varying proportions of proteins during prenatal, post-natal and adult stages of existence as the demands of the heart vary, as well as during pathological conditions of intermittent high altitude hypoxia, over- or under-nourishment and diabetes (Pelouch et al., 1994).

Although the basic components of the ECM have been elucidated, much is still unknown as to how ECM functions in health and disease or in its use as a scaffold (Pelouch et al., 1994; Valentin et al., 2009). Synthesis of ideal biomimetic ECM in the laboratory has so far proved impossible as the characterization of the organization of the structural and functional molecules of the ECM is still lacking (Sreejit and Verma, 2013, Benders et al., 2013). Thus, as Albert Einstein once wrote, "Look deep, deep into nature, and then you will understand everything better," it is clear that if scientists are to design better scaffolds to mimic the myocardiac environment, then a good understanding of nature's example, ECM, is needed.

In a number of studies, ECM has also been shown to promote the proliferation of cardiomyocytes and thus improving numbers of this cell type which is difficult to obtain clinically (Ieda et al., 2009; French et al., 2012; Kuhn et al., 2007; Ott et al., 2007).

2.6 Size Exclusion Chromatography

Size exclusion chromatography (SEC), also called gel filtration (GF) or permeation, is a well-established, classical chemical engineering separation technique. In general, it involves the use of an inert gel, or media which has pores that allow mixtures of macromolecules to be separated based on their hydrodynamic volumes relative to the average pore size (Barth et al., 1994). Large molecules will follow the path of least resistance which normally excludes entering the pores of the media, and thus are eluted out first. Medium-sized and smaller particles elute slower as they diffuse through the pores. The whole process is aided by use of a GF buffer which provides the necessary bulk motion to move the particles down the column and fractions may be collected to produce a chromatogram that may be analyzed via UV-Vis Spectroscopy. Similar to other forms of chromatography, SEC also has a stationary phase (liquid in between the pores) and a mobile phase (liquid outside the particles) (Amersham).

The processes involved in SEC include hydrodynamic and stress induced diffusion, the polarization effect, multipath, enthalpic and soft body interactions (Dondi et al., 2002). Convective mass transport and obstructive diffusion have also been cited as influences in the flow of particles through a column (Potschka, 1993). A number of other factors also influence the resolution, or degree of separation obtained, and these include sample volume, column dimensions, particle size, flow rate, media selectivity and ratio of the sample volume to the column volume (Amersham).

Different types of media are available for use in this procedure ranging from dextrose-based gels like Sepharose to dextran-based gels like Sephadex. Each gel has a unique exclusion limit which is the molecular weight above which all the analyte particles are excluded from entering the gel pores and thus will not be separated (Sigma-Aldrich). In addition to exclusion

limits, other factors considered when choosing media are pressure stability, pH, chemical composition, and particle-size ranges.

2.7 Cells for Tissue Engineering

Presently, a number of cell sources are being studied for their use in tissue engineering applications. These include but are not limited to: cardiomyocytes, embryonic stem cells, mesenchymal stem cells and fibroblasts (Li et al., 1999; Levenberg et al., 2003; Krupnick et al., 2001; Li et al., 2000). Each cell comes with its own set of advantages and disadvantages including the difficulty of controlling differentiation or transdifferentiation into cardiomyocytes (Leor et al., 2005).

Although cardiomyocytes are difficult to obtain and proliferate, they have been identified as the most suitable cell type to use in tissue engineering as they naturally adapt to the same biochemical and physiological setting in cardiac tissue (Leor et al., 2005). Within the study of cardiomyocytes for this purpose, it has also been found that fetal and neonatal cardiomyocytes are better suited for this application as they thrive better than adult cardiomyocytes (Reinecke et al., 1999).

2.8 Summary

The design of ideal biomimetic scaffolds for use in cardiac tissue engineering has been shown to be a viable alternative to the treatment of MI. Studies have shown that the use of ECM in these constructs is advantageous as ECM is an ideal scaffold found in nature. Along with this, it has been shown that not only does ECM work well as a scaffold material, but it also promotes the proliferation of cardiomyocytes. Identifying crucial peptides in the ECM responsible for this proliferation will aid studies into creating better scaffolds and also increasing cardiomyocyte populations available for the scaffolds.

3.0 Methods

3.1 ECM Digestion

The procedure followed here was one that has already been established and tested in the lab. The ECM studied was from adult domestic porcine myocardium obtained from a local butchery and from adult Sprague Dawley rat myocardium, isolated following an approved Tufts University IACUC protocol.

To obtain the rat hearts, adult Sprague Dawley rats were euthanized by carbon dioxide asphyxiation followed by diaphragm perforation and surgical excision of the myocardium using a scalpel. The rat carcass was then frozen and stored for appropriate disposal while the heart was washed twice in 1% phosphate buffered saline (PBS) solution to remove blood. The heart was then cut into small chunks and de-celled in 1% sodium dodecyl sulfate (SDS) solution for 48 hours on an orbital at room temperature, with the SDS being changed every 24 hours. We then rinsed the resulting tissue in a 0.5% of Triton X-100 (Sigma-Aldrich) for another 24 hours. Lastly, the tissue was then rinsed three times in deionized (DI) water for periods of time ranging from 6 to 24 hours, followed by freezing at -20 ºC overnight in DI water. Lyophilization followed thereafter for 24 hours and dry ECM obtained.

Porcine ECM was obtained using a similar procedure, except that the myocardium was obtained without any carcass and a laboratory blender had to be used to chop the heart into manageable sizes.

To create an ECM solution at approximately 10 mg/mL, a solution of 1mg/ml pepsin in 0.1 M hydrochloric acid (HCl) was prepared and added to the dry tissue at a mass ratio of 10:1 (tissue:pepsin). The pepsin was allowed to digest the ECM for 12-24 hours on a stir plate at 350 – 700 rpm, with periodic observations to check on the process. When a semi-clear solution with no visible chunks of ECM present was obtained, we then stopped the pepsin action by adding 1

M NaOH at a volume equal to 10% that of the pepsin-HCl used. The solution was then stored at -20 ºC until needed.

3.2 Phase I: Column Setup and Operation

Modified portions of two protocols were applied to suit the desired experimental setup: Instructions for Sephadex Media (GE Healthcare) and Protein Purification by Gel Filtration Chromatography (Institute of Molecular Development LLC). A Disposable Column Trial Pack (Thermo Scientific) was obtained. Initially, we used Sephadex G-100 (GE Healthcare), a dextran based media. The media, provided in powder form was stated by the manufacturer to swell to 15-20 mL per gram of dry gel. Thus, a mass of 0.553 g was swelled in a filtered buffer of 0.05 M Tris-HCl (pH 6.8) for a 2 column setup of volumes 2.3 cm^3 and 5.5 cm^3. The gel was allowed to swell for 72 hours at room temperature in a volume of buffer equal to the total column volume plus 30%. Supernatant was then decanted and the gel re-suspended in buffer to make a 75% suspension which was then degassed in a vacuum chamber. The slurry was poured into the 2 columns, thus packing the media by gravity flow. The gel was allowed to settle for 20 minutes at the required bed volume.

Samples at volumes equal to 5% and 2% of the total bed volume of the 2.3 cm^3 and 5.5 cm^3 columns, respectively, were prepared. Each sample was run through the column in turn with the time it took for an entire bed volume to flow through being noted. Five fractions were collected from each column and examined using a Nanodrop 2000 spectrophotometer (Thermo Scientific) and absorbance at 280 nm recorded. The fractions were then lyophilized for 5 days and the resulting dry tissue was re-dissolved in 0.348 M acetic at an estimated concentration of 10 mg/ml. We then ran the fractions through an SDS-PAGE gel and Imperial Stain (Thermo Scientific).

3.3 Preparing SDS-PAGE Gels

The procedure we followed for setting up and running gels is one that is also well established in the lab. To make 4 resolving gels at an acrylamide concentration of 10%, the protocol calls for the mixing of 9.96 mL of DI water, 13.5 mL of 1 M tris solution (pH 8.8), 360 µL of 10% SDS solution and 12 mL of 30% acrylamide (manufacturer). A 10% solution of ammonium persulfate (APS) was created by adding 60 mg of solid APS (Sigma Aldrich) to 600 µL of DI water. Four 1.5mm gel plates were set up on the gel casting frame and onto a stand (Bio Rad). When all was set, the final ingredients: 360 µL of 10% APS solution and 36 µL tetramethylethylenediamine (TEMED) (Sigma Aldrich) were then added to the resolving gel mixture, inverted 3 times and then pipetted into the space between the plates. The protocol recommends letting the gel set for 15-30 minutes under a thin layer of 70% isopropyl alcohol. While the gel set, the stacking gel was prepared by mixing 11.04 mL of DI water, 1.875 mL of 1 M tris solution (pH 6.8), 150 µL of 10% SDS solution and 1.95 mL of 30% APS. When the resolving gel had set, the isopropyl alcohol was then poured out; 150 µL of 10% APS and 15 µL of TEMED were added to the stacking gel mixture, inverted thrice and added to the plates, on top of the resolving gel. A 10-well comb was then placed in the stacking gel and after another 15-30 minute wait, the protocol finally calls for the wrapping of the gels in a paper towel and saran wrap and storing overnight in a fridge at 4 ºC.

3.4 Electrophoresis

Each sample was prepared by taking 30 µL of ECM fraction and adding 2.5 µL of 2 M dithiothreitol (Thermo Scientific) (30.85 mg in 100 µL of DI water) and 7.5 µL of 4x sample buffer (3 mL of 1 M tris solution (pH 6.8), 4 mL of 10% SDS, 2mL glycerol, 40 mg bromophenol blue and 2 mL DI water). A sample of ECM stock solution was also prepared. The samples, while closed tightly, were heated on a heat block at 100 ºC for 5 minutes and then

vortexed and centrifuged. Combs were removed from the gels and the gels were loaded onto the vertical electrophoresis apparatus (Bio-Rad). A 1 L solution of 1x running buffer (a 10% solution of 30.3 g tris Base, 144 g glycine, 10 g SDS, all in 1 L DI water and at pH 8.3) was added to the setup, between 2 gels and in the surrounding container to the "2-Gel" line. Each 40 µL sample was loaded into a well using gel loading tips for the pipette, always reserving one well for the ECM stock sample and one for 5 µL of the full range ladder (GE Healthcare). Once the lid was secured, ensuring the correct connections to a power supply, the apparatus was allowed to run at a constant voltage of 100 V for 10 minutes and then ramped up to 200 V for another 30-45 minutes. The gels were stopped and removed when the samples were close to the bottom edge.

3.5 Staining a Gel

For some of the gels, a Zinc Reversible Stain Kit (Thermo Scientific) was used. After electrophoresis, gels were placed in a tray with enough Zinc Stain to cover the gels and placed on a shaker for 10 minutes. The Zinc Stain was then replaced with Zinc Developer for 1-2 minutes. Stain development was then visualized against a dark background. Development was stopped by washing the gels in DI water and then pictures were taken. Zinc eraser was then used to destain the gels on the shaker and then gels were rinsed twice in DI water before running the Imperial Protein Stain.

A modified version of the Imperial Protein Stain (Thermo Scientific) protocol was used. After electrophoresis, gels were placed in clean trays and rinsed thrice in DI water for 5 minutes. The stain bottle was shaken and enough stain to cover the gels was poured into the trays. Gels were then left on a shaker at room temperature for 24 hours. We removed the excess stain and washed the gel once with DI water. The gel was then left in fresh DI water on the shaker at room temperature for another 24 hours and pictures of the gels were taken at the end of this time period.

3.6 Running a Bicinchoninic Acid (BCA) Protein Assay

The Pierce® BCA Protein Assay Kit was used along with the manufacturer's protocol. Standards were created using diluted porcine ECM, rat ECM and bovine serum albumin (BSA) at 2 mg/mL. For a working range of 20-2000 µg/mL, the protocol calls for the following dilution scheme, which was applied to all standards:

Vial	Volume of Diluent (µL)	Volume and Source of Standard (µL)	Final Concentration (µ/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B solution	750
E	325	325 of vial C solution	500
F	325	325 of vial E solution	250
G	325	325 of vial F solution	125
H	400	100 of vial G solution	25
I	400	0	0 (Blank)

Table 1**:** Recipe and concentrations for the BCA assay standard

The diluent used for preparing the standards was the same buffer used in chromatography, 0.05 M Tris-HCl (pH 6.8) and vortexing was done after each dilution. Enough BCA Working Reagent (WR) was prepared using the following formula: (# standards + # unknowns) x (2 replicates) x (200 µL of WR per sample) = total volume of WR required. A WR mixture was made by mixing 50 parts BCA Reagent A with 1 part BCA Reagent B, with both reagents being provided in the kit. Centrifuge tubes enough to hold all the samples and standards were setup and filled with 400 µL of WR. By carefully using a pipette, 50 µL of each standard and unknown was then added to the centrifuge tubes and vortexed. A 96-well plate with a clear bottom was then filled with 225 µL volumes of each sample, each sample having 1 replicate. The plate was then covered and incubated in an oven at 37 ºC for 30 minutes and then cooled to room temperature. Absorbance was then measured at 562 nm on a plate reader. After subtracting the

average absorbance for the blanks, standard curves of average absorbance vs. concentration (in μg/mL) were then plotted and sample concentrations determined.

3.7 Phase 2: Column Setup and Operation

A protocol similar to that used in Phase 1 was applied with modifications adopted from the protocol: Amersham Gel Filtration Calibration Kits Product Booklet (GE Healthcare). A glass column with internal diameter 11 mm and a height of 300 mm (with 250 mL reservoir) was obtained. The Sephadex G-100 gel was weighed out at the mass needed to swell enough to fill the column (~28.5 cm^3) and dissolved in a volume of 0.05 M tris-HCl buffer (pH 6.8) that was 3 times the expected bed volume and allowed to swell in a water bath at 90 ºC for 5 hours. The supernatant was removed and the gel re-suspended in buffer to make a 75% suspension which was allowed to settle for 20 minutes. After excess buffer was removed by suction, the gel was re-suspended in a volume equal to the settled bed volume and was then degassed and allowed to equilibrate to the temperature of column operation (room temperature). A glass column was mounted vertically onto a stand. With the outlet closed, buffer was then poured into the column to a height of 5 cm, followed by the gel suspension, which was poured onto a glass rod whose end touched the inner wall of the column till the gel reached the required bed volume. A 1 cm layer of buffer was pipetted on top of the gel and the gel was allowed to settle for 5 minutes. The outlet was then opened and the column washed with 2 bed volumes of buffer by gravity flow. The outlet was closed and the column inspected using a light illuminating it from behind.

A Gel Filtration Molecular Weight Markers Kit for Molecular Weight 12,000-200,000 Da (Sigma Aldrich) was obtained with 6 proteins: cytochrome c from horse heart (12.4 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa), albumin from bovine serum (66 kDa), alcohol dehydrogenase from yeast (150 kDa), β-amylase from sweet potato (200 kDa) and blue

dextran (2,000 kDa). According to the manufacturer's protocol, each protein was dissolved in 0.05 M tris-HCl buffer (pH 6.8) containing 5% glycerol to the following concentrations:

Protein	**Concentration**
Blue dextran	2 mg/mL
Albumin	10 mg/mL
Alcohol dehydrogenase	5 mg/mL
β-Amylase	4 mg/mL
Carbonic anhydrase	3 mg/mL
Cytochrome c	2 mg/mL

Table 2: Concentrations of standard proteins run through the column

Starting with blue dextran, a sample volume equal to 2% of the bed volume was applied to the column and immediately afterwards, fraction collection began. Small volumes of buffer were gently added to the top of the column while the column ran using a drop pipette. Each fraction collected was ~1 mL and a total of 20 fractions were collected. Similarly, the other proteins were also applied to the column at the same sample volume and speed, and 30 fractions of 1 mL each were collected. The following proteins were mixed and run together on the column: cytochrome c and β-amylase, carbonic anhydrase and alcohol dehydrogenase; the albumin was run separately. The absorbance of each fraction at 280 nm was measured on a spectrophotometer and each fraction was also put through the BCA protein assay to determine protein concentration. Finally a selectivity curve of log molecular weight vs. V_e/V_o was plotted. The fractions were then analyzed via SDS-PAGE and imperial staining.

To fractionate the porcine ECM, sample volumes equal to 2% of the bed volume and at concentrations of 10 mg/ml were run through the column. Twelve fractions, each at 5 ml, were collected from these runs. To concentrate the proteins once more, the fractions were then frozen overnight and then lyophilized for another 24 hours before being re-solubized in 500 µL volumes of DI water. The samples were then subjected to a BCA assay to determine protein concentrations, using porcine ECM as its own standard.

3.8 Phase 3: Column Setup and Operation

A similar protocol to Phases 1 and 2 was followed but this time using new pre-swollen media, Toyopearl HW-55F (Tosoh), which has a larger fractionation range and is shipped in a 20% ethanol suspension. Setup was done following variations of protocols used in the Toyopearl Instruction Manual (Tosoh) and the Amersham Gel Filtration Calibration Kits Product Booklet (GE Healthcare). To remove fines from the pre-swollen media, the settled gel was first stirred vigorously to re-suspend the resin. We required 30 mL of resin for our 11 mm ID column and thus, 40 mL of suspension was measured out and 0.05 M tris-HCl buffer (pH 6.8) was added to make a total volume 4 times the resin volume. The resin was stirred vigorously and then left to settle for 20 minutes, followed by decantation of the supernatant. The bottom of the column was wet with buffer to a height of 2-4 cm before the resin was carefully poured in and allowed to settle under gravity for 20 minutes. The column was then equilibrated by running 2-3 volumes of buffer through it.

To obtain a molecular weight standard curve, the Gel Filtration Molecular Weight Markers Kit for Molecular Weight 12,000-200,000 Da (Sigma Aldrich) was run through the column in a manner similar to the Phase 2 method. Two additional proteins: apoferritin from horse spleen (443 kDa) (Sigma Aldrich) and bovine thyroglobulin (669 kDa) (Sigma Aldrich) were also run through the column at 10 mg/ml and 8 mg/ml concentrations, respectively. Similar to Phase 2 operation, samples were diluted in 0.05 M tris-HCl buffer (pH 6.8) containing 5% glycerol and volumes equal to 2% of the settled column bed volume pipetted into the column. Immediately afterwards, fraction collection began. The proteins were run through the column in the following order: blue dextran (21 fractions), cytochrome c and β-amylase (28 fractions), carbonic anhydrase and alcohol dehydrogenase (30 fractions), albumin and thyroglobulin (30 fractions) and apoferritin (30 fractions). There was an overnight time-delay of 12 hours between

the runs for carbonic anhydrase and albumin, in which the column was left standing with the resin suspended in buffer. The following day the rest of the proteins were run through the column, as well as 2 vials of adult rat ECM (800 µL each). The collected fractions of the standard proteins were then subjected to a BCA assay versus a BSA standard curve to determine protein concentrations.

3.9 Coating ECM onto Tissue Culture Plastic

The protocol followed for coating ECM, seeding and staining cells, imaging and analysis was one developed by Corin Williams. The goal was to coat each well on a 48 well plate with approximately 37 mg of ECM and have each condition tested in triplicates. For our purposes, since we had very low protein concentrations in our fractions, we decided to aim for 20 mg of protein in each well and to achieve this, ECM fractions were paired up into 6 conditions as follows: fractions 1 and 2 together, 3 and 4 together, and so forth. Using sterile technique and working in a biological hood, two 48-well plates were coated with ECM volumes necessary to give 20 mg of protein in each well. To fill the plates, 3 sets of porcine ECM fractions were used and 9 wells were coated with stock porcine ECM. The coated plates were then left open and in the hood to dry overnight.

For control purposes, some wells were not coated with ECM to allow cardiomyocytes to grow on tissue culture plastic (TCP). Additionally, collagen I and poly-L-Lysine (Sigma Aldrich) were also used as conditions. Native ECM contains collagen in large quantities and poly-L-Lysine is a molecule that enhances cell adhesion to TCP (Yavin and Yavin 1974). Poly-L-Lysine was also setup under 2 conditions where one would have normal myo media and another would have serum-free media.

3.10 Cell Culture

Cell Procurement: Neonatal cardiomyocytes were isolated from Sprague Dawley pups that were decapitated followed by a sternotomy. Cells obtained were suspended in media and counted using a hemocytometer. Populations of approximately 75,000 cells were seeded into each well and supplemented with 500 µL of media each.

Cell Media and Feeding: The cardiomyocytes were fed serum-free myocyte media so as to avoid the serum masking the effects of ECM on proliferation. For every 100 ml of the media, we had 1% 10,000 U/mL Penicillin/Streptomycin (Gibco), 0.5% ITS Liquid Media Supplement (Sigma Aldrich), 200 mg BSA (Sigma Aldrich) and 50% Ham's F12 Nutrient Mixture (Invitrogen) in Dulbecco's Modified Eagle Media (Gibco). The media was prepared at room temperature and stored at 4ºC.

The cells were fed on day 2 with the serum-free media, while the wells set aside for Poly-L-Lysine (with serum) were fed with media containing 15% fetal bovine serum (Gibco). The cells were cultured for a total of 5 days.

3.11 Immunohistochemistry

Fixing: To fix the cells for imaging, plates were washed twice using 1X PBS before 200 µL of cold methanol was added to each well. The plates were then placed on an orbital at 4ºC for 10 minutes. Methanol was pipetted off and the cells washed 3 times with 1X PBS, with 5 minutes being allowed for each wash on the orbital at room temperature. The plates were then stored in 1X PBS overnight.

Blocking: To block unwanted binding sites on the proteins, 200 µL of 5% donkey serum (Sigma Aldrich) in 0.1% BSA in PBS was added to each well and allowed to shake at room temperature for 30 minutes.

Primary Antibodies: Two primary antibodies were applied to the wells: cardiac α-actinin (mouse) (Santa Cruz) and p-Histone H3 (Ser 10)-R (rabbit) (Santa Cruz) (PHH3). Each was made in a 1:250 dilution in 0.1% BSA in PBS and mixed together. For each well, 150 µL of the primary solution was added and left on an orbital for 1 hour, followed by 3 rinses with 1X PBS (5 minutes each on the orbital).

Secondary Antibodies: Cy 3 Goat Anti-Mouse (Molecular Probes) and Alexa Fluor 488 Goat Anti-Rabbit (Invitrogen) were prepared in the dark at 1:250 dilutions with 0.1% BSA in PBS. Hoescht nuclear stain was also added with the secondaries at a 1:1000 concentration. Again, 150 µL of the solution was added to each well and left shaking on an orbital for 2 hours, followed by 3 more 1X PBS rinses for a total of 15 minutes.

Imaging: Fluorescent images of the plates were taken using an Olympus IX81 Motorized Inverted Microscope (Olympus Corporation). Its multidimensional analysis function was used to take 10 pictures in red (TRITC), green (GFP) and blue (DAPI) at 10 pre-assigned spots on each well.

Analysis: Images obtained were analyzed using CellProfiler software (Broad Institute) running an algorithm (pipeline) developed by Corin Williams and Joshua Resnikoff. The program counted cells, which it identified as objects with diameters ranging from 4 to 80 pixels. Threshold values were assigned and masks created to reduce interference by background signals and the numbers of cardiomyocytes and proliferating myocytes were quantified. Further analysis of the data obtained from CellProfiler was done using SigmaPlot software (Systat Software Inc.) in which one way ANOVA tests were run. A p-vlaue of 0.05 was used as the baseline for statistical significance, with data being significant if their $p<0.05$. If data was found to be significant, then the Tukey method was used to compare it with other conditions in the group, also using $p<0.05$ as a benchmark for significance.

4.0 Results

4.1 Developing a Protocol & Determining Concentrations

Protein concentrations were determined using the Pierce BCA protein assay kit. The bicinchoninic acid (BCA) assay (Smith et al., 1985) operates via two reactions: firstly is the reduction of Cu^{2+} to Cu^{+} by peptide bonds at the cysteine, cysteine, tryptophan and tyrosine residues, with the amount of Cu^{2+} reduced being proportional to the concentration of protein present in the solution. The second reaction involves the formation of a complex chelate between the Cu^{+} and BCA, resulting in a purple product that absorbs light at 562 nm (Wiechelman et al., 1988; Olsen and Markwell, 2007). Results shown in Figure 1 suggested that regular BCA standard proteins BSA and collagen I do not give an accurate estimate of ECM protein concentrations, but they did show that digested ECM may be used as its own standard to give a more accurate picture when running the assay. Of interest was that the estimates given by the BSA standard came out almost twice as large as those predicted by the ECM standard, while those predicted by collagen I were closer to the actual ECM standard.

Trying out different methods of preparing the ECM, either freezing it with liquid nitrogen or just lyophilizing it did not show any significant differences in concentrations (Figure 2). The standard curves obtained from either treatment were almost identical for samples from the porcine left and right ventricles. However, the curve for nitrogen treated right ventricle was slightly different form the rest (Figure 3). The method of lyophilizing was thus used for all succeeding experiments. Additionally, the ECM obtained from either of the cardiac ventricles was shown to behave in the same manner (Figures 4 and 5).

When results from the different ventricles, prepared by either method are plotted together and compared with the BSA and collagen I standard curves (Figure 4), a similar trend of ECM doing well as its own standard and how the source or treatment of the ECM does not make a

difference may be clearly seen. Lastly, Figure 5 shows the slight effect of having pepsin in the resulting ECM stock solution.

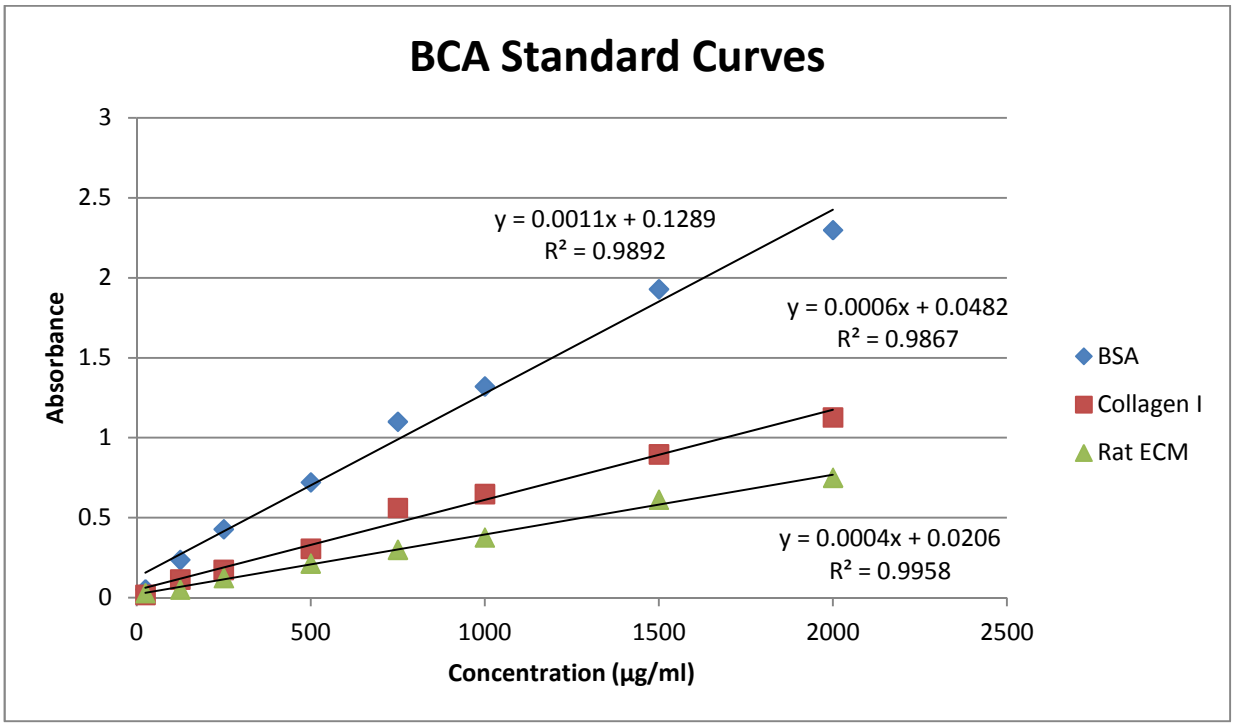


Figure 1: BCA curves obtained using different standards

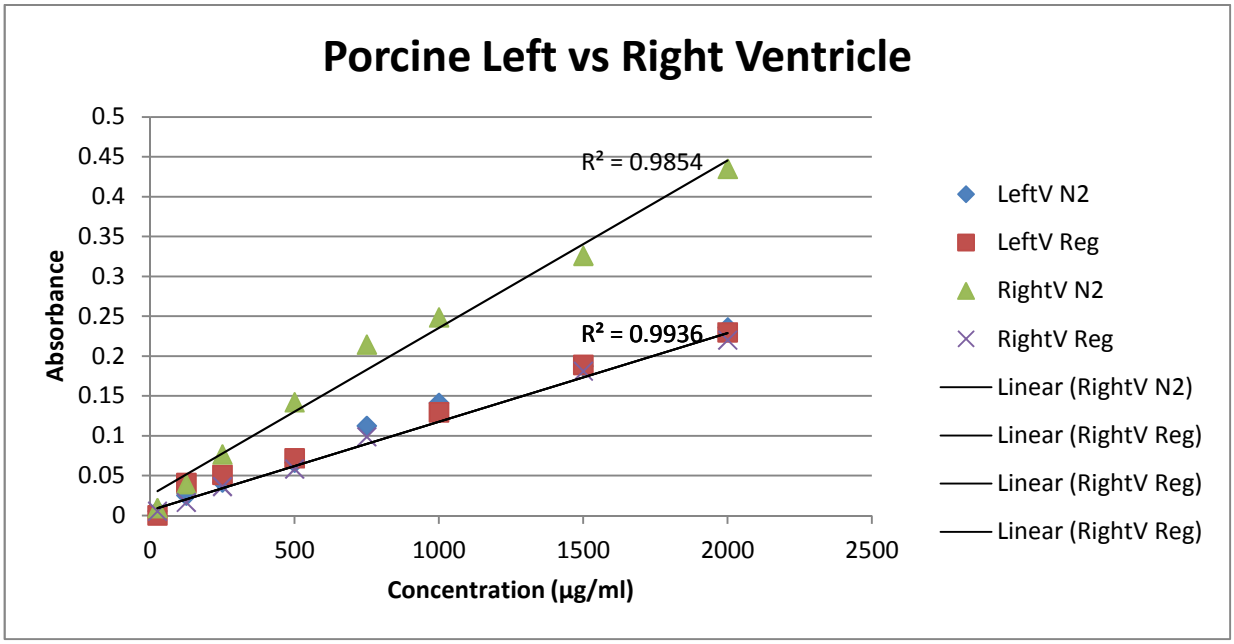


Figure 2: BCA standard curves for ECM obtained from the left and right porcine ventricles, treated with nitrogen or regular lyophilizing

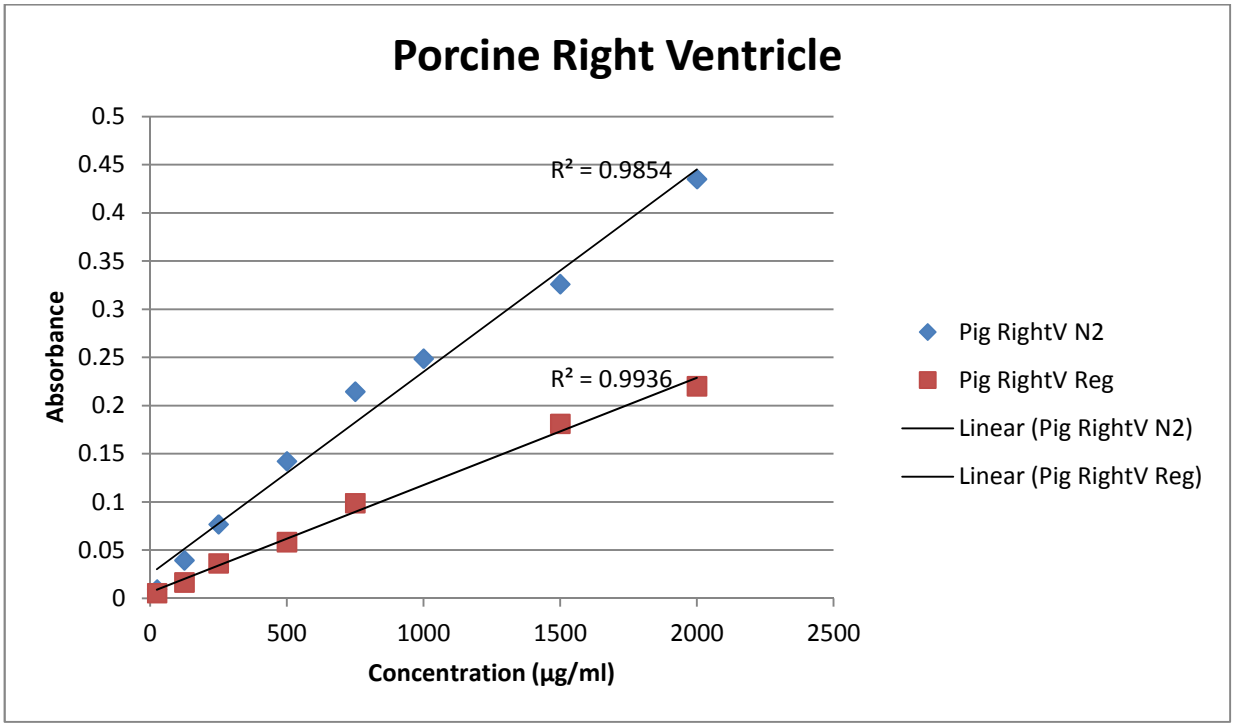


Figure 3: BCA standard curves for nitrogen and regular lyophilizing treatment for right ventricle ECM

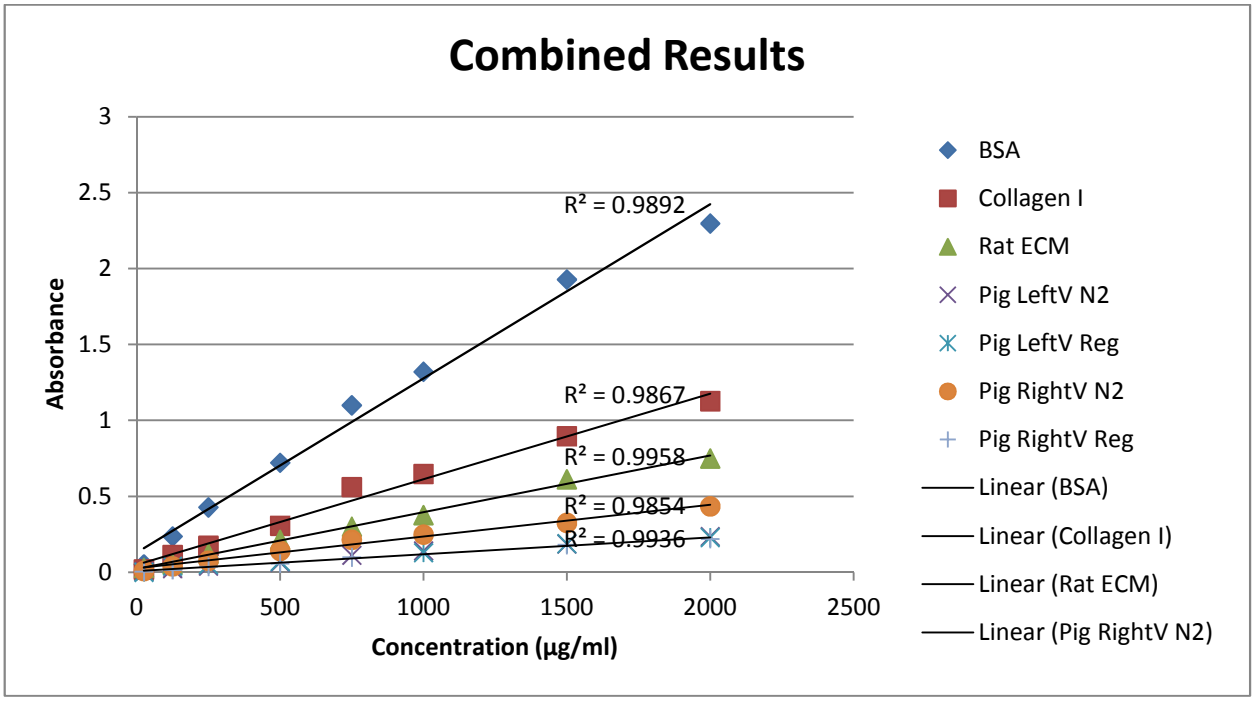


Figure 4: Combined BCA standard curves for porcine ECM from both right and left ventricles treted with nitrogen or regular lyophilizing.

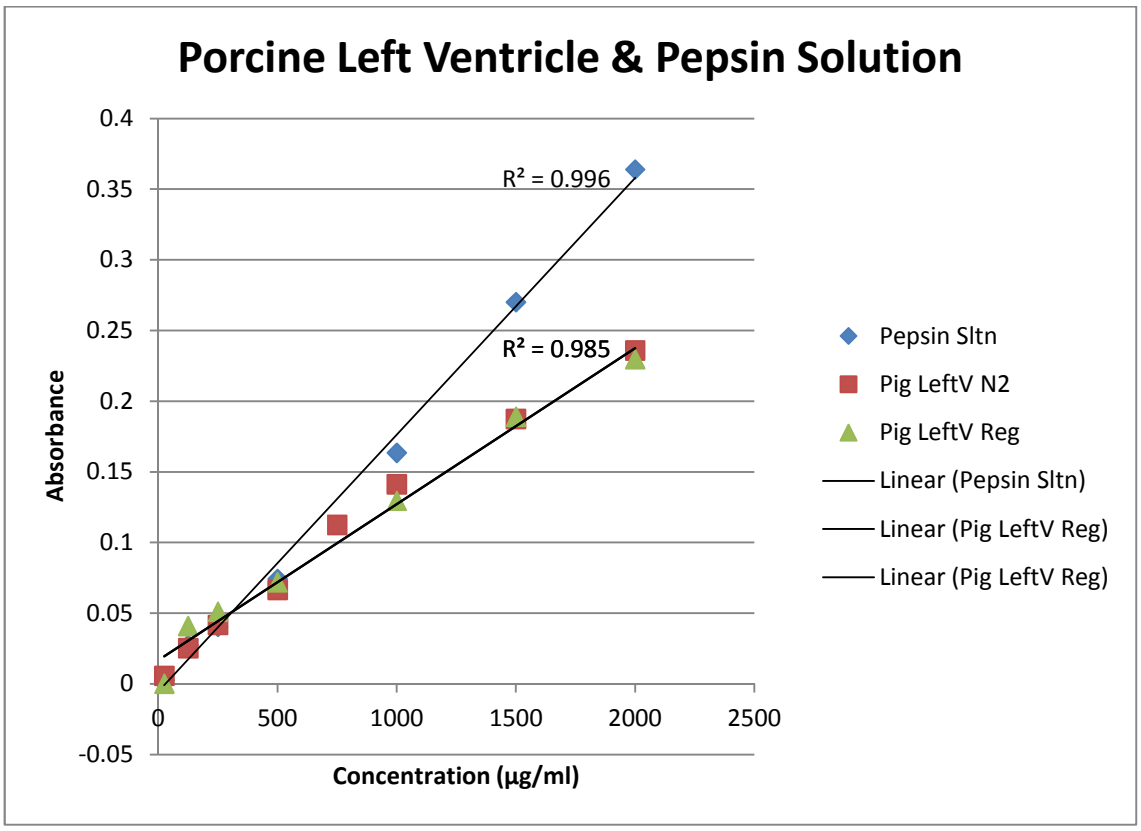


Figure 5: BCA standard curves for porcine ECM versus a stock solution of pepsin

4.2 Fractionation Results: Phase I

Fractions obtained from the 2 trial columns showed that indeed the ECM fractions had been significantly digested and that the Sephadex G-100 media was able to separate the proteins to some extent (Figure 6). The small column had a bed volume of 2.3 cm^3 and the large column was 5.5 cm^3. A larger portion of the proteins, 918 µg/mL for the small column and 724 µg/mL for the large column were collected in the Wash fractions as compared to the other fractions and these are thought to be very small peptides. The BCA results also showed a presence of some proteins in the very first fractions (Fraction A) collected from the columns, which was not expected.

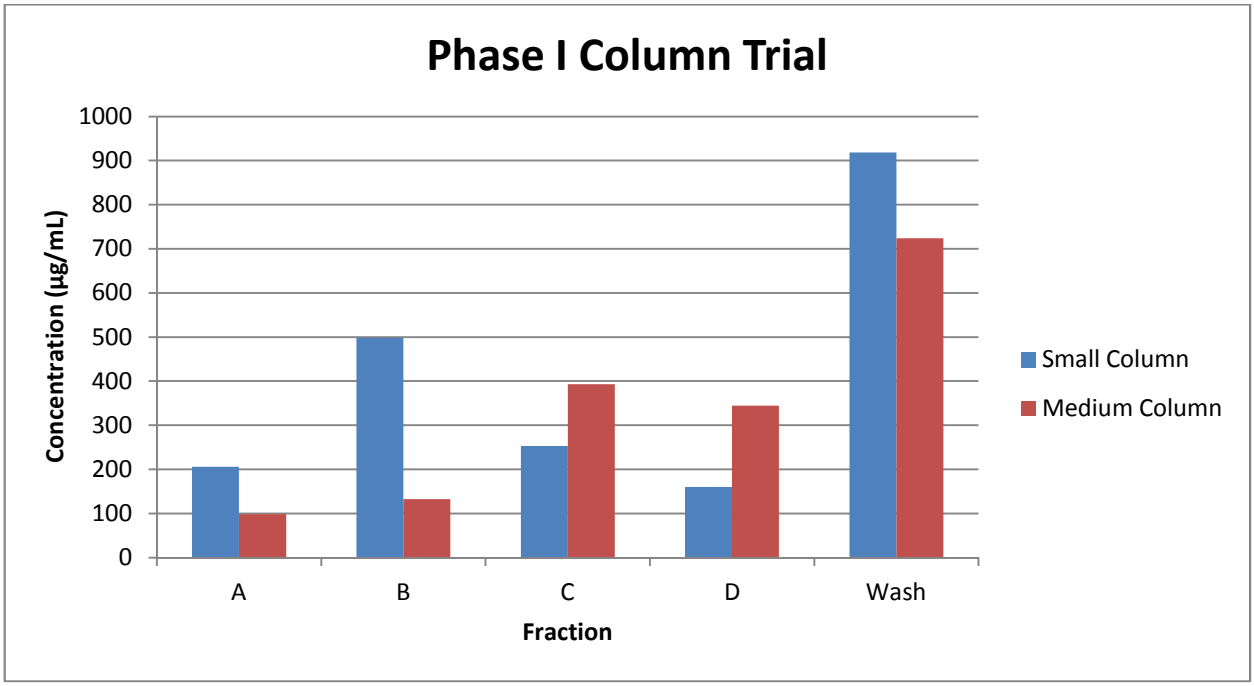


Figure 6: Fractions obtained using a small column (2.3 cm^3) and a medium column (5.5 cm^3)

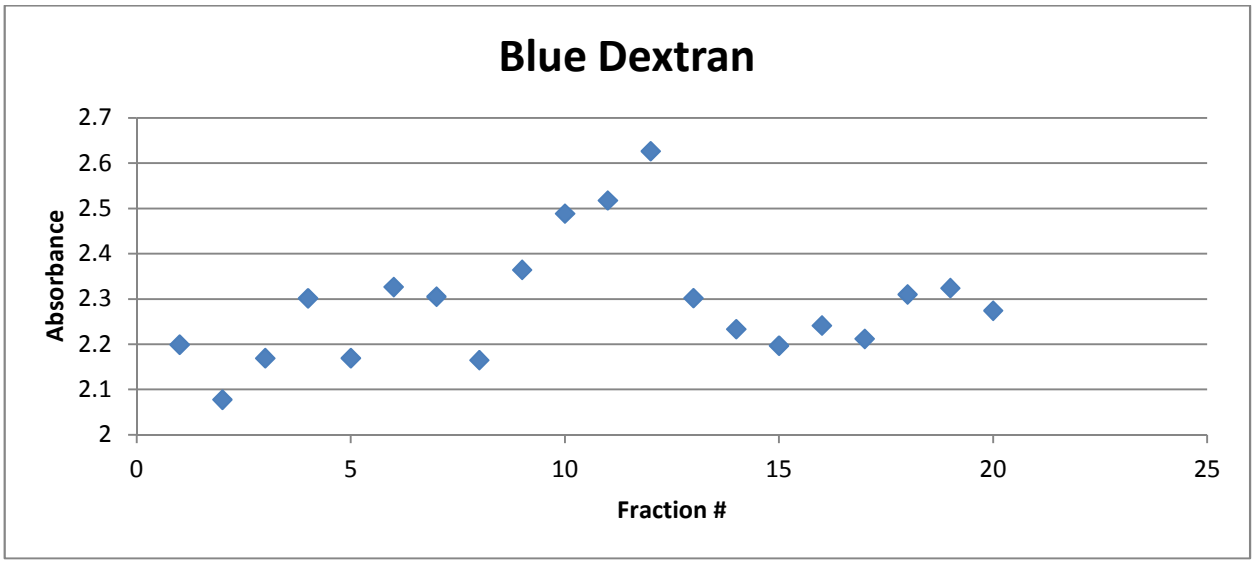
4.3 Fractionation Results: Phase II

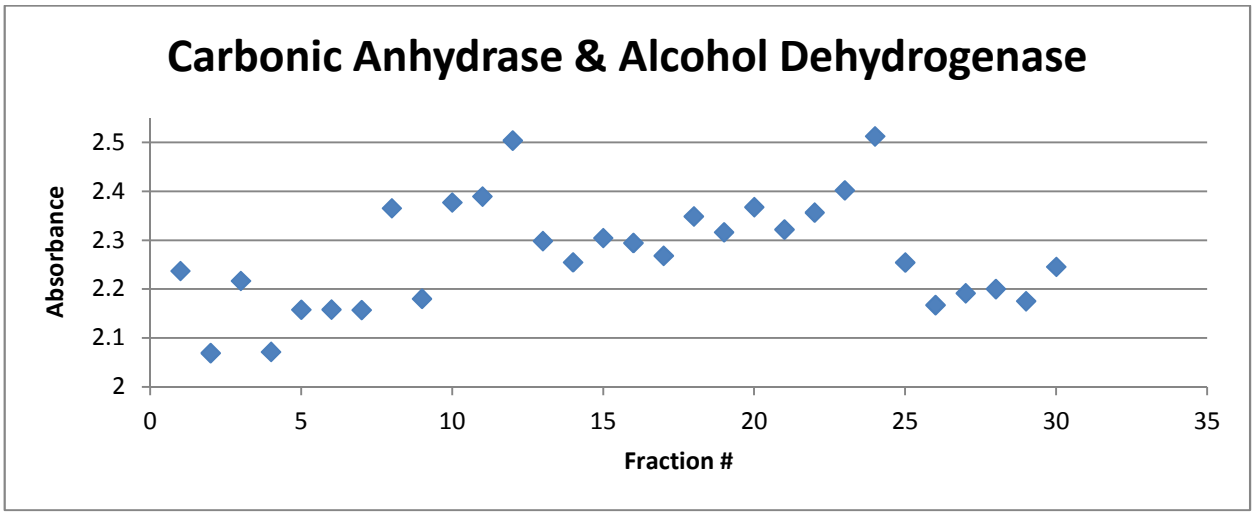
The fractions obtained from the Phase II trials were first placed in a spectrophotometer and readings for their absorbance at 280 nm are shown in Figure 7. Each fraction for the protein standards was 1mL in volume. The data showed no consistency and the peaks for each fraction were not clearly discernible. The same fractions were then subjected to the BCA protein assay and then run through the spectrophotometer again, but this time clearer peaks and trends could be discerned (Figure 8).

The concentrations shown were determined using BSA as a standard in the BCA assay. Some of the concentrations calculated were noted to be negative but despite this, the chromatograms showed clearer peaks and better resolution. The dextran (2000 kDa), which was run individually in the column gave a well resolved peak, from which a void volume (V_o) of 12 mL was obtained. Chromatograms for albumin (66 kDa), cytochrome c (12.4 kDa) and β-amylase (200 kDa) all showed good resolution from the column and values for their elution

volumes (V_e) were easily determined. However, the chromatogram for carbonic anhydrase (29 kDa) and alcohol dehydrogenase (150 kDa) were poorly resolved by the Sephadex resin as Figure 8 shows their peaks to be stacked together.

A plot of the selectivity curve for the resin using these results reveals a logarithmic curve for the points correlating to albumin, cytochrome c and β-amylase (Figure 9). As further evidence of the failure of the resin to resolve the carbonic anhydrase and alcohol dehydrogenase, the curve stretches to regions beyond the linear portion of the selectivity curve.





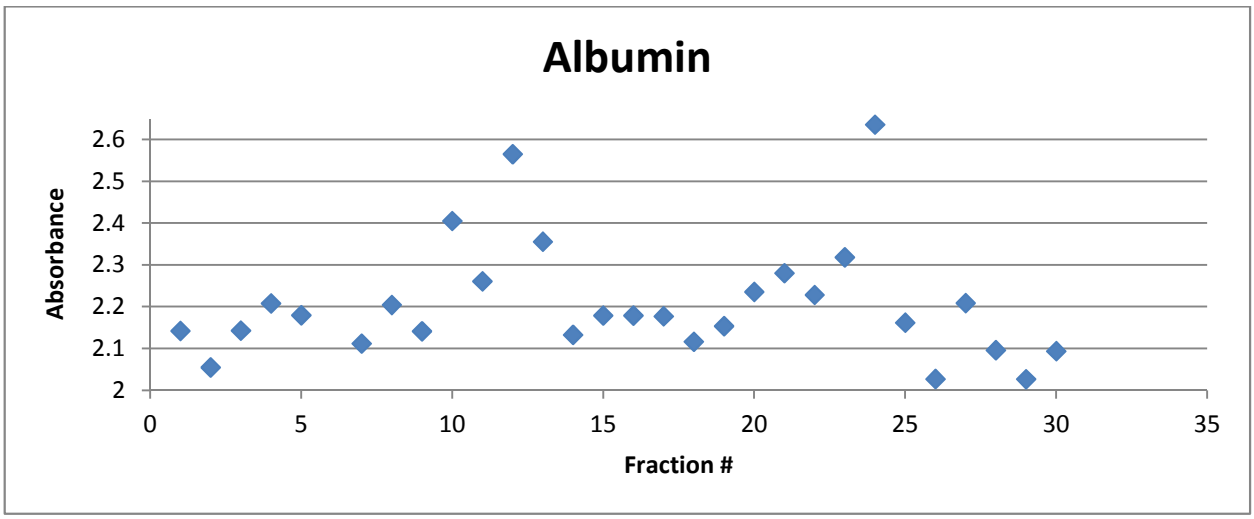
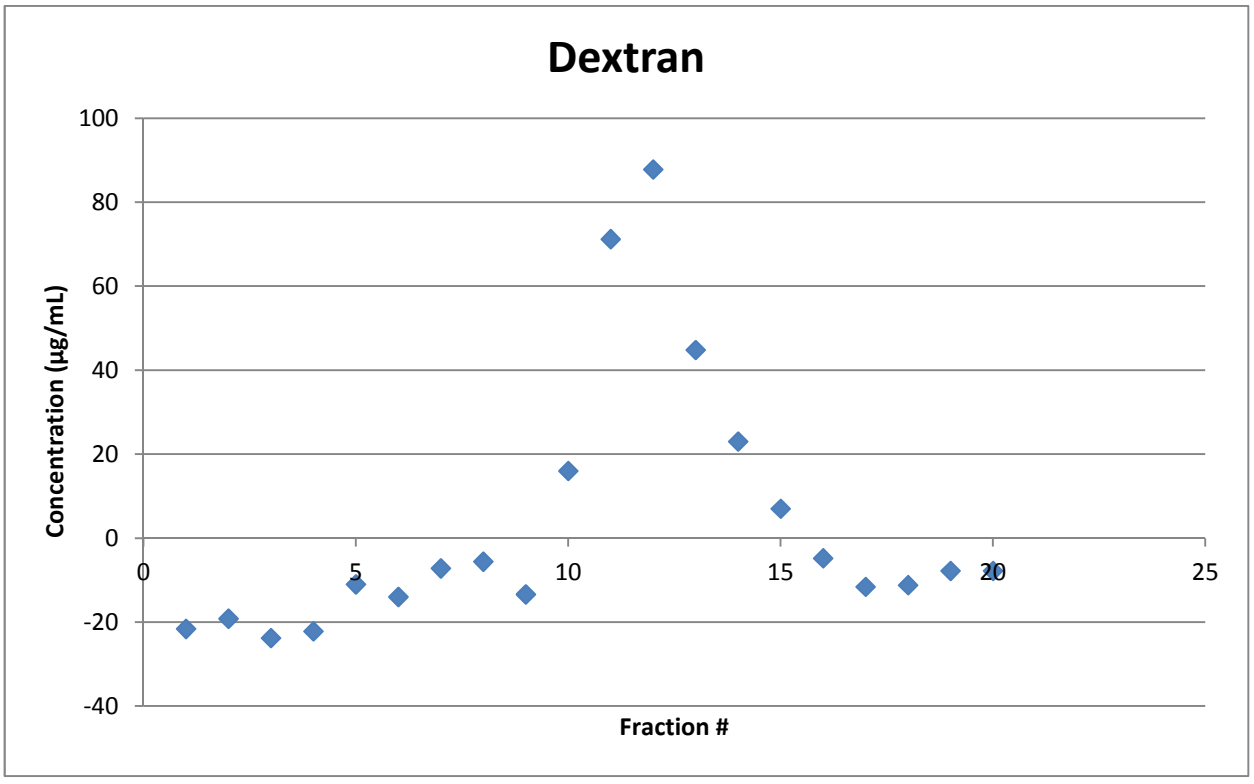
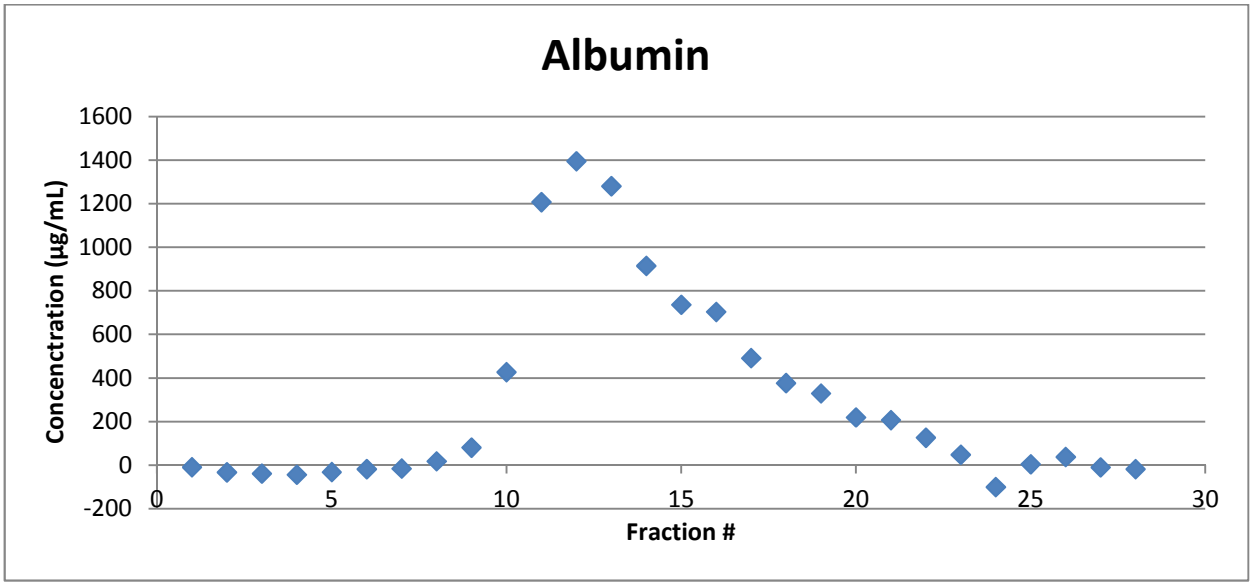
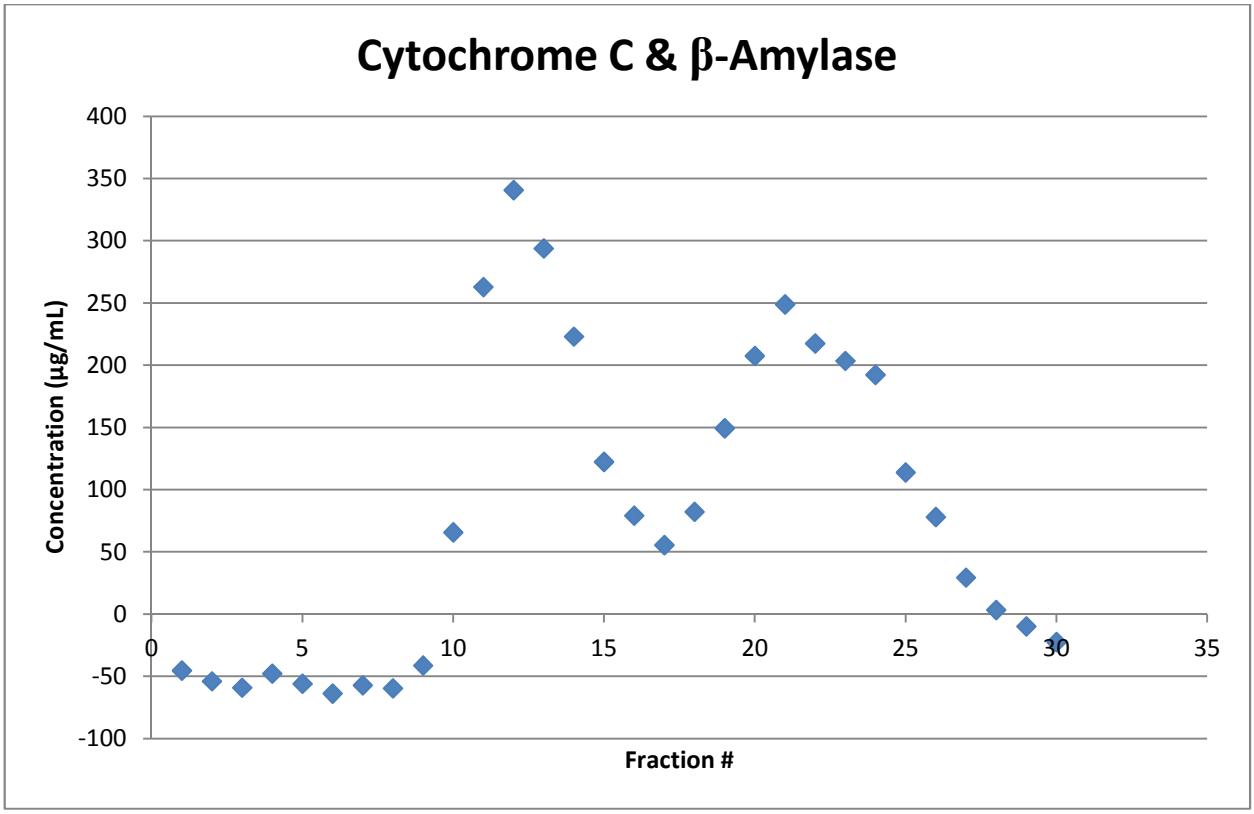


Figure 7: Absorbance readings for phase II fractions at 280 nm




Albumin
Concenctration (µg/mL)
1600
1400
1200
1000
800
600
400
200
0
-200
0
5
10
15
20
25
30
Fraction #


Cytochrome C & β-Amylase
Concentration (µg/mL)
400
350
300
250
200
150
100
50
0
-50
-100
0
5
10
15
20
25
30
35
Fraction #

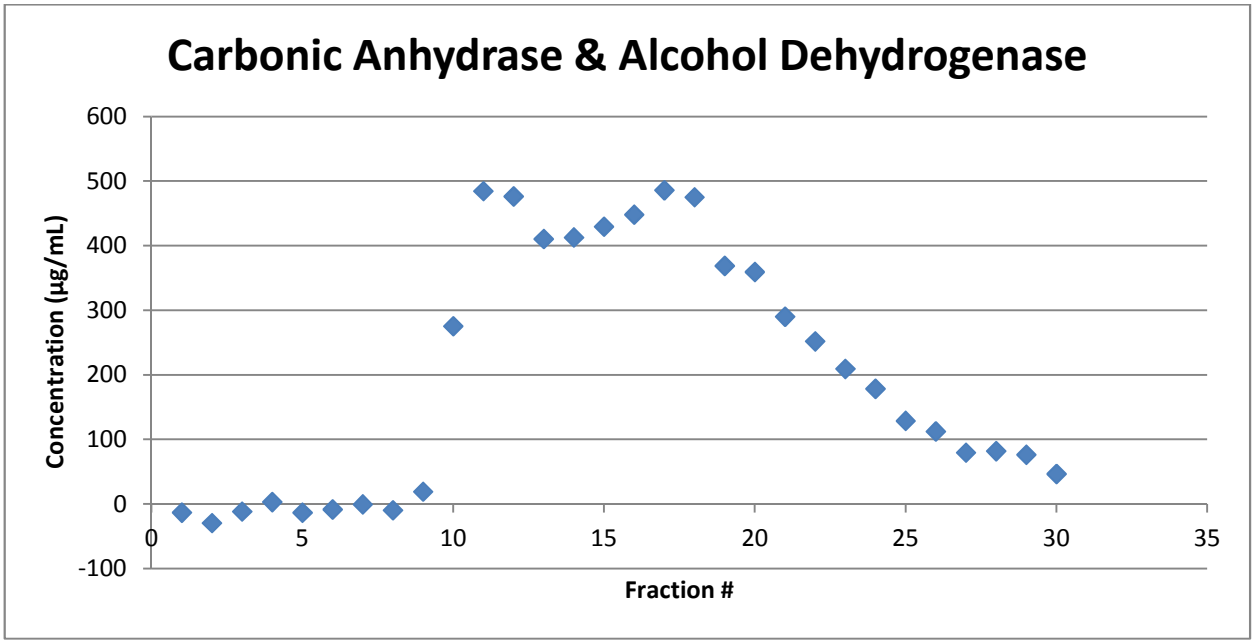
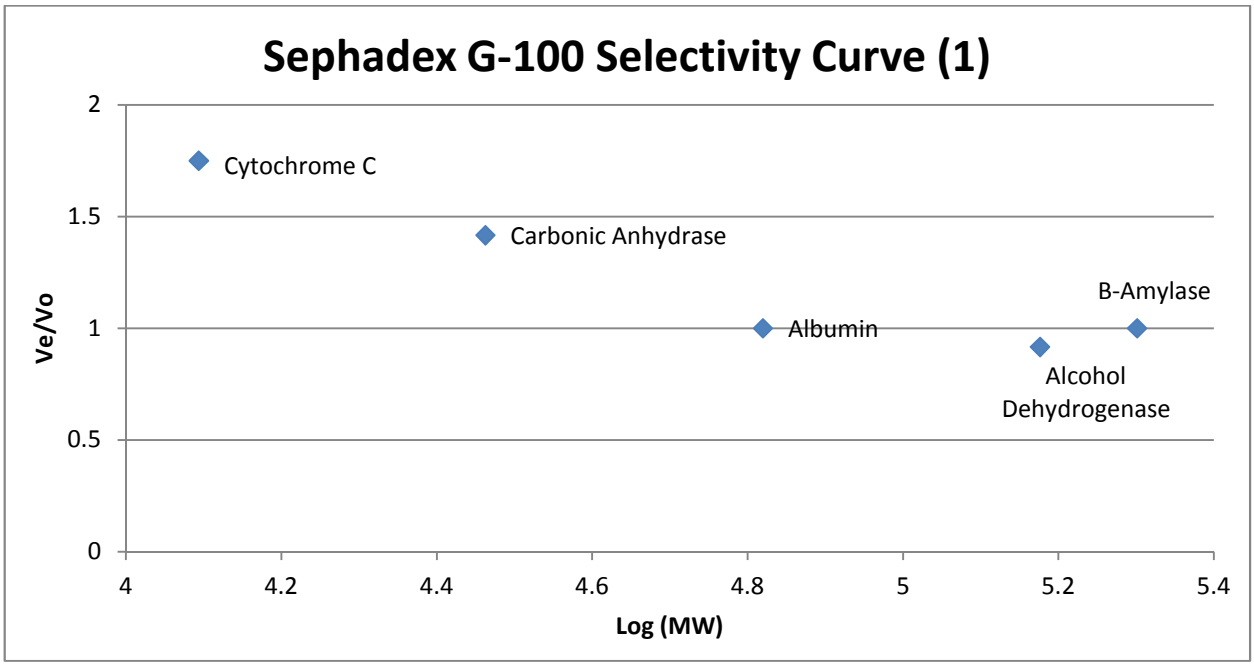


Figure 8: BCA results for fractions of standard proteins obtained in phase II



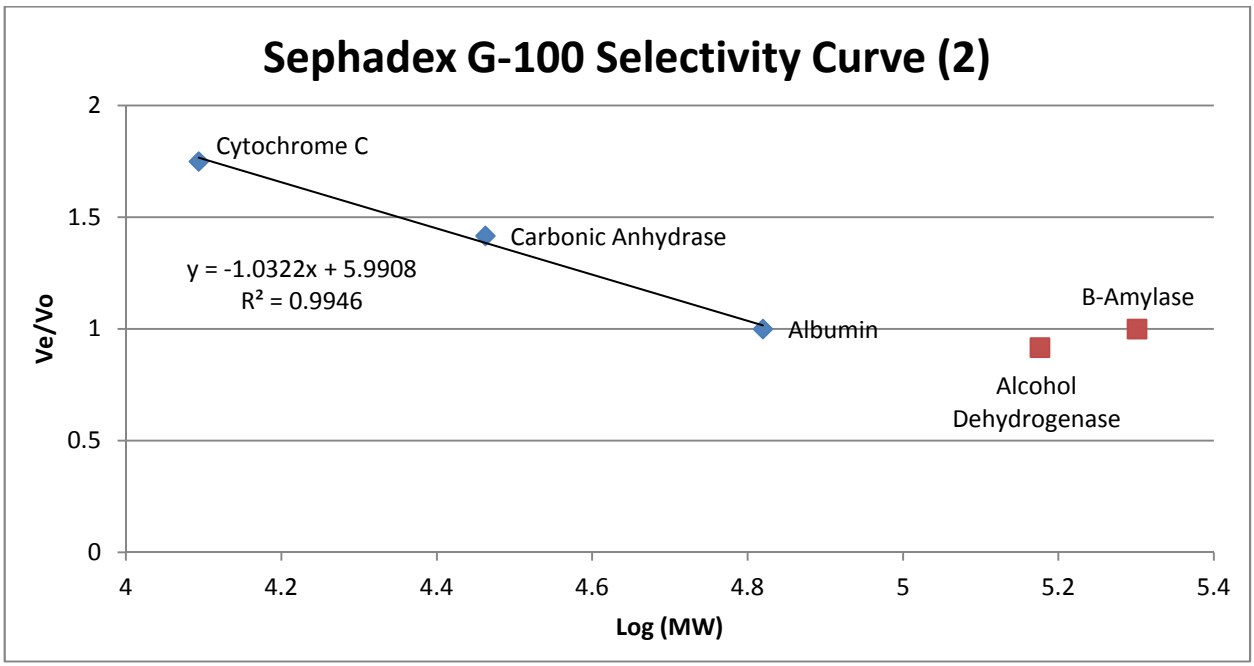
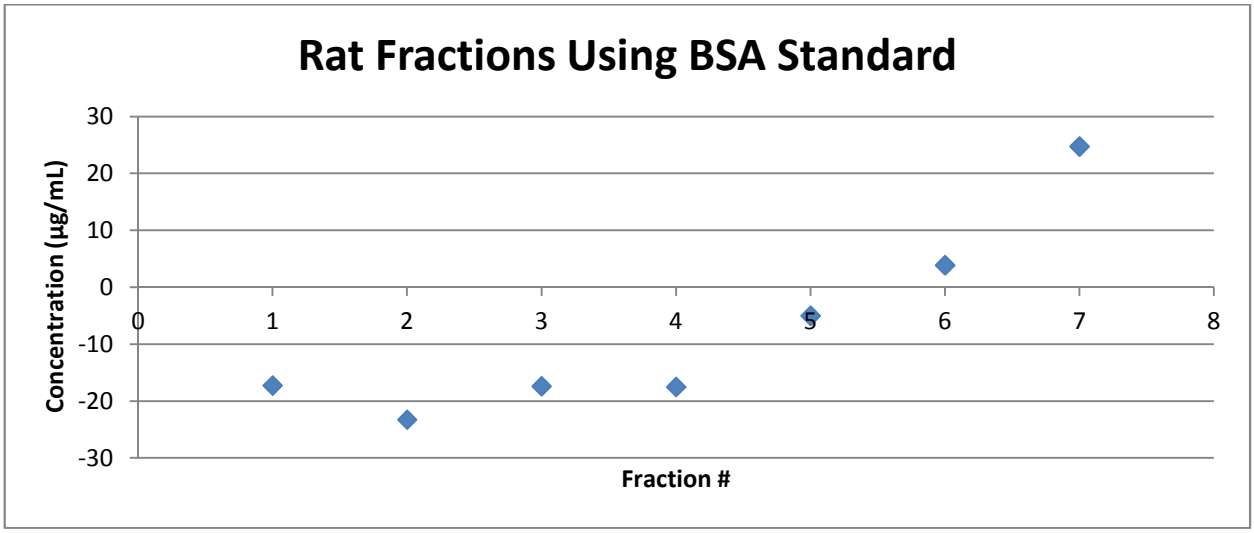


Figure 9: Selectivity curves obtained using V_e values from standard proteins

As further testament of how BSA cannot be used to reliably predict the concentrations of the rat ECM, plots of fraction concentrations obtained from the BSA standard and the ECM standard are compared in Figure 10. The BSA standard was found to underestimate the actual concentration present. Each fraction of ECM had a volume of 5ml, with a total volume of 60 mL being collected from the column. Such large fractions were necessitated because the SEC column can only work with sample volumes of less than 5% of the total bed volume, which results in very dilute fractions being obtained. Since the end goal of this experiment was to coat the fractions onto tissue culture plastic, we decided that 5 mL fractions would collect enough protein to be useful in the coating and staining studies.

Figure 11 shows the concentrations of porcine ECM fractions collected using porcine ECM as its own BCA assay standard. The fractions with the highest concentration were between fractions 3 and 4, and fraction 7. These are the same fractions that were used later in the coating and imaging analysis. (Figures for Tubes 1 and 4 are in Appendix section)



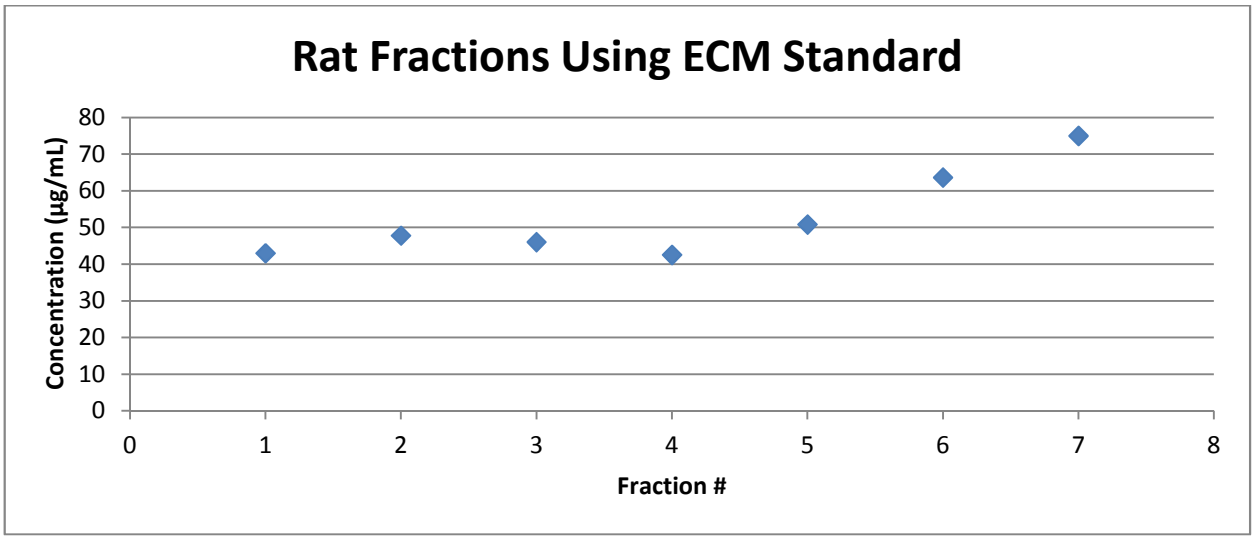
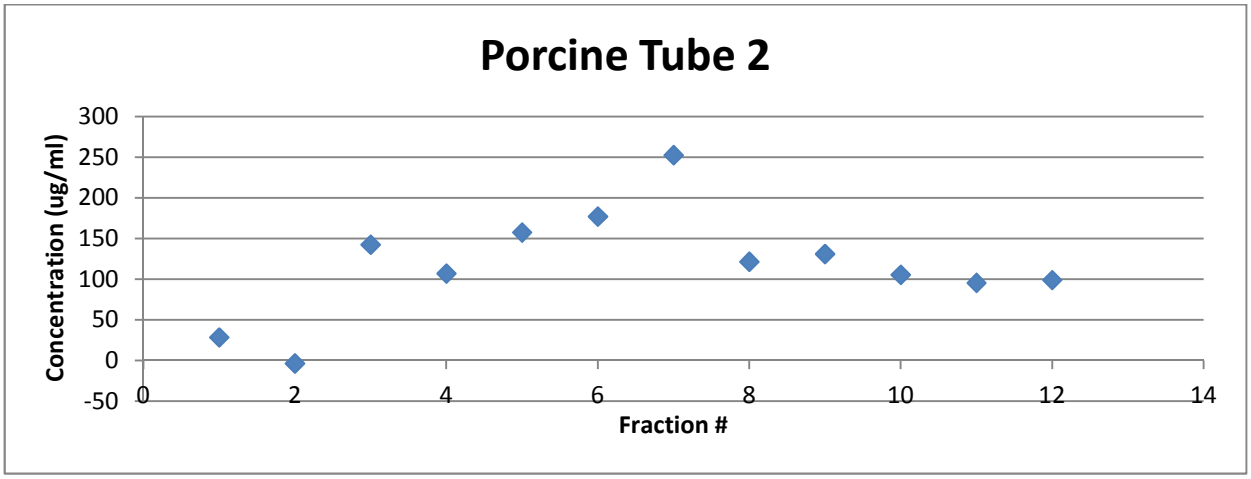
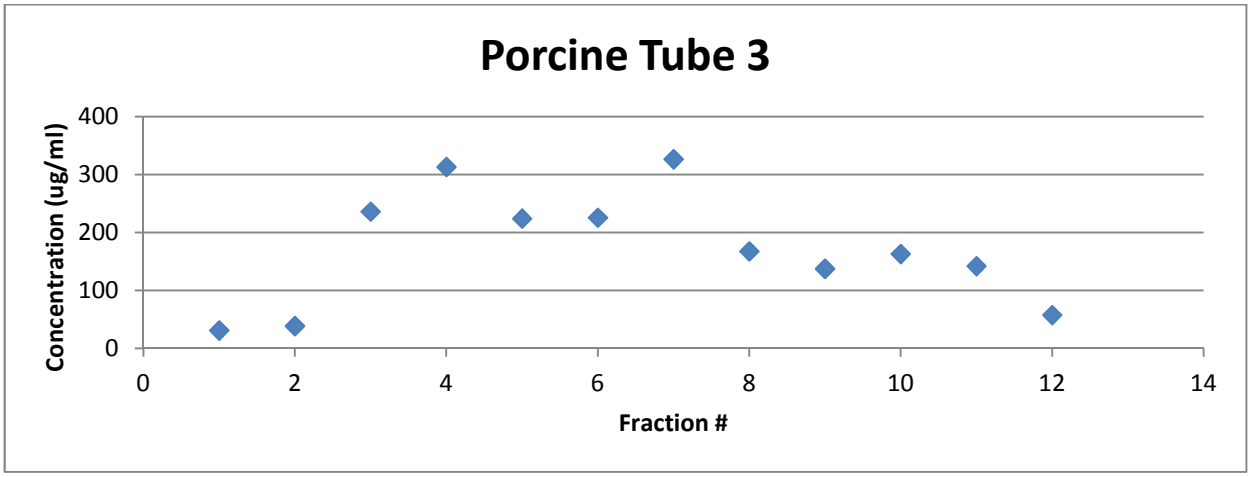


Figure 10: BCA results for concentrations of rat ECM fractions using BSA and ECM as standards





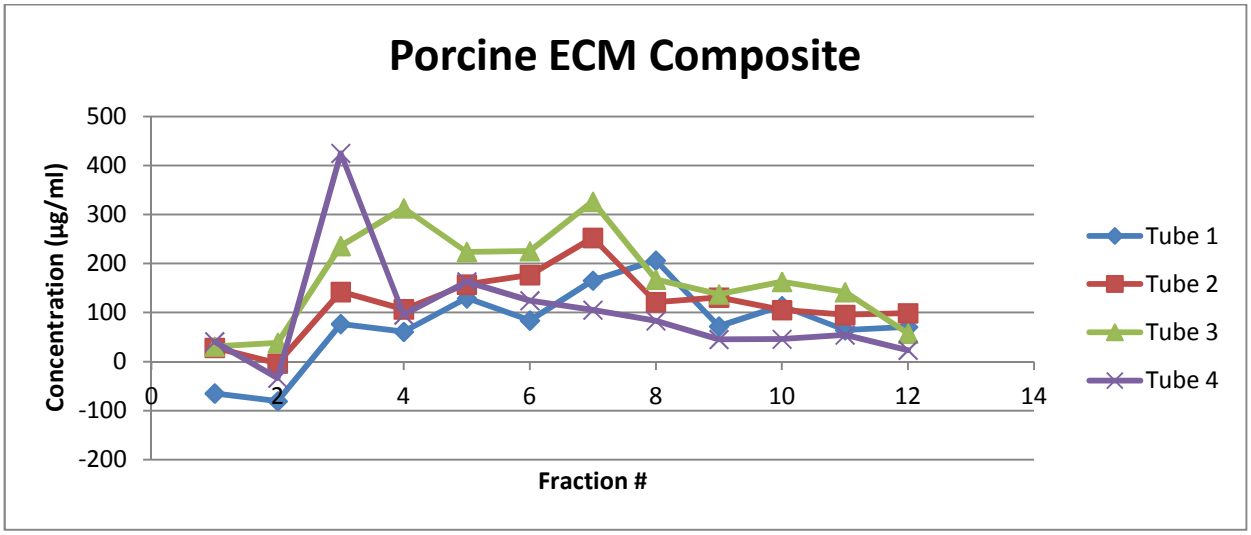
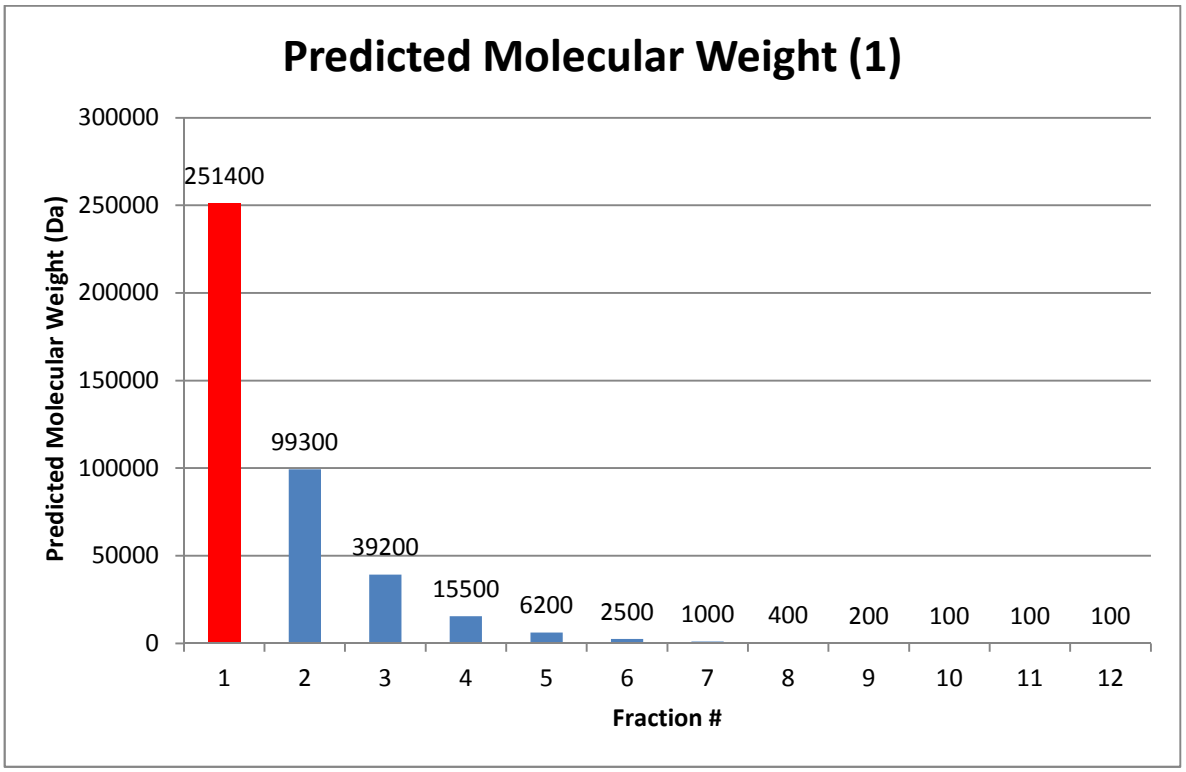


Figure 11: BCA results of concentrations of porcine fractions collected using ECM as a standard

Using the selectivity curve equation in Figure 9, a plot of the estimated average molecular weight obtained in each fraction was plotted (Figure 12). The values shown in this plot were rounded up to the nearest 100 Da as this was how much we could trust the accuracy of our resin. Since the manufacturer recommends the accuracy of our resin to be for proteins up to molecular weight of 100 kDa, values beyond this (marked in red in Figure 12) were taken to be possibly inaccurate. Also from this experiment, fractions obtained beyond fractions 7 and 8 were predicted to have molecular weights of below 1 kDa, which meant that they were tiny fragments and that we also could not accurately predict their weight as it was too small for our setup to

detect. Thus, the selectivity curve gave us values we could trust for fractions 2 to 7, with fraction 2 having peptides averaging 99.3 kDa and fraction 7 averaging 1 kDa.



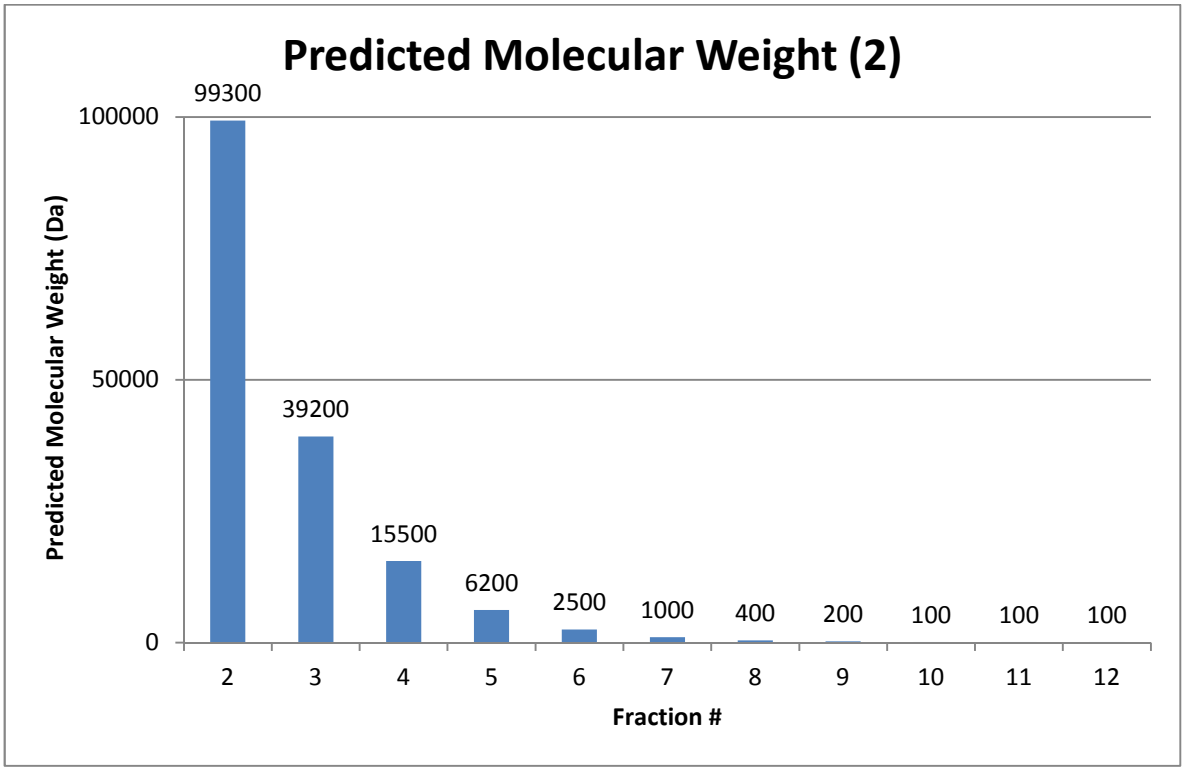


Figure 12: Molecular weights predicted to be in each fraction, calculated using the equation from Figure 9

Some experiments were setup to confirm the molecular weight estimates of Figure 12 using gel electrophoresis and imperial stain results but as the result of Figure 13 shows, this method could not detect any protein in the samples. In Figure 13, the full range molecular weight ladder which has protein markers in the range 12 – 225 kDa is clearly seen on the far right hand side. The well just to the left of that belonged to the stock sample of ECM and the single band obtained was thought to be from the pepsin used to digest the ECM. The rest of the gel did not show any other bands. More gel electrophoresis results are shown in the Appendix.

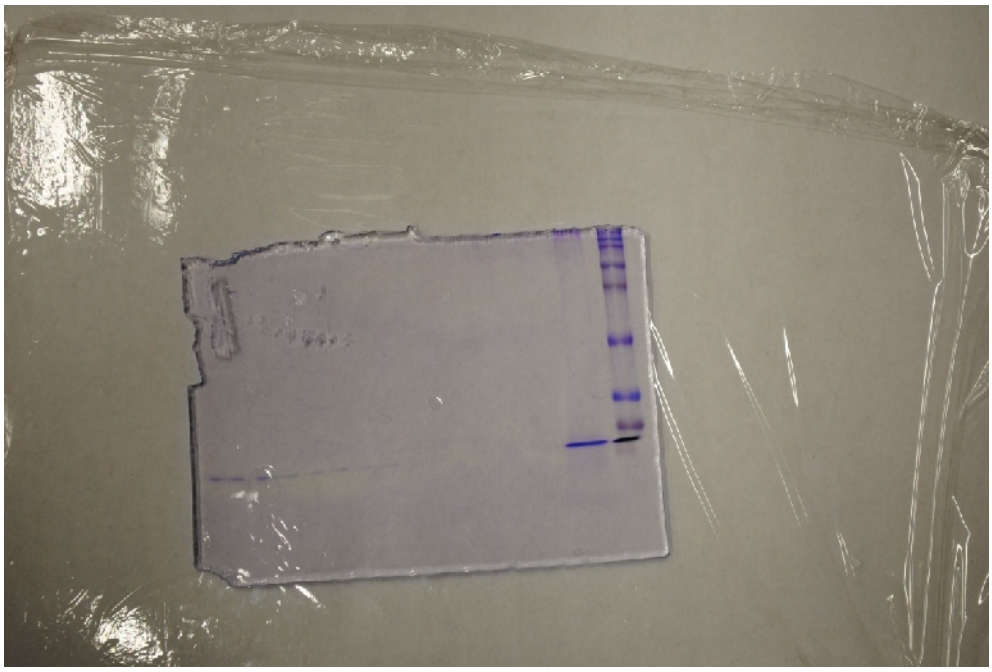
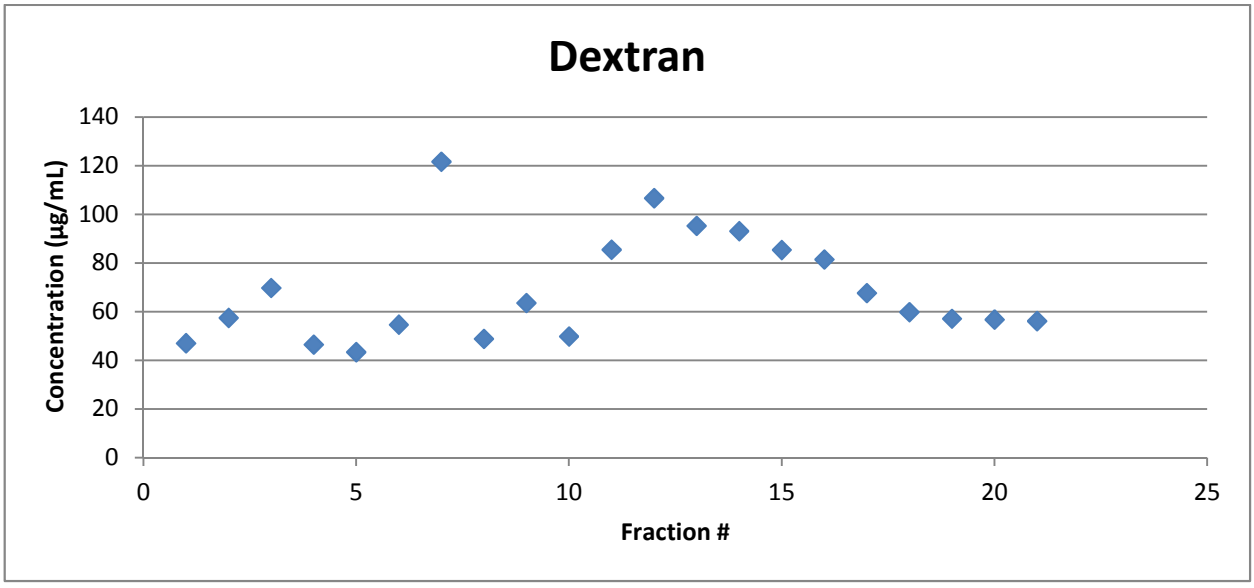


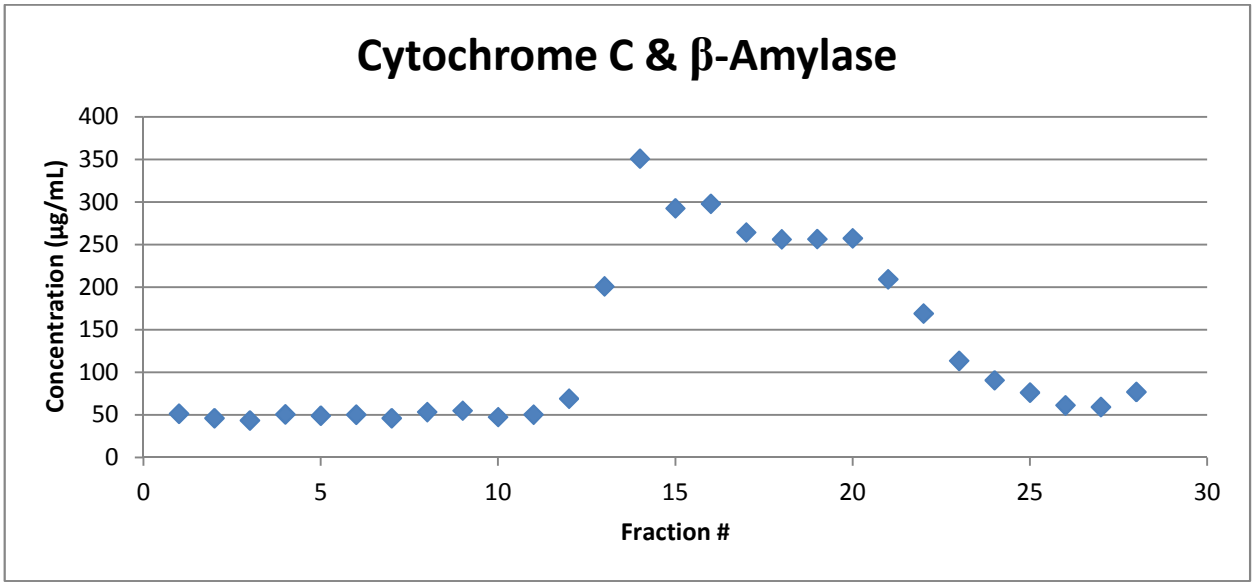
Figure 13: Sample result of an imperial stain on a PAGE gel. The ladder is on the far right, followed by a single band from the stock solution. No bands observed for fractions in the rest of the wells.

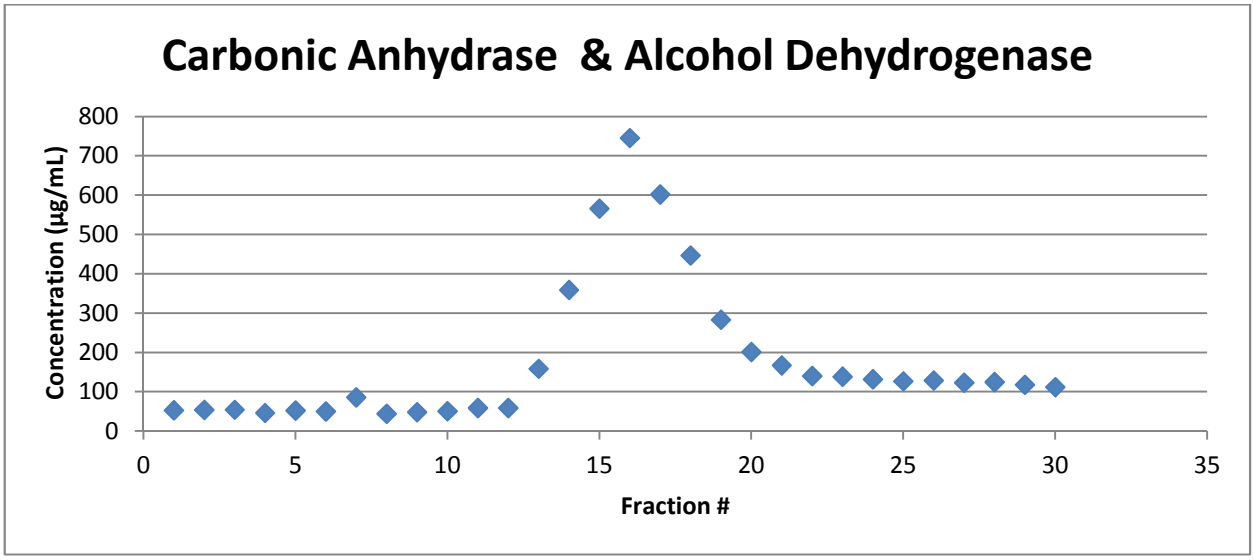
4.4 Fractionation Results: Phase III

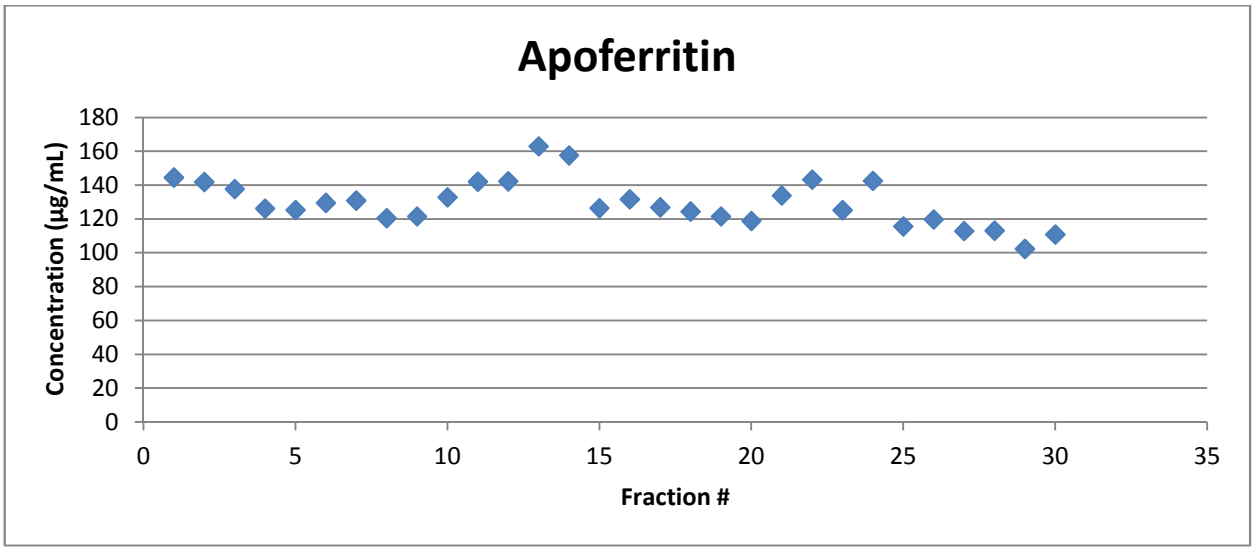
The Phase III trials were done using a Toyopearl HW-55F resin instead of the Sephadex G-100 to increase the obtainable exclusion range from 4 - 100 kDa to 1 – 200 kDa. The same column used in Phase II was used and the fractions obtained were then subjected to the BCA assay for analysis. As Figure 14 shows, the resin did not clearly resolve the standard proteins. The chromatogram for blue dextran (2000 kDa) did not reveal a distinct peak as well as those for cytochrome c (12.4 kDa) and β-amylase (200 kDa). Even more evident is the chromatogram for

carbonic anhydrase (29 kDa) and alcohol dehydrogenase (150 kDa) (Figure 14) which only gave 1 peak instead of the 2 that were expected. A similar situation was observed for the albumin (66 kDa) and thyroglobulin (669 kDa) fractions. The reading s obtained for apoferritin (443 kDa) did not show any discernible peak at all.









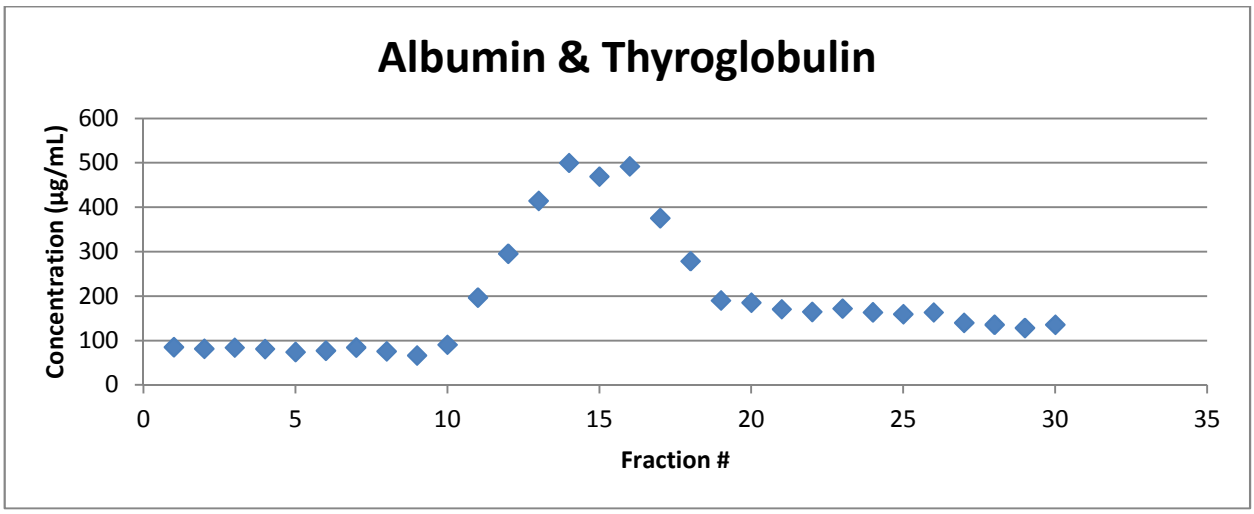


Figure 14: BCA results of concentrations of fractions obtained in phase III

4.4 Effects of ECM Fractions on Cardiomyocyte Proliferation

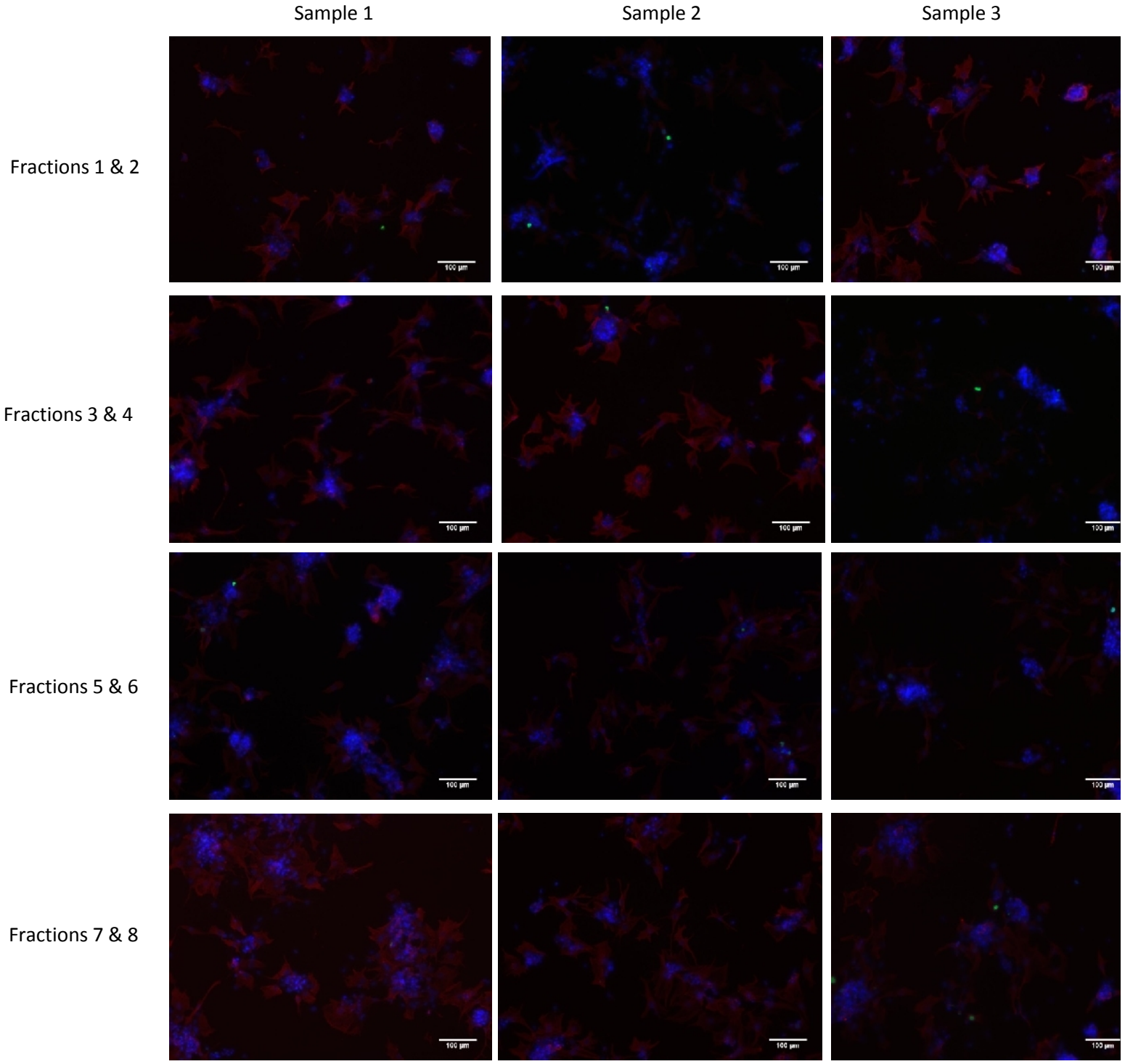
The proliferation of neo-natal cardiomyocytes cultured on the ECM, poly-L-lysine (with and without serum), collagen I and on tissue culture plastic was determined qualitatively using immunohistochemistry. The various conditions were probed for cardiac α-actinin and p-Histone H3 (PHH3). The α-actinin is a monoclonal IgG responsible for binding to actin present in the cytoskeleton and other structures of many cell types, and this particular actinin is specific for cardiac actin (Santa Cruz). The PHH3 is a polyclonal IgG that binds specifically to Histone H3, which is an essential protein in the formation of nucleosomes by DNA that becomes phosphorylated at the end of the prophase of cell cycle mitosis (Fernandez-Garcia et al., 2010; Drobic et al., 2010). Hoescht nuclear stain was used to stain all nuclei present, while the α-actinin stained all cardiomyocytes and PHH3 responding only to cells fixed amidst undergoing mitosis, thus proliferating. Standards used in this experiment were tissue culture plastic (TCP), poly-L-lysine (PLL) and collagen I. All the cells were cultured in serum-free media except when stated.

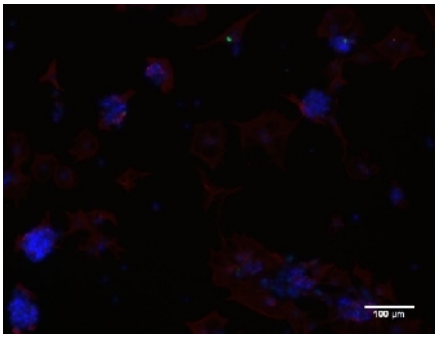
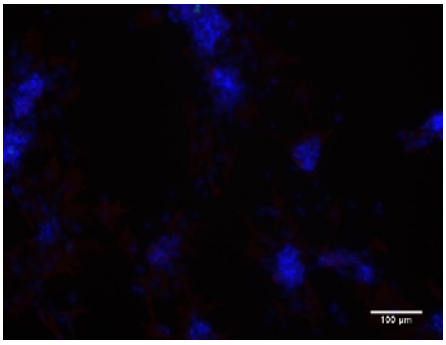
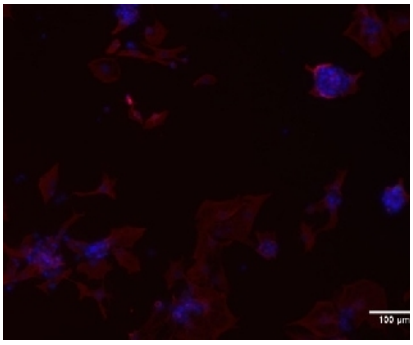
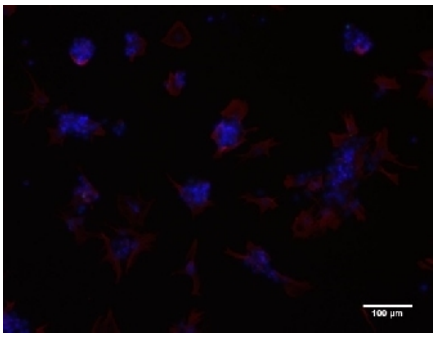
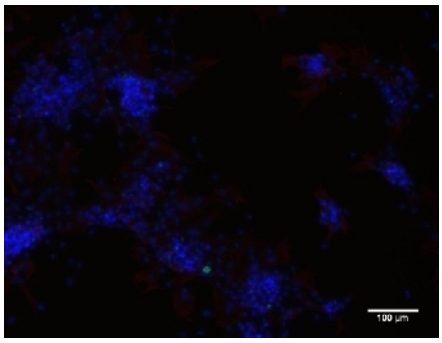
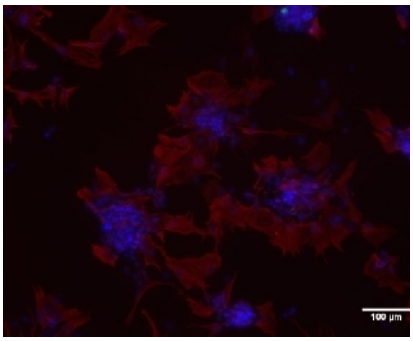
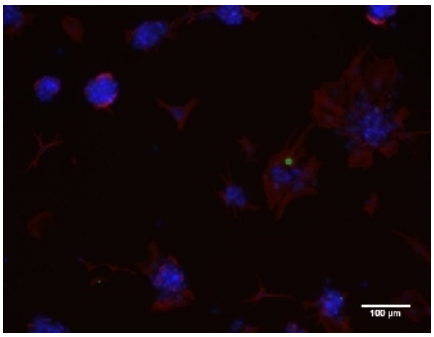
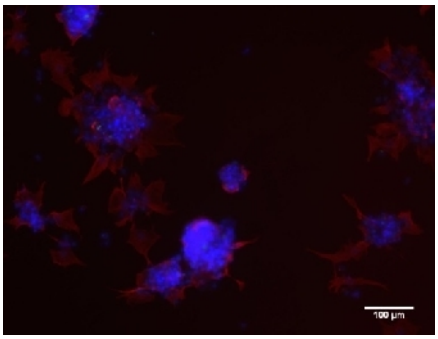
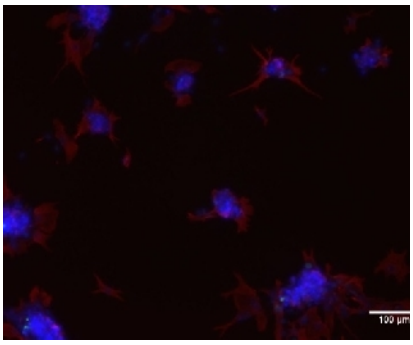
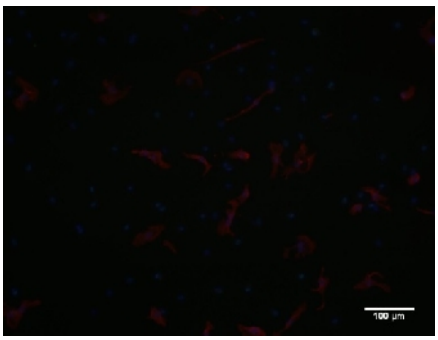
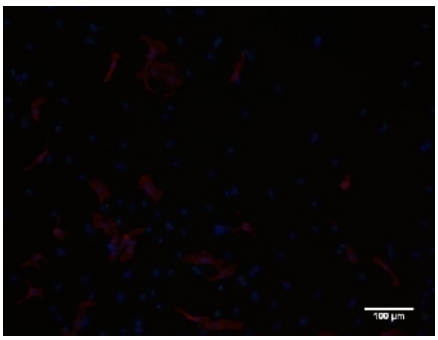
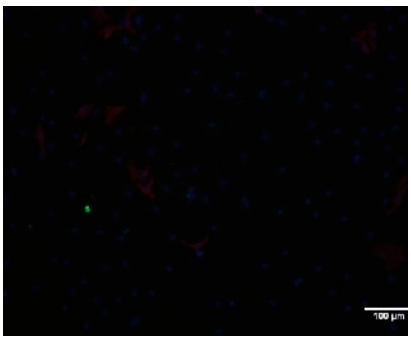
Immunohistochemistry:

All the fractions and standards observed tested positive for α-actinin and PHH3, just at varying levels as seen in representative images shown in Figure 15. In the over-layed images shown, all nuclei are stained blue, with red being the cardiomyocyte actin filaments stained by α-actinin and the rare green stains representing any PHH3 stained cell. Overall, all the fractions showed a dense packing of cells further suggesting that cell confluency may have been reached. As already mentioned with the quantitative results, the fractions all exhibited good conditions for cardiomyocyte proliferation, with denser images being seen for the medium range fractions, especially when compared to the sparsely populated standards.

The TCP had a significant overall cell population, but few cardiomyocytes. This was expected as cardiomyocytes do not attach well to TCP alone, thus making this a good standard for comparison. Similar results were obtained for the poly-L-lysine with serum-free media. For the samples with serum, as expected, a lot of cells proliferated over the period tested but since this was also on TCP, there still remained only a few cardiomyocytes.

The images for collagen I are moderately dense, but to a degree that seems less than that of the fractionated peptides, suggesting that despite collagen I being an essential component of ECM, there are other factors also at play, and these factors may have some influence from the sizes of various peptides.


Sample 1
Sample 2
Sample 3
Fractions 1 & 2
Fractions 3 & 4
Fractions 5 & 6
Fractions 7 & 8

	Sample 1	Sample 2	Sample 3
Fractions 9 & 10			
Fractions 11 & 12			
Stock Fractions			
TCP Fractions			

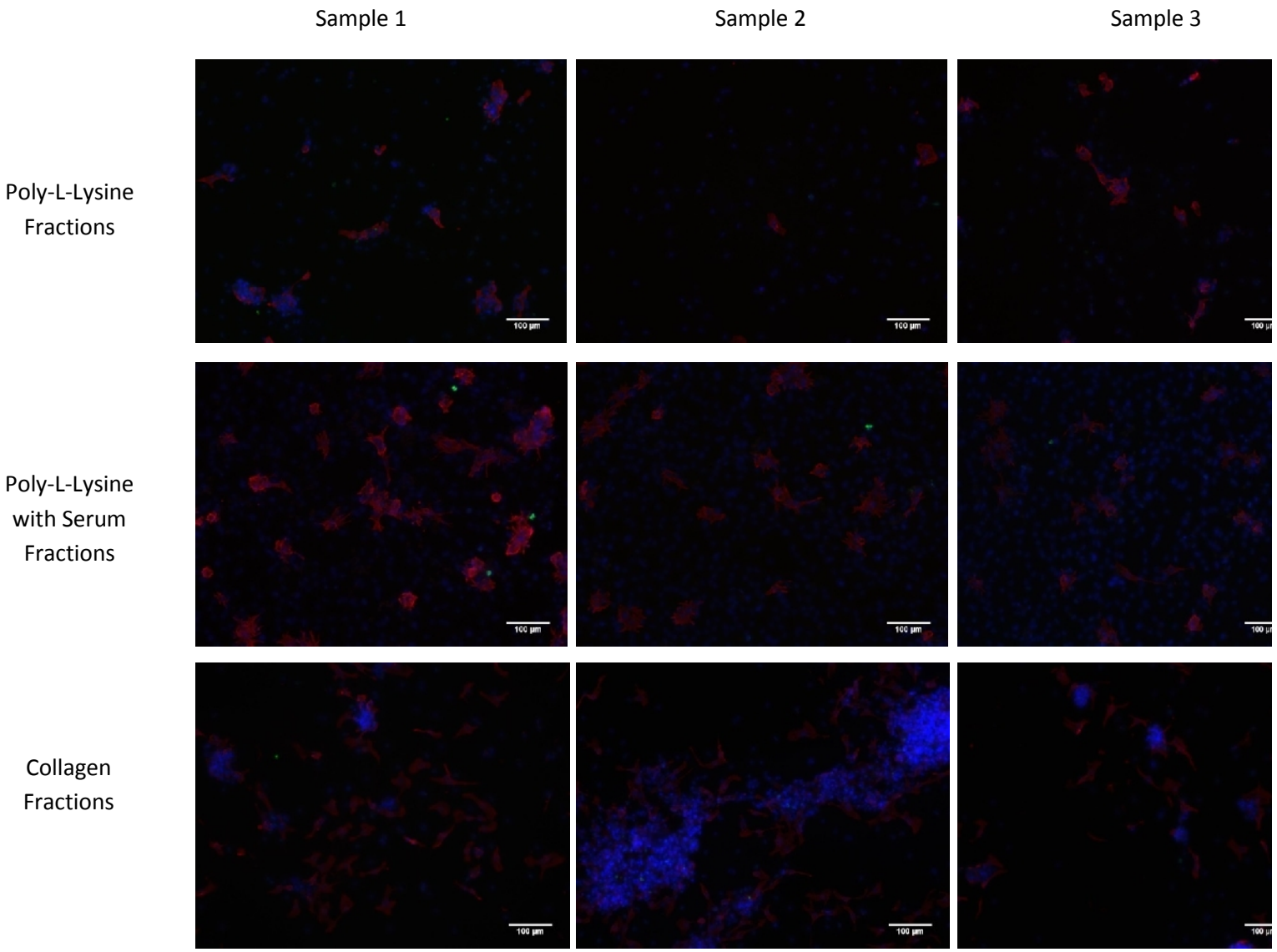
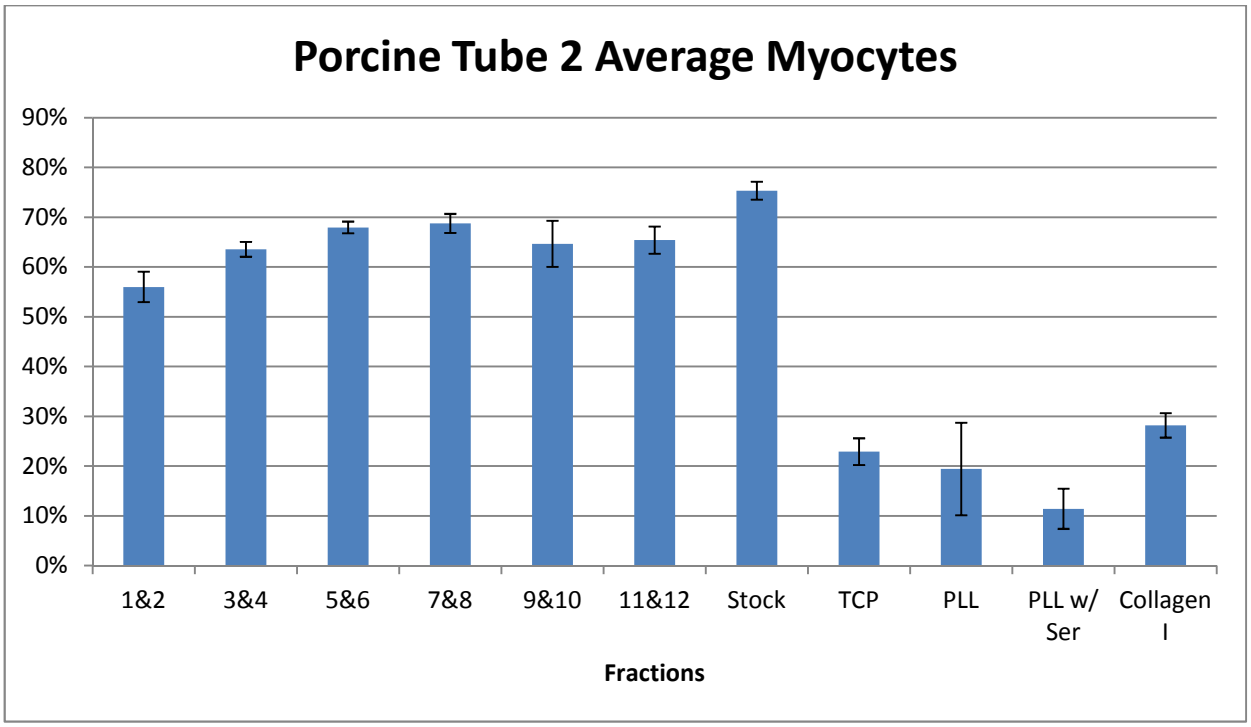


Figure 15: Representative images of α-actinin (red), PHH3 (green) and Hoescht (blue) for cardiomyocytes cultured on ECM coated tissue culture plastic. Scale bar = 100 µm

Quantitative Analysis:

The numbers of cells and cardiomyocytes present was determined using a customized CellProfiler pipeline developed by Joshua Resnikoff and Corin Williams. Once set, the system automatically removed background fluorescence and counted cells stained with Hoescht (DAPI), α-actinin (TRITC) and PHH3 (GFP). Figure 16 shows the result of the percent population of cardiomyocytes that were detected. Across the fractions, at least half of the cells counted were found to be cardiomyocytes, but only less than half of those found on the TCP, poly-L-lysine and collagen I. There as a statistically significant difference in the ANOVA results for the different conditions, with $p<0.05$. Pairwise comparison of the conditions showed the mid-range fractions (3&4, 5&6 and 7&8) displaying some significant difference to the standard conditions, but there were inconclusive results when compared to the low and high range fractions.

The percentage of cells that were fixed during mitosis was rather very small. As all conditions were fixed after 5 days, the cells may possibly have been approaching confluency and thus would slow down their proliferation. For numbers pertaining to proliferation, values were counted manually as CellProfiler was unable to distinguish proliferating cells from other background emissions. A few of the images obtained from the microscope had not stained properly and thus had artifacts that disturbed the pipelines methodology. However, for all conditions tested for the porcine ECM, the percentage of proliferating cells (both cardiomyocytes and non-cardiomyocytes) was found to be below 1% throughout (Figure 17). Although the numbers showed statistical difference between the fractions and the standards ($p<0.05$), there was no significance to the data obtained when each fraction was compared with another one. Of note however was the observation that of those few proliferating cells, at least half of them were cardiomyocytes for each of the fractions, but no more than 32% were cardiomyocytes on the standards, with collagen being the only exception with an average of 64% cardiomyocytes.



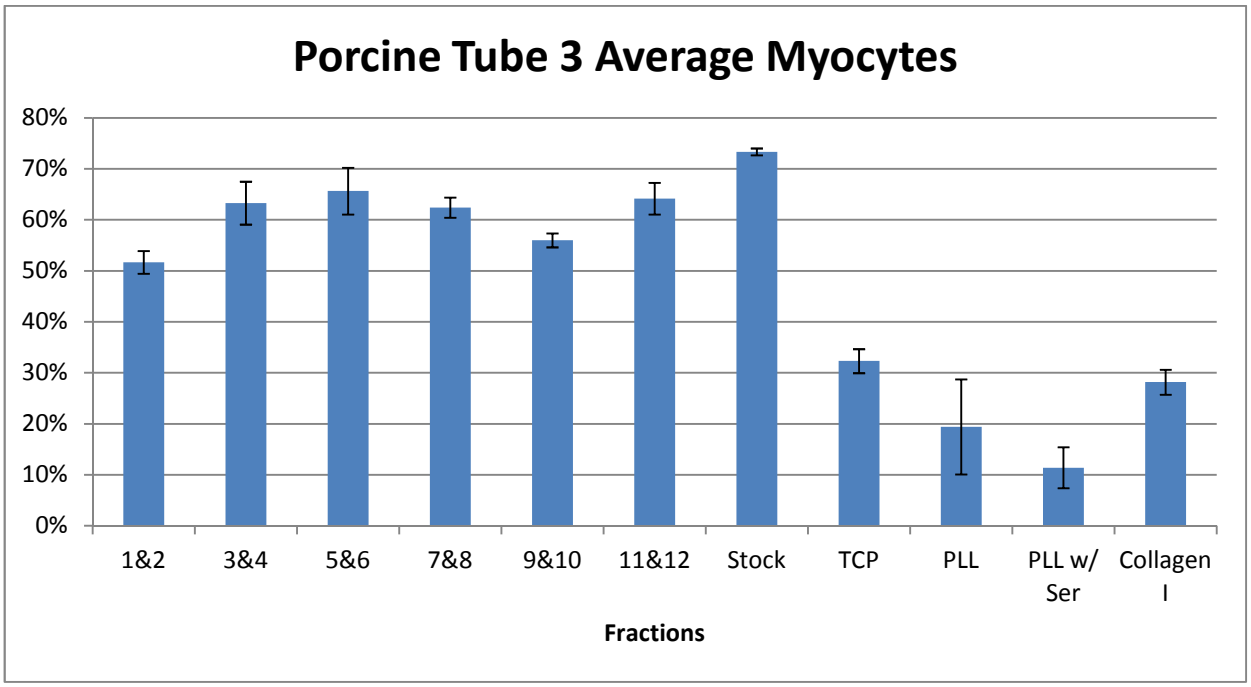
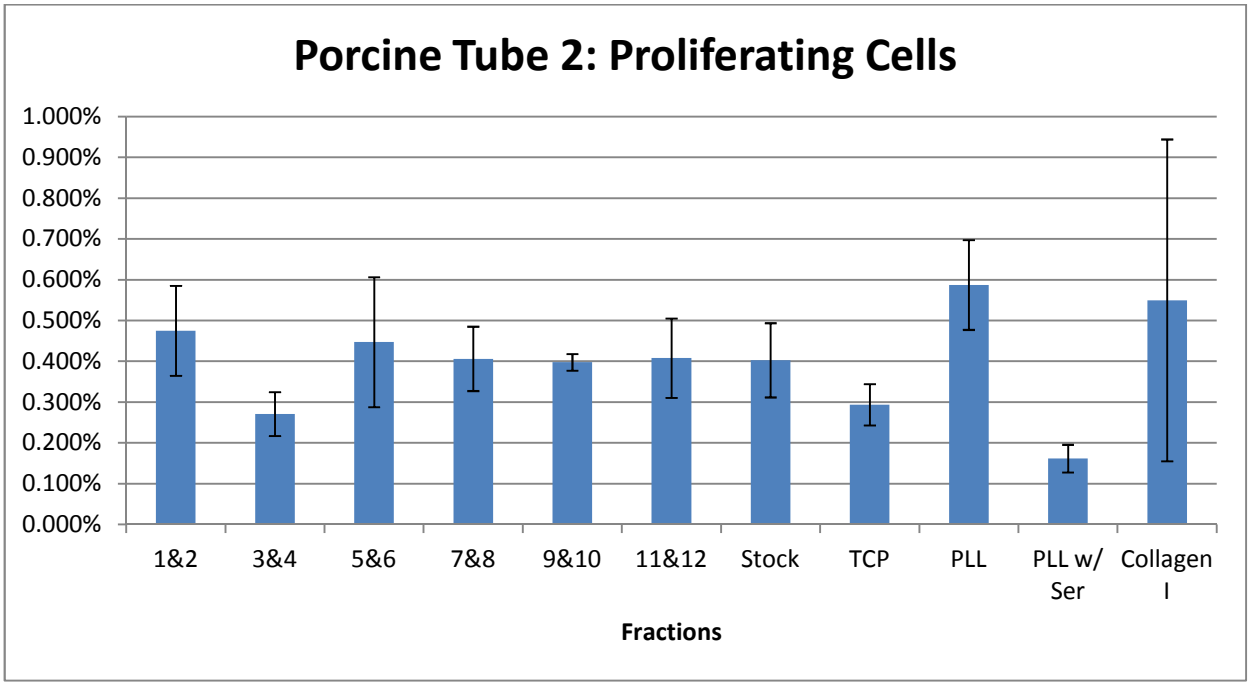


Figure 16: Average number of cardiomyocytes as a percent of total cells present for each condition for 2 samples of porcine ECM



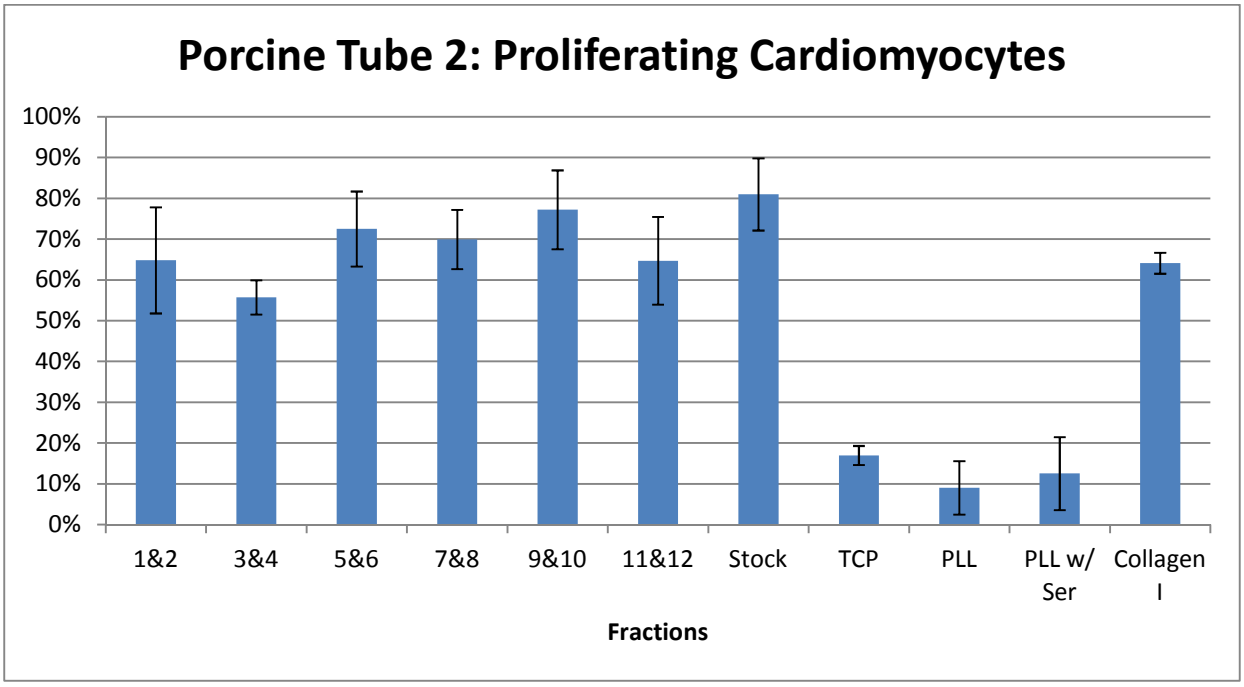


Figure 17: Percentage of all proliferating cells on all conditions and the percent of those cells proliferating that were cardiomyocytes

5.0 Discussion

The goal of this study was to devise a purification procedure for the fractionation of ECM proteins and polypeptides. In developing this protocol, adult porcine and adult Sprague-Dawley rat cardiac tissue was de-celled and digested using pepsin. The digested peptides were then run through a size-exclusion chromatography column with two different resins in order to separate the peptides by their respective sizes. Fractions obtained from these runs were then analyzed using a protein assay kit and using the selectivity curves of one of the resins, an attempt to determine the respective molecular weights of each fraction was made. Gel electrophoresis and imperial staining were also employed in an effort to estimate molecular weights.

The fractions obtained thus allowed us to test what effect peptide size may have on cardiomyocyte proliferation when the cells are cultured on tissue culture plastic coated with each fraction and compared with pure collagen, poly-L-lysine and regular plastic.

5.1 Development of a Fractionation Protocol

Determining ECM concentration: The first challenge to developing this protocol was to find an accurate and reliable method of determining how much ECM we had present in each solution. The protocol currently followed in our lab to digest de-celled cardiac tissue is done under the calculation that the resulting ECM will be at 10 mg/mL, though this has not been verified as the digested ECM is made up of a diverse mixture of proteins and peptides. The BCA assay was chosen as a good assay to monitor concentration but its weakness comes from the fact that the assay uses a purified singular protein, such as BSA to create standard curve. This is normally sufficient for most tests which the assay is run under but since ECM is known to contain a lot of other proteins and in varying proportions, using a singular protein fails to give an accurate estimate of a solution's actual concentration.

A hypothesis we decided to test was if digested ECM itself would work in creating a more accurate standard curve. Working under the assumption that our stock solution of ECM was at the nominal 10 mg/mL concentration and then diluting it accordingly, results showed that indeed ECM may be used as a standard when running the BCA assay. Not only can it be a standard, but the results also highlight the difference in the readings made from each standard curve. Based on the slopes of these lines (Figure 1), it is seen that BSA and collagen overestimate the concentrations in a given ECM solution. Thus, even though collagen I is a component of native ECM, collagen does not best approximate it for concentration readings in the BCA assay.

With the knowledge that ECM can be used to create a more accurate standard curve for itself, we then proceeded to examine whether there is any difference in the ECM samples obtained from different chambers of the heart. Since the right ventricle of the myocardium is responsible for pumping deoxygenated blood from the body while the left ventricle pumps oxygenated blood from the lungs, we wished to ensure that there are no differences exhibited in the ECM from both ventricles when running a BCA assay. To study this, we digested ECM from both ventricles using two slightly different methods: one the method already described for lyopholization and one where we froze the ECM with liquid nitrogen and pulverized it with mortar and pestle before digesting both setups with pepsin solution.

As the results obtained showed, the standard curves obtained for the right ventricle for both nitrogen and regular treatments gave almost identical curves. Added onto this, the curve for the left ventricle regular treatment also showed a close relation to the one for the right ventricle ECM. The left ventricle nitrogen treated curve was slightly different from the rest but when all the curves are plotted onto one graph as shown in Figure 4, the difference is quite negligible, especially compared to the curves for collagen I and BSA. Another interesting discovery we

made during this comparison was that the curves for rat ECM and porcine ECM are also slightly different form each other. This may be explained when the sizes of the different animals is taken into consideration. As ECM changes composition with age, so too is there a slight difference in the BCA standard curves given by ECM from two different species.

To check how much of an influence the pepsin itself was having on the readings, we also ran a standard curve for pure pepsin and the results confirmed that pepsin does indeed contribute to the readings. This was to be expected sine pepsin is a protein and the BCA assay would tag its macromolecules as well, but we concluded that this influence was rather small and since all samples to be tested were going to be digested in pepsin, then they would all have a consistent trend in how they are affected by the presence of the pepsin.

Phase I: Sephadex G-100 resin was used to develop the protocol. The resin itself has a fractionation range of 4 – 100 kDa which was thought to be a good estimate of the size range of the peptides to be studied, as evidenced by previous SDS-PAGE studies done by other members in the lab which showed that our digested ECM fractions have polypeptide mostly falling into this range.

The use of a trial column was decided upon as a check to see whether the resin can indeed fractionate digested ECM. Figure 6 shows the concentrations of 5 fractions obtained from 2 runs in columns of 3.3 cm^3 (small) and 5.5 cm^3 (large). Of particular interest, the results from these runs suggest the presence of a lot of small peptides which had concentrations of 918 μg/mL and 724 μg/mL for the small and large wash fractions respectively, which is higher than for any other fraction collected.

5.2 Determination of Molecular Weight Range of Fractions

Phase II: The phase II trials were conducted in a full-sized column, 11 mm ID and 300 mm tall. Using the Sephadex G-100 resin and following the procedure described in the Methods

section, standard proteins from a kit were run through the column and analyzed to determine a selectivity curve for the resin, which could then be used to estimate the molecular weight ranges of the ECM fractions. The protocol that was modified and followed in the experiment called for the use of a UV-Vis Spectrophotometer to read the absorbance of each fraction directly and construct a chromatogram from these data, but as the results showed, this approach would not yield any useful data as none of the peaks were either clear or resolved. A clear example of this is seen in Figure 7 from which I expected to obtain 2 discernible peaks for carbonic anhydrase and alcohol dehydrogenase, but instead obtained a mixture of readings that did not point to any peak.

Upon coming across this unexpected result, it was decided to try running the same samples I had run in the spectrophotometer, first by running a BCA assay and then back through the spectrophotometer. The results of this approach, shown in Figure 8 gave a much clearer picture of how the fractionation had occurred. Using these results, clear peaks could be discerned from which V_o and V_e values could be calculated to create the selectivity curve shown in Figure 9. As seen in Figure 8, the resin performed exceptionally well for proteins that fell within its range of 4 - 100 kDa, clearly resolving the 2 proteins, cytochrome c (12.4 kDa) and β-amylase (200 kDa). Although the β-amylase falls beyond the range for Sephadex G-100, the peaks were resolved as cytochrome c is much smaller and thus took much longer to flow through the column.

Also as expected, for the runs involving carbonic anhydrase (29 kDa) and alcohol dehydrogenase (150 kDa), the peaks were not as clearly resolved as we would have liked and we attributed this to the larger size of the alcohol dehydrogenase and also how both proteins have a smaller gap in weight and thus would elute closer to each other.

A look at the chromatogram for albumin (66 kDa) showed some level of tailing occurring in the column and this may have been due to some non-equilibrium conditions being present inside the resin. Another possible reason for this may be that there was some level of non-covalent interactions present between albumin and the resin material, which is dextran based. Such interactions like van der Waals attractions, although minimized because of the design of the resin, still do exist and if present to a significant amount with a particular protein, are the causes of tailing in chromatograms. The chromatogram for dextran (2000 kDa) was much sharper and clearly did not experience the same trouble flowing through the column.

Analysis of the selectivity curve (Figure 9) generated from the chromatograms also confirms the expected result that the resin would not be accurate for alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa) as these proteins fall beyond the trusted linear range of the curve. Figure 9 (2) shows the same selectivity curve without the two proteins and clearly shows a linear logarithmic curve, as was expected for this region of the selectivity curve.

Predicting Molecular Weights: Using the equation obtained from the solubility curve, an attempt was made to predict the average molecular weight of peptides that were collected from the column (Figure 12). The results showed a steady decline in the molecular weights as the fraction number increases as expected.

A point of caution that was noted when using this graph, the fractions used to determine the solubility curve were 1 mL each, the bed volume of the column was 30 cm^3 and the ECM fractions were 5 mL each with a total of 12 fractions or 60 mL. A good understanding of size-exclusion chromatography already suggested a problem with the setup of the column as all proteins eluted from the column are expected to be eluted within a volume *less* than the bed volume of 30 cm^3. But as can be seen in our results, we still had proteins eluting at volumes of

up to 60 cm^3. Although this does not discredit the validity of the findings, it does highlight a need for further analysis of the resin used and column construction.

Another unexpected result obtained was the presence of proteins in the very first fraction collected from the column. This may be attributed to a number of reasons especially the volatility of the BCA assay when used with the tris-HCl buffer we used in our experiments and also residual proteins that may have gotten leftover from previous runs.

Despite these discrepancies, Figure 12 was very useful in giving a good estimate of the sizes of the peptides that are present in the fractions. The estimated range in size was 6.2 kDa going down to peptides less than 1 kDa. As mentioned in the results section, the predicted molecular weights could only be relied on for fractions 2 to 7 as the other fractions gave molecular weights that are beyond what the linear region of the selectivity curve can accurately determine. The predictions were also useful in giving an idea of molecular weights as applying gel electrophoresis to the fractions proved to be disappointing . The results from the imperial stains did not show any significant protein being present despite multiple adjustments being made to the setup to make it more sensitive. One of the reasons attributed to this failure was the highly diluted nature of the fractions obtained which may have kept concentrations at levels below those that gel electrophoresis and imperial staining are sensitive to.

Phase III: A new resin was employed for phase 3 of the experiment as a way to broaden the fractionation range obtainable from the column and use a resin which may have less non-covalent interactions with the peptides. Similar standard proteins to those used in phase 2 were run through the column in an effort to plot a new selectivity curve. Unfortunately, the separation process did not happen as expected and thus the BCA results did not give a clear peak for any of the proteins. A number of factors had been changed in the setup of the new resin which include swelling time and length of time needed to settle but we did not have time to troubleshoot the

resin. As the results show, the resin was not well-packed and thus probably had channeling issues and possibly air bubbles as well.

5.3 Effects of ECM Fractions on Cardiomyocyte Proliferation

As can be clearly seen from the pictures shown in Figure 15, the cardiomyocytes proliferated more on the ECM fractions as compared to the standards. This also goes to confirm previous studies by other labs that ECM promotes proliferation (Ieda et al., 2009). A key result observed in this experiment was the effect of serum on the growth of organisms. Serum is known to increase proliferation of cells and thus all the conditions tested were run with serum-free media so as to narrow down the number of factors influencing the cardiomyocytes. As expected, the poly-L-lysine (PLL) sample with serum showed a large number of cells being present but since there wasn't any ECM for cardiomyocytes to bind to, the cardiomyocyte population remained very low.

The results of this experiment did prove that cardiomyocytes need components present in the ECM for them to proliferate as there was a statistically significant number of more cardiomyocytes on ECM and collagen I than in the other standards.

Since the cells are seeded onto the plates as an impure mixture of cardiomyocytes and other cells that include undifferentiated myoblasts, and fibroblasts, the proliferation of these other cells is was confirmed by the statistical analysis performed on the data. Specifically, the overall number of cardiomyocytes on the collagen sample was relatively low compared to the ECM fractions, but a close look at the number of proliferating cardiomyocytes on the sample show that a significant number of cardiomyocytes were indeed proliferating. This result points to the likelihood of how the non-cardiomyocytes had been proliferating much faster than the cardiomyocytes.

5.4 Conclusions

The results of this study showed that size-exclusion chromatography may be successfully used to fractionate digested ECM, with the fractions varying in size. The fractions obtained using this methodology were characterized by their concentration and molecular weight. To achieve this characterization, it was also noted that the BCA assay may be used to analyze fractions obtained from a column and that ECM can be used as its own standard. A study of the proliferation of cardiomyocytes on these fractions showed that significantly more cardiomyocytes were present on the fractions than on the standards. Overall, fractions 5 to 7 (with molecular weights of about 1 kDa to 6.2 kDa) were seen to closely resemble proliferation data obtained from unfractionated ECM, suggesting that these may be the fractions to focus future studies on as they may have the specific protein I sought to find.

6.0 Future Directions

The impact of native ECM on tissue engineering constructs will continue to increase as more of its function and components are better understood. Future studies may focus on a number of observations made in this project which, if successful could lead to the creation of better constructs and an understanding of how we can use ECM to influence cardiomyocyte proliferation. In its global application, such knowledge could someday lead to more clinically relevant techniques of inducing myocardial regeneration. Lessons learned in this study may be applied in other size-exclusion chromatography setups for fractionating ECM, while new insights gained as a result of cardiomyocyte proliferation may be used in other separation techniques.

The process of digesting ECM is of importance to the success of any separation technique that is executed afterwards. In our experiment, we used pepsin digestion, but pepsin is a nondiscriminatory enzyme which cleaves peptides randomly. Its use is convenient for most studies but the inability to control how much digestion occurs can greatly influence the results. Digesting ECM for specified times also proved to give some level of variation in the ECM obtained. Alternatively, future studies may use urea instead of pepsin to digest the ECM and this should hopefully give more batch-to-batch consistency.

With the fractions obtained in the size-exclusion chromatography, more studies can be done as to what importance any fraction may hold in promoting proliferation or differentiation in stem cells. As stem cells are also another key aspect of EHT, learning more about which specific peptide or peptides influence their differentiation into cardiomyocytes would go a long way in improving constructs.

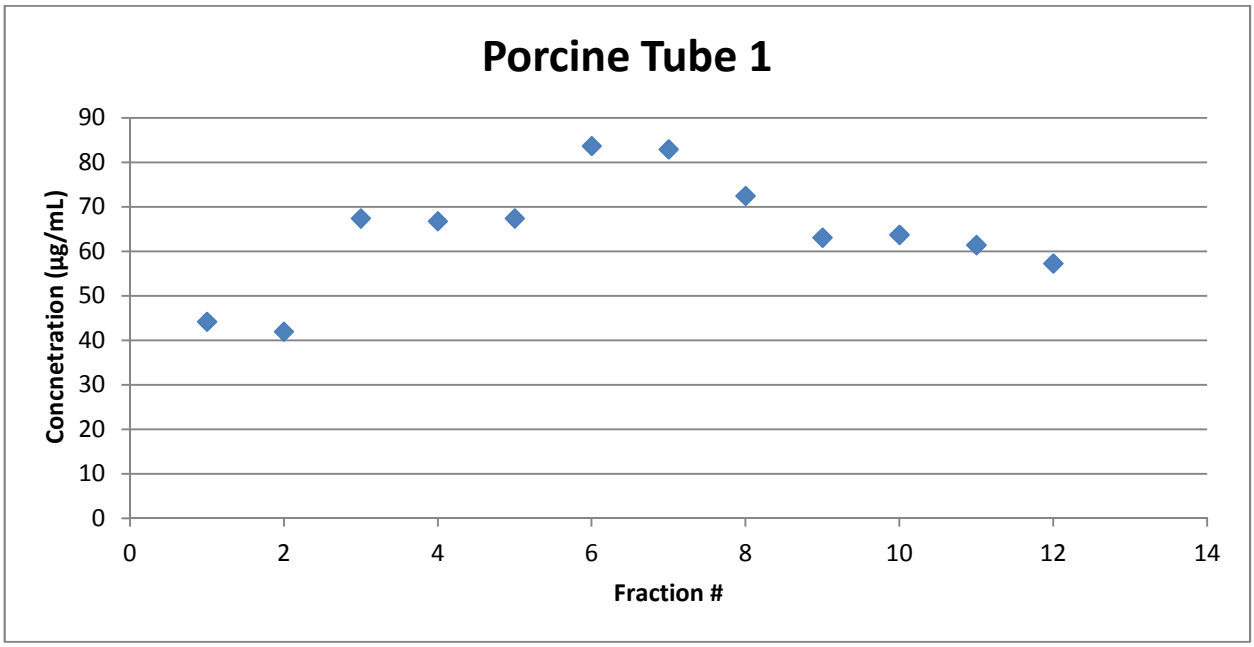
Lastly, the fractionation of ECM based on other characteristics may prove to be worthwhile. In this study, molecular weight was the key point of interest but other properties that

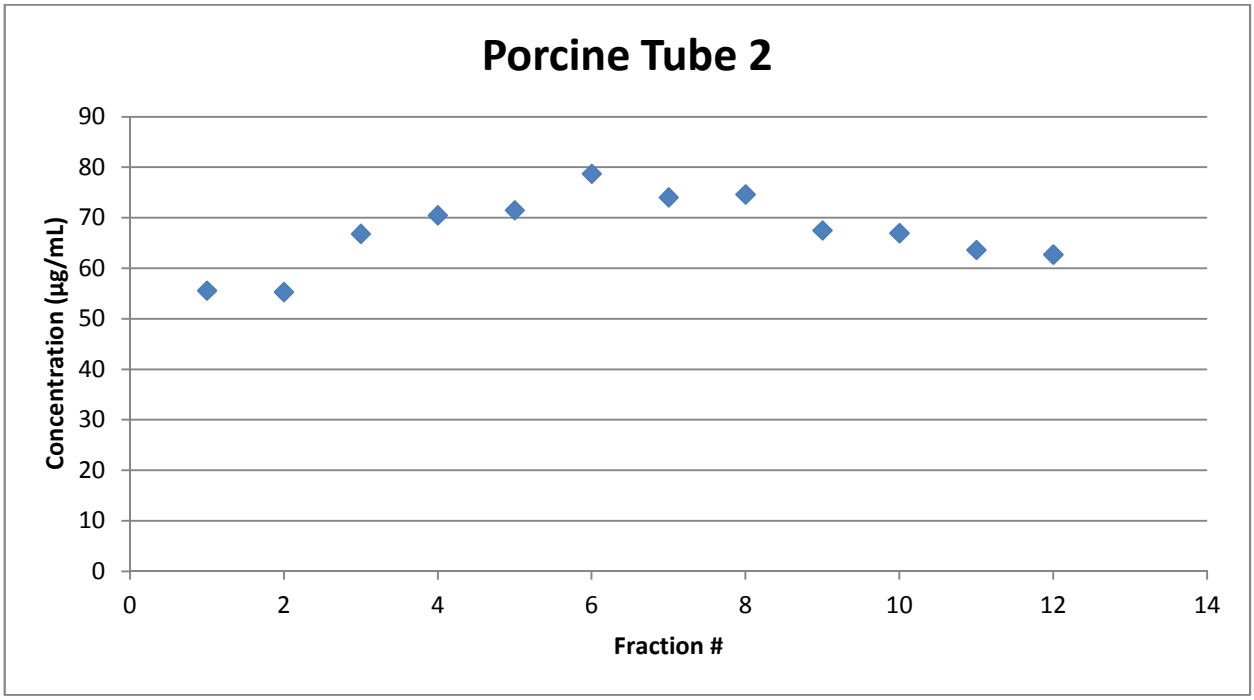
are utilized in the protein purification industry include charge, hydrophobicity and affinity. Fractionating digested ECM via some of these methods would help elucidate more features of the peptides responsible for proliferation and differentiation. With greater insight into these peptides, researchers may then be able to produce these peptides in large quantities and save on using uncharacterized batches of ECM in the hope that each batch has the necessary ingredient needed.

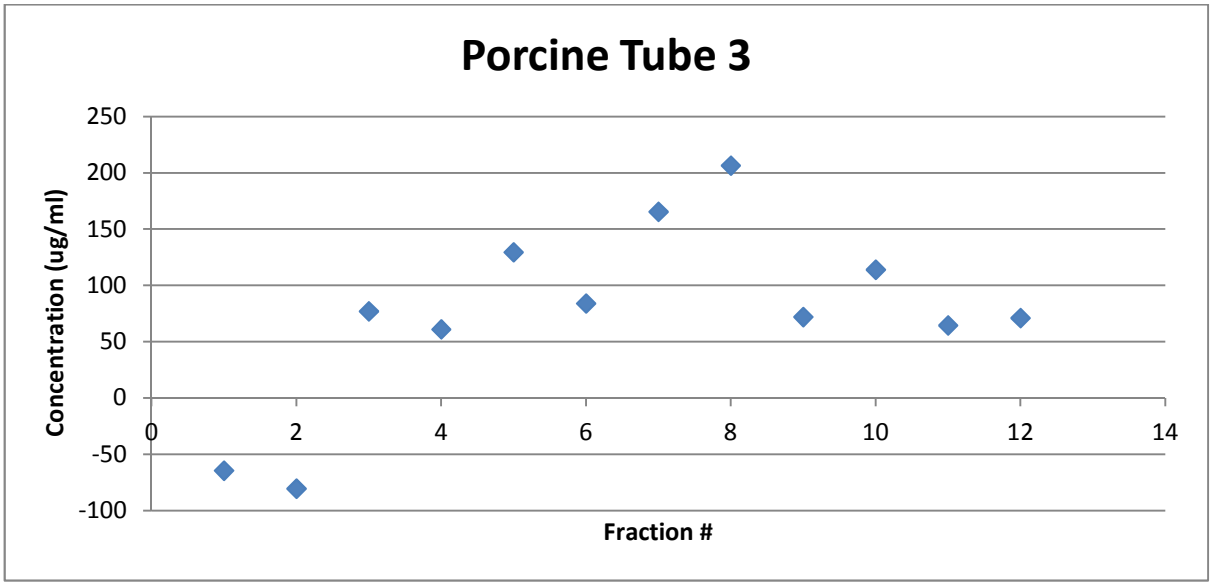
7.0 Appendix

7.1 Extra Figures

This section of the appendix highlights extra figures of results obtained in the various phases and trials of developing a protocol and characterizing fractions.







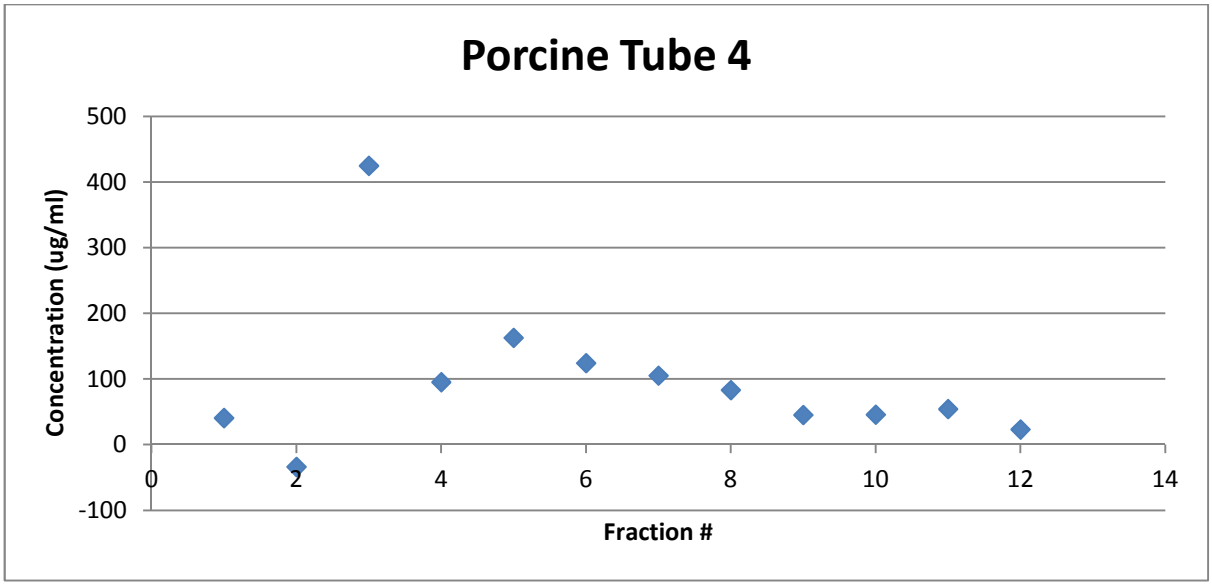


Figure 18: BCA results of concentrations of 4 samples of porcine ECM

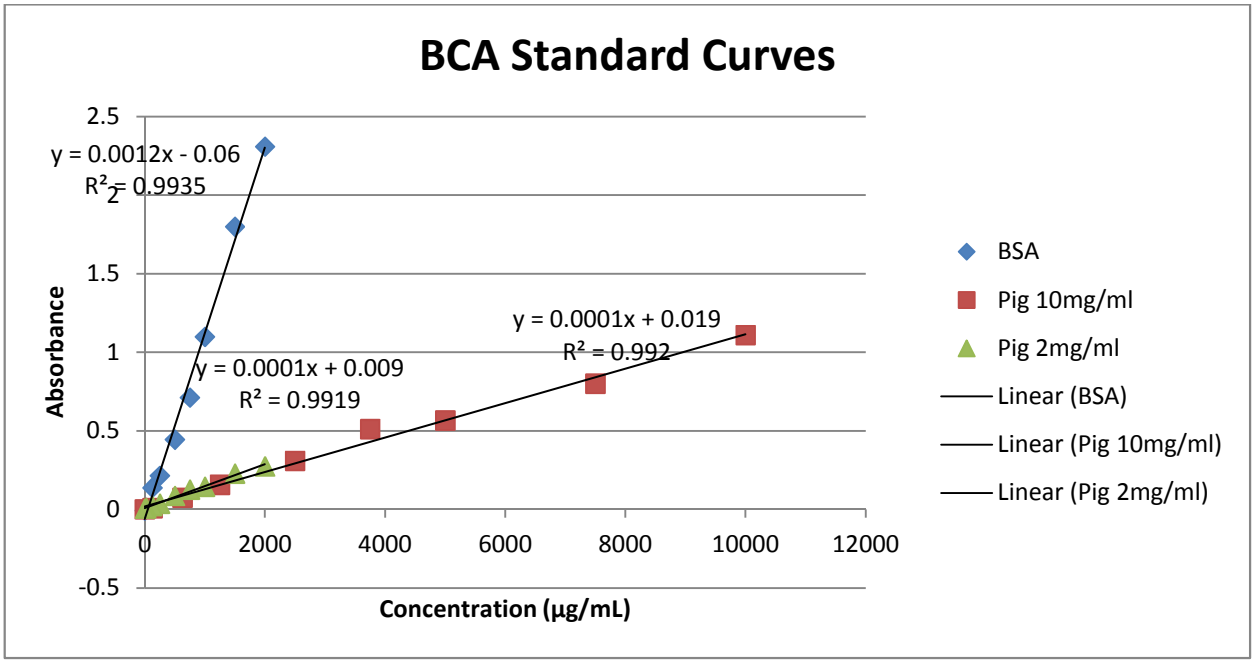
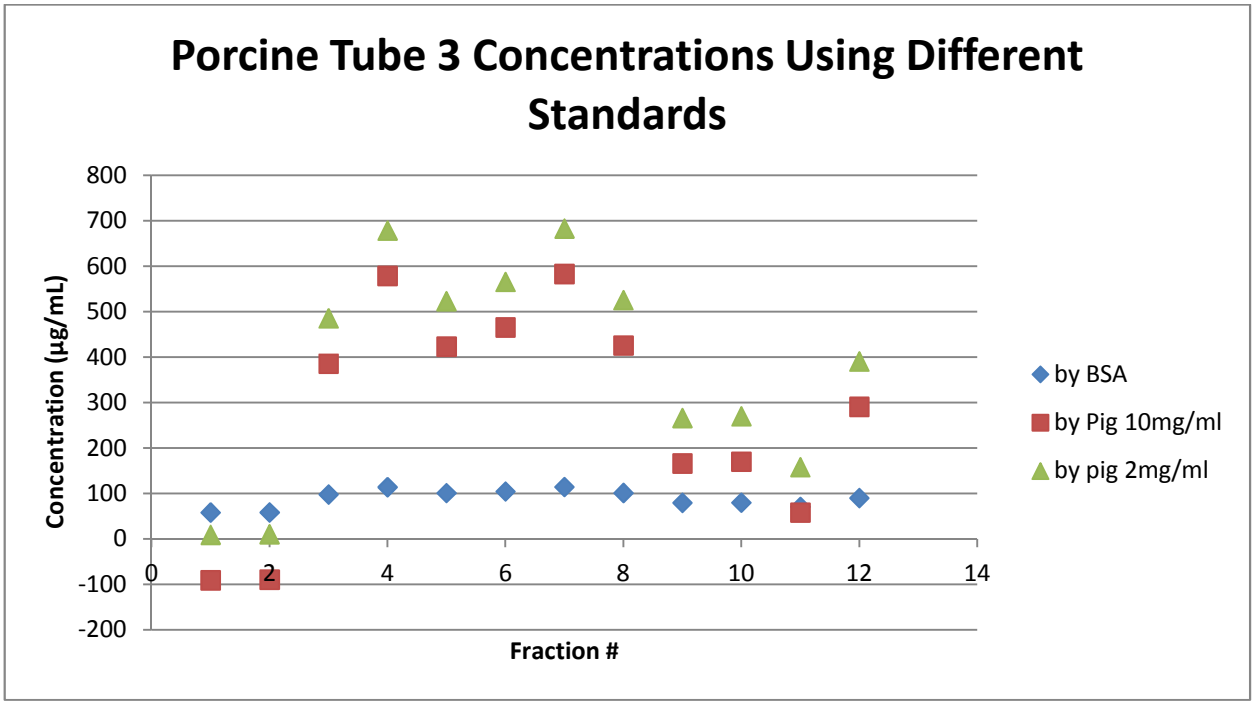


Figure 19: BCA standard curves comparing BCA and porcine ECM



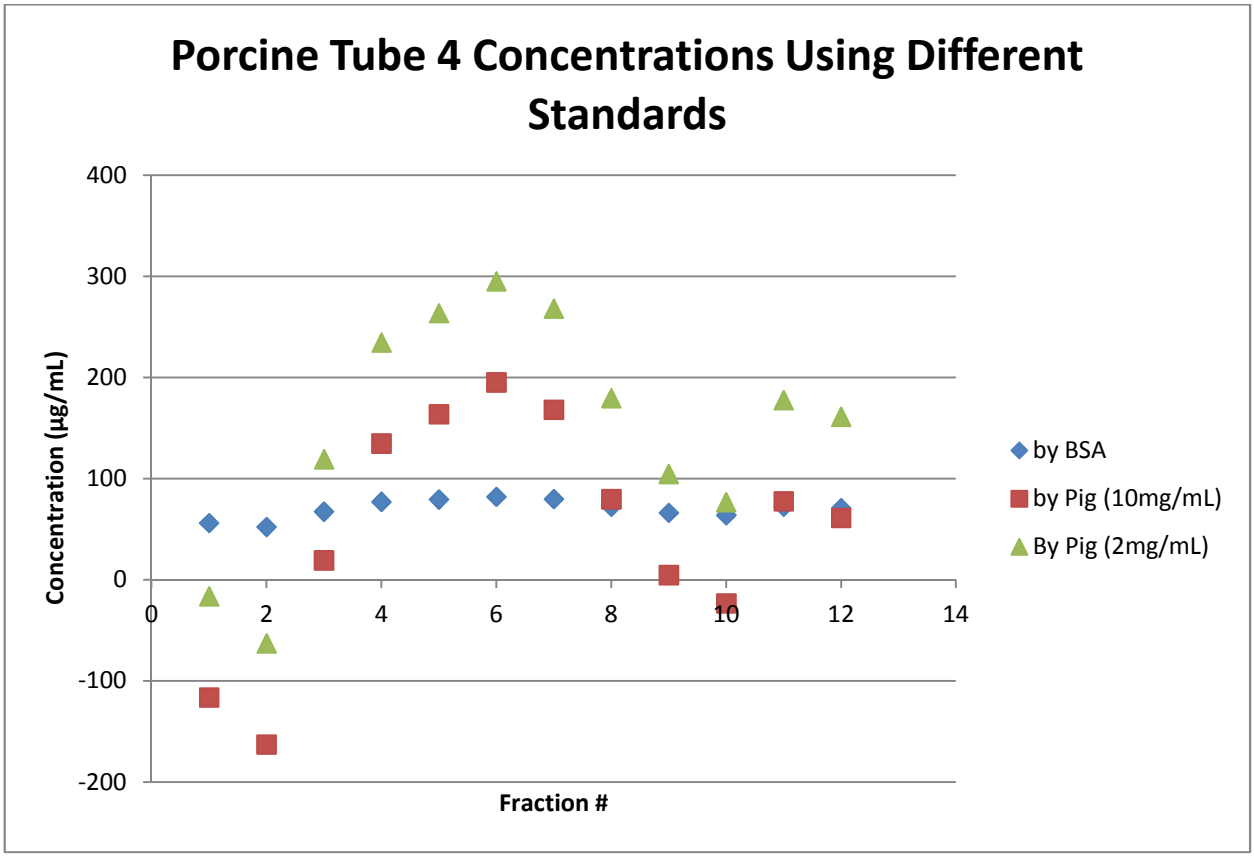
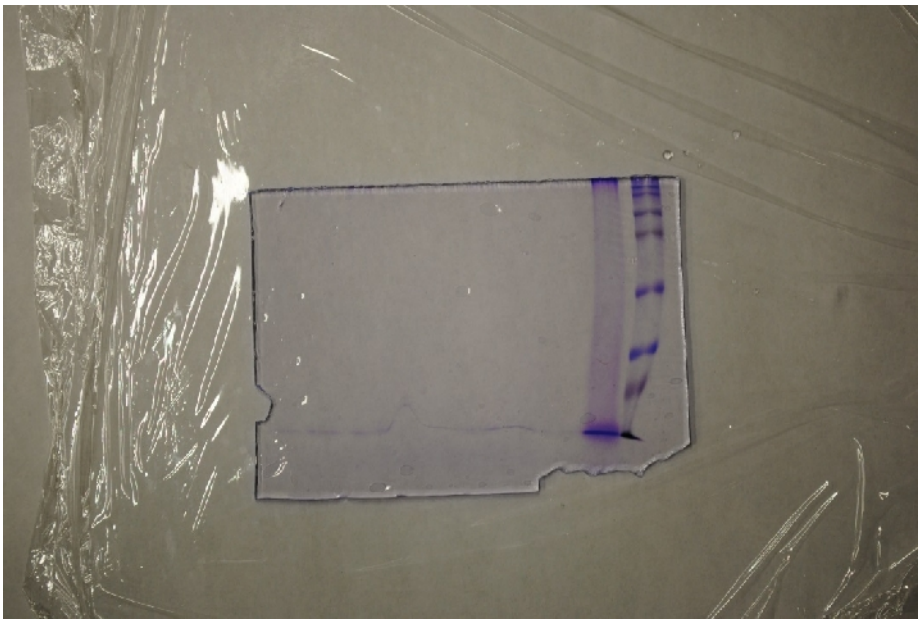


Figure 20: BCA results showing concentrations of fractions obtained using different BCA standards



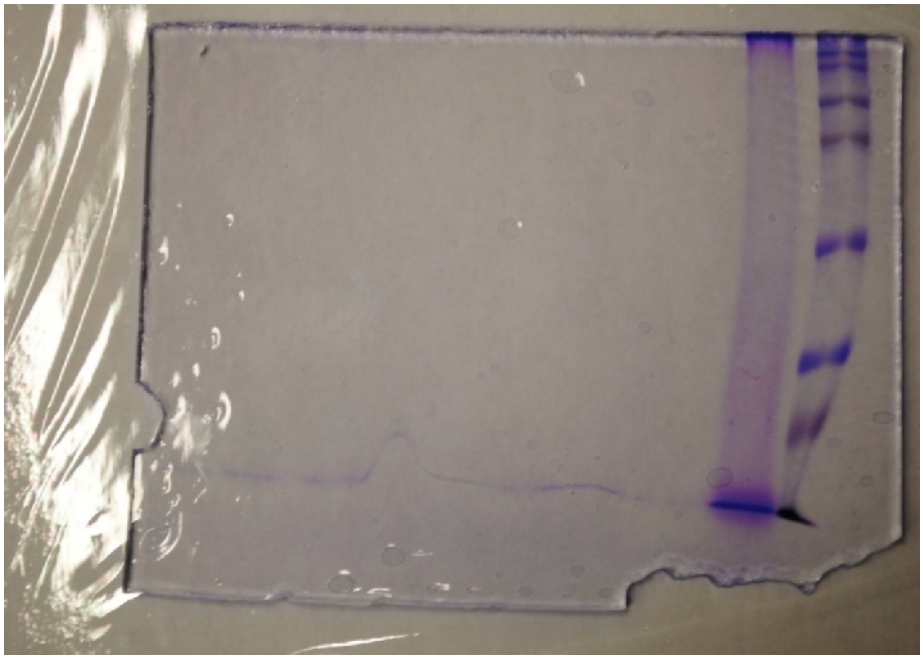


Figure 21: Imperial Stain results of PAGE gels showing the ladder, a single band for the stock solution and no bands for the fractions

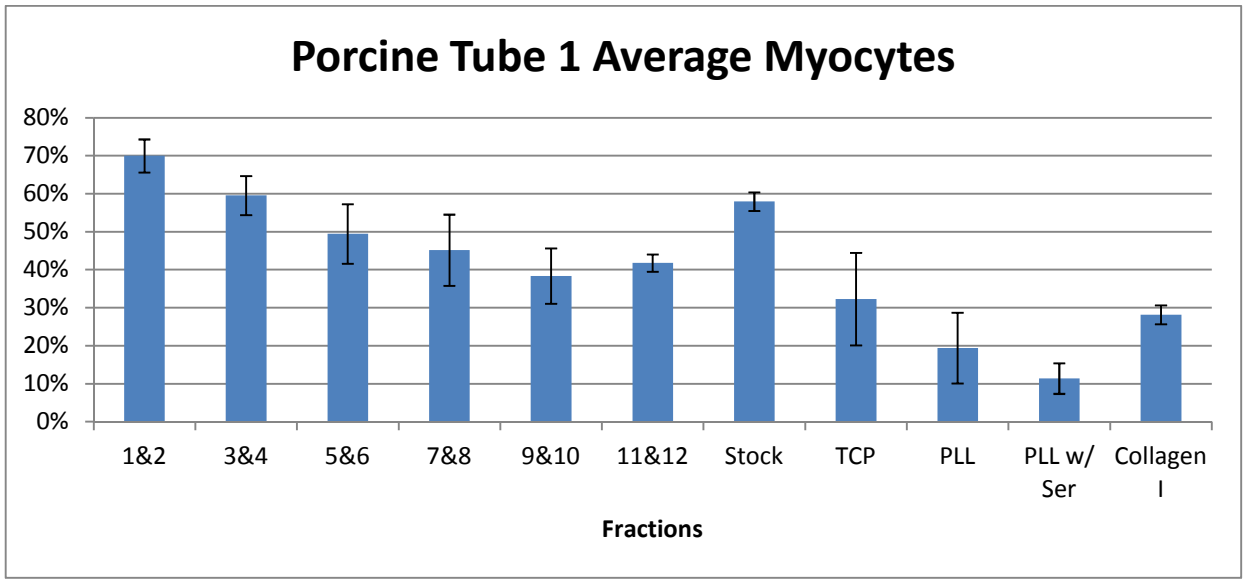


Figure 22: Average number of cardiomyocytes as a percentage of all cells present at each condition

7.2 Statistical Analysis

This section of the appendix contains the ANOVA results obtained using SigmaPlot on the results obtained from CellProfiler. One way ANOVA tests were run for three sets of fractions of porcine ECM on how many of all the cells present were cardiomyocytes. Extra tests were run on the percent proliferating cells and proliferating cardiomyocytes for one tube. $p<0.05$ was used as the baseline for statistical significance and a Tukey test was run on any sets that showed significance.

a. Porcine Tube 3 Samples: % Cardiomyocytes

One Way Analysis of Variance Wednesday, April 17, 2013, 4:05:56 PM

Data source: Data 1 in Notebook2

Group Name	N	Missing	Mean	Std Dev	SEM
1&2	3	0	0.517	0.0272	0.0157
3&4	3	0	0.633	0.0514	0.0297
5&6	3	0	0.656	0.0559	0.0323
7&8	3	0	0.624	0.0243	0.0140
9&10	3	0	0.560	0.0167	0.00962
11&12	3	0	0.642	0.0379	0.0219
Stock	3	0	0.733	0.00841	0.00485
TCP	3	0	0.323	0.0288	0.0166
PLL No Serum	5	0	0.194	0.104	0.0465
PLL Serum	4	0	0.114	0.0465	0.0233
Collagen 1	2	0	0.282	0.0349	0.0247

Source of Variation	DF	SS	MS	F	P
Between Groups	10	1.601	0.160	54.554	<0.001
Residual	24	0.0704	0.00293		
Total	34	1.671			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.200: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Stock vs. PLL Serum	0.619	11	21.169	<0.001	Yes
Stock vs. PLL No Serum	0.539	11	19.278	<0.001	Yes
Stock vs. Collagen 1	0.452	11	12.916	<0.001	Yes
Stock vs. TCP	0.410	11	13.115	<0.001	Yes
Stock vs. 1&2	0.216	11	6.921	0.002	Yes
Stock vs. 9&10	0.174	11	5.548	0.022	Yes

Stock vs. 7&8	0.109	11	3.494	0.368	No
Stock vs. 3&4	0.101	11	3.215	0.481	Do Not Test
Stock vs. 11&12	0.0917	11	2.931	0.606	Do Not Test
Stock vs. 5&6	0.0770	11	2.462	0.801	Do Not Test
5&6 vs. PLL Serum	0.542	11	18.537	<0.001	Yes
5&6 vs. PLL No Serum	0.462	11	16.525	<0.001	Yes
5&6 vs. Collagen 1	0.375	11	10.714	<0.001	Yes
5&6 vs. TCP	0.333	11	10.653	<0.001	Yes
5&6 vs. 1&2	0.139	11	4.459	0.112	No
5&6 vs. 9&10	0.0965	11	3.086	0.537	Do Not Test
5&6 vs. 7&8	0.0323	11	1.031	1.000	Do Not Test
5&6 vs. 3&4	0.0235	11	0.753	1.000	Do Not Test
5&6 vs. 11&12	0.0146	11	0.468	1.000	Do Not Test
11&12 vs. PLL Serum	0.528	11	18.036	<0.001	Yes
11&12 vs. PLL No Serum	0.448	11	16.002	<0.001	Yes
11&12 vs. Collagen 1	0.360	11	10.295	<0.001	Yes
11&12 vs. TCP	0.319	11	10.185	<0.001	Yes
11&12 vs. 1&2	0.125	11	3.991	0.209	Do Not Test
11&12 vs. 9&10	0.0819	11	2.618	0.740	Do Not Test
11&12 vs. 7&8	0.0176	11	0.563	1.000	Do Not Test
11&12 vs. 3&4	0.00889	11	0.284	1.000	Do Not Test
3&4 vs. PLL Serum	0.519	11	17.732	<0.001	Yes
3&4 vs. PLL No Serum	0.439	11	15.684	<0.001	Yes
3&4 vs. Collagen 1	0.351	11	10.041	<0.001	Yes
3&4 vs. TCP	0.310	11	9.901	<0.001	Yes
3&4 vs. 1&2	0.116	11	3.706	0.292	Do Not Test
3&4 vs. 9&10	0.0730	11	2.333	0.845	Do Not Test
3&4 vs. 7&8	0.00872	11	0.279	1.000	Do Not Test
7&8 vs. PLL Serum	0.510	11	17.434	<0.001	Yes
7&8 vs. PLL No Serum	0.430	11	15.372	<0.001	Yes
7&8 vs. Collagen 1	0.342	11	9.792	<0.001	Yes
7&8 vs. TCP	0.301	11	9.622	<0.001	Yes
7&8 vs. 1&2	0.107	11	3.428	0.393	Do Not Test
7&8 vs. 9&10	0.0643	11	2.055	0.921	Do Not Test
9&10 vs. PLL Serum	0.446	11	15.238	<0.001	Yes
9&10 vs. PLL No Serum	0.366	11	13.075	<0.001	Yes
9&10 vs. Collagen 1	0.278	11	7.954	<0.001	Yes
9&10 vs. TCP	0.237	11	7.567	<0.001	Yes
9&10 vs. 1&2	0.0429	11	1.373	0.995	Do Not Test
1&2 vs. PLL Serum	0.403	11	13.770	<0.001	Yes
1&2 vs. PLL No Serum	0.323	11	11.540	<0.001	Yes
1&2 vs. Collagen 1	0.235	11	6.726	0.003	Yes
1&2 vs. TCP	0.194	11	6.194	0.008	Yes
TCP vs. PLL Serum	0.209	11	7.148	0.002	Yes
TCP vs. PLL No Serum	0.129	11	4.615	0.090	No
TCP vs. Collagen 1	0.0415	11	1.186	0.998	Do Not Test
Collagen 1 vs. PLL Serum	0.168	11	5.054	0.047	Yes
Collagen 1 vs. PLL No Serum	0.0876	11	2.734	0.692	Do Not Test
PLL No Serum vs. PLL Serum	0.0800	11	3.114	0.524	No

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

b. Porcine Tube 2 Samples: % Cardiomyocytes

One Way Analysis of Variance Wednesday, April 17, 2013, 4:08:23 PM

Data source: tube 2 in Notebook2

Group Name	N	Missing	Mean	Std Dev	SEM
1&2	3	0	0.560	0.0372	0.0215
3&4	3	0	0.635	0.0184	0.0106
5&6	3	0	0.679	0.0144	0.00831
7&8	3	0	0.687	0.0235	0.0136
9&10	3	0	0.646	0.0566	0.0327
11&12	3	0	0.654	0.0334	0.0193
Stock	3	0	0.753	0.0220	0.0127
TCP	3	0	0.229	0.0328	0.0189
PLL No Serum	5	0	0.194	0.104	0.0465
PLL Serum	4	0	0.114	0.0465	0.0233
Collagen 1	2	0	0.282	0.0349	0.0247

Source of Variation	DF	SS	MS	F	P
Between Groups	10	1.947	0.195	69.018	<0.001
Residual	24	0.0677	0.00282		
Total	34	2.015			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.200: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Stock vs. PLL Serum	0.639	11	22.282	<0.001	Yes
Stock vs. PLL No Serum	0.559	11	20.386	<0.001	Yes
Stock vs. TCP	0.524	11	17.096	<0.001	Yes
Stock vs. Collagen 1	0.472	11	13.753	<0.001	Yes
Stock vs. 1&2	0.193	11	6.306	0.006	Yes
Stock vs. 3&4	0.118	11	3.843	0.249	No
Stock vs. 9&10	0.107	11	3.486	0.370	Do Not Test
Stock vs. 11&12	0.0994	11	3.241	0.470	Do Not Test
Stock vs. 5&6	0.0740	11	2.412	0.819	Do Not Test
Stock vs. 7&8	0.0659	11	2.150	0.899	Do Not Test
7&8 vs. PLL Serum	0.573	11	19.983	<0.001	Yes
7&8 vs. PLL No Serum	0.493	11	17.982	<0.001	Yes
7&8 vs. TCP	0.458	11	14.946	<0.001	Yes
7&8 vs. Collagen 1	0.406	11	11.829	<0.001	Yes
7&8 vs. 1&2	0.127	11	4.156	0.169	No
7&8 vs. 3&4	0.0519	11	1.693	0.977	Do Not Test
7&8 vs. 9&10	0.0410	11	1.335	0.996	Do Not Test
7&8 vs. 11&12	0.0334	11	1.091	0.999	Do Not Test
7&8 vs. 5&6	0.00801	11	0.261	1.000	Do Not Test
5&6 vs. PLL Serum	0.565	11	19.704	<0.001	Yes
5&6 vs. PLL No Serum	0.485	11	17.690	<0.001	Yes
5&6 vs. TCP	0.450	11	14.685	<0.001	Yes
5&6 vs. Collagen 1	0.398	11	11.596	<0.001	Yes

5&6 vs. 1&2	0.119	11	3.895	0.234	Do Not Test
5&6 vs. 3&4	0.0439	11	1.432	0.993	Do Not Test
5&6 vs. 9&10	0.0329	11	1.074	0.999	Do Not Test
5&6 vs. 11&12	0.0254	11	0.829	1.000	Do Not Test
11&12 vs. PLL Serum	0.540	11	18.818	<0.001	Yes
11&12 vs. PLL No Serum	0.460	11	16.762	<0.001	Yes
11&12 vs. TCP	0.425	11	13.855	<0.001	Yes
11&12 vs. Collagen 1	0.372	11	10.854	<0.001	Yes
11&12 vs. 1&2	0.0940	11	3.065	0.546	Do Not Test
11&12 vs. 3&4	0.0185	11	0.602	1.000	Do Not Test
11&12 vs. 9&10	0.00751	11	0.245	1.000	Do Not Test
9&10 vs. PLL Serum	0.532	11	18.556	<0.001	Yes
9&10 vs. PLL No Serum	0.452	11	16.489	<0.001	Yes
9&10 vs. TCP	0.417	11	13.610	<0.001	Yes
9&10 vs. Collagen 1	0.365	11	10.635	<0.001	Yes
9&10 vs. 1&2	0.0865	11	2.820	0.654	Do Not Test
9&10 vs. 3&4	0.0110	11	0.357	1.000	Do Not Test
3&4 vs. PLL Serum	0.521	11	18.174	<0.001	Yes
3&4 vs. PLL No Serum	0.441	11	16.089	<0.001	Yes
3&4 vs. TCP	0.406	11	13.253	<0.001	Yes
3&4 vs. Collagen 1	0.354	11	10.315	<0.001	Yes
3&4 vs. 1&2	0.0755	11	2.463	0.800	Do Not Test
1&2 vs. PLL Serum	0.446	11	15.541	<0.001	Yes
1&2 vs. PLL No Serum	0.366	11	13.335	<0.001	Yes
1&2 vs. TCP	0.331	11	10.790	<0.001	Yes
1&2 vs. Collagen 1	0.278	11	8.112	<0.001	Yes
Collagen 1 vs. PLL Serum	0.168	11	5.154	0.040	Yes
Collagen 1 vs. PLL No Serum	0.0876	11	2.789	0.668	No
Collagen 1 vs. TCP	0.0528	11	1.538	0.988	Do Not Test
TCP vs. PLL Serum	0.115	11	4.006	0.205	No
TCP vs. PLL No Serum	0.0349	11	1.272	0.997	Do Not Test
PLL No Serum vs. PLL Serum	0.0800	11	3.176	0.497	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

c. Porcine Tube 1 Samples: % Cardiomyocytes

One Way Analysis of Variance Wednesday, April 17, 2013, 4:09:49 PM

Data source: tube 1 in Notebook2

Group Name	N	Missing	Mean	Std Dev	SEM
1&2	3	0	0.700	0.0533	0.0308
3&4	3	0	0.596	0.0631	0.0364
5&6	3	1	0.495	0.111	0.0783
7&8	3	0	0.452	0.115	0.0662
9&10	2	0	0.384	0.103	0.0727
11&12	3	0	0.418	0.0277	0.0160
Stock	3	0	0.580	0.0297	0.0171
TCP	3	0	0.229	0.0328	0.0189
PLL No Serum	5	0	0.194	0.104	0.0465
PLL Serum	4	0	0.114	0.0465	0.0233
Collagen 1	2	0	0.282	0.0349	0.0247

Source of Variation	DF	SS	MS	F	P
Between Groups	10	1.155	0.115	21.309	<0.001
Residual	22	0.119	0.00542		
Total	32	1.274			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.200: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
1&2 vs. PLL Serum	0.586	11	14.734	<0.001	Yes
1&2 vs. PLL No Serum	0.506	11	13.304	<0.001	Yes
1&2 vs. TCP	0.471	11	11.078	<0.001	Yes
1&2 vs. Collagen 1	0.418	11	8.799	<0.001	Yes
1&2 vs. 9&10	0.316	11	6.650	0.004	Yes
1&2 vs. 11&12	0.282	11	6.635	0.004	Yes
1&2 vs. 7&8	0.248	11	5.833	0.015	Yes
1&2 vs. 5&6	0.205	11	4.317	0.141	No
1&2 vs. Stock	0.120	11	2.830	0.650	Do Not Test
1&2 vs. 3&4	0.104	11	2.454	0.803	Do Not Test
3&4 vs. PLL Serum	0.481	11	12.110	<0.001	Yes
3&4 vs. PLL No Serum	0.401	11	10.560	<0.001	Yes
3&4 vs. TCP	0.367	11	8.624	<0.001	Yes
3&4 vs. Collagen 1	0.314	11	6.604	0.005	Yes
3&4 vs. 9&10	0.212	11	4.456	0.117	No
3&4 vs. 11&12	0.178	11	4.181	0.168	Do Not Test
3&4 vs. 7&8	0.144	11	3.379	0.415	Do Not Test
3&4 vs. 5&6	0.101	11	2.122	0.905	Do Not Test
3&4 vs. Stock	0.0160	11	0.376	1.000	Do Not Test
Stock vs. PLL Serum	0.465	11	11.708	<0.001	Yes
Stock vs. PLL No Serum	0.385	11	10.139	<0.001	Yes
Stock vs. TCP	0.351	11	8.248	<0.001	Yes

Stock vs. Collagen 1	0.298	11	6.267	0.008	Yes
Stock vs. 9&10	0.196	11	4.119	0.182	Do Not Test
Stock vs. 11&12	0.162	11	3.805	0.265	Do Not Test
Stock vs. 7&8	0.128	11	3.002	0.575	Do Not Test
Stock vs. 5&6	0.0849	11	1.786	0.966	Do Not Test
5&6 vs. PLL Serum	0.381	11	8.443	<0.001	Yes
5&6 vs. PLL No Serum	0.301	11	6.902	0.003	Yes
5&6 vs. TCP	0.266	11	5.592	0.022	Yes
5&6 vs. Collagen 1	0.213	11	4.091	0.188	No
5&6 vs. 9&10	0.111	11	2.130	0.903	Do Not Test
5&6 vs. 11&12	0.0769	11	1.617	0.983	Do Not Test
5&6 vs. 7&8	0.0428	11	0.900	1.000	Do Not Test
7&8 vs. PLL Serum	0.338	11	8.498	<0.001	Yes
7&8 vs. PLL No Serum	0.258	11	6.782	0.003	Yes
7&8 vs. TCP	0.223	11	5.246	0.038	Yes
7&8 vs. Collagen 1	0.170	11	3.582	0.339	Do Not Test
7&8 vs. 9&10	0.0681	11	1.433	0.993	Do Not Test
7&8 vs. 11&12	0.0341	11	0.802	1.000	Do Not Test
11&12 vs. PLL Serum	0.304	11	7.640	<0.001	Yes
11&12 vs. PLL No Serum	0.224	11	5.885	0.014	Yes
11&12 vs. TCP	0.189	11	4.443	0.119	No
11&12 vs. Collagen 1	0.136	11	2.864	0.635	Do Not Test
11&12 vs. 9&10	0.0340	11	0.716	1.000	Do Not Test
9&10 vs. PLL Serum	0.270	11	5.984	0.012	Yes
9&10 vs. PLL No Serum	0.190	11	4.356	0.134	No
9&10 vs. TCP	0.155	11	3.258	0.464	Do Not Test
9&10 vs. Collagen 1	0.102	11	1.961	0.939	Do Not Test
Collagen 1 vs. PLL Serum	0.168	11	3.719	0.292	No
Collagen 1 vs. PLL No Serum	0.0876	11	2.012	0.929	Do Not Test
Collagen 1 vs. TCP	0.0528	11	1.110	0.999	Do Not Test
TCP vs. PLL Serum	0.115	11	2.890	0.624	Do Not Test
TCP vs. PLL No Serum	0.0349	11	0.918	1.000	Do Not Test
PLL No Serum vs. PLL Serum	0.0800	11	2.292	0.857	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

d. % Proliferating Cardiomyocytes (Tube 2)

One Way Analysis of Variance Wednesday, May 01, 2013, 9:53:04 PM

Data source: Data 1 in Notebook1

Group Name	N	Missing	Mean	Std Dev	SEM
1&2	3	0	0.648	0.159	0.0920
3&4	3	0	0.557	0.0515	0.0297
5&6	3	0	0.725	0.113	0.0650
7&8	3	0	0.699	0.0889	0.0513
9&10	3	0	0.772	0.118	0.0683
11&12	3	0	0.647	0.132	0.0760
Stock	3	0	0.810	0.108	0.0626
TCP	3	0	0.170	0.0287	0.0166
PLL NO ser	3	0	0.0905	0.0804	0.0464
PLL Serum	3	0	0.125	0.109	0.0631
Collagen 1	2	0	0.641	0.0363	0.0256

Source of Variation	DF	SS	MS	F	P
Between Groups	10	2.180	0.218	20.522	<0.001
Residual	21	0.223	0.0106		
Total	31	2.403			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Stock vs. PLL NO ser	0.719	11	12.086	<0.001	Yes
Stock vs. PLL Serum	0.684	11	11.498	<0.001	Yes
Stock vs. TCP	0.640	11	10.752	<0.001	Yes
Stock vs. 3&4	0.253	11	4.244	0.158	No
Stock vs. Collagen 1	0.169	11	2.535	0.772	Do Not Test
Stock vs. 11&12	0.163	11	2.734	0.692	Do Not Test
Stock vs. 1&2	0.162	11	2.714	0.700	Do Not Test
Stock vs. 7&8	0.110	11	1.854	0.957	Do Not Test
Stock vs. 5&6	0.0846	11	1.422	0.993	Do Not Test
Stock vs. 9&10	0.0375	11	0.630	1.000	Do Not Test
9&10 vs. PLL NO ser	0.682	11	11.455	<0.001	Yes
9&10 vs. PLL Serum	0.647	11	10.867	<0.001	Yes
9&10 vs. TCP	0.602	11	10.122	<0.001	Yes
9&10 vs. 3&4	0.215	11	3.614	0.330	Do Not Test
9&10 vs. Collagen 1	0.131	11	1.972	0.937	Do Not Test
9&10 vs. 11&12	0.125	11	2.104	0.909	Do Not Test
9&10 vs. 1&2	0.124	11	2.084	0.913	Do Not Test
9&10 vs. 7&8	0.0728	11	1.223	0.998	Do Not Test
9&10 vs. 5&6	0.0471	11	0.791	1.000	Do Not Test
5&6 vs. PLL NO ser	0.635	11	10.664	<0.001	Yes
5&6 vs. PLL Serum	0.600	11	10.076	<0.001	Yes
5&6 vs. TCP	0.555	11	9.331	<0.001	Yes
5&6 vs. 3&4	0.168	11	2.823	0.654	Do Not Test

5&6 vs. Collagen 1	0.0841	11	1.264	0.997	Do Not Test
5&6 vs. 11&12	0.0781	11	1.312	0.996	Do Not Test
5&6 vs. 1&2	0.0769	11	1.293	0.997	Do Not Test
5&6 vs. 7&8	0.0257	11	0.432	1.000	Do Not Test
7&8 vs. PLL NO ser	0.609	11	10.232	<0.001	Yes
7&8 vs. PLL Serum	0.574	11	9.644	<0.001	Yes
7&8 vs. TCP	0.530	11	8.899	<0.001	Yes
7&8 vs. 3&4	0.142	11	2.391	0.824	Do Not Test
7&8 vs. Collagen 1	0.0584	11	0.878	1.000	Do Not Test
7&8 vs. 11&12	0.0524	11	0.880	1.000	Do Not Test
7&8 vs. 1&2	0.0512	11	0.861	1.000	Do Not Test
1&2 vs. PLL NO ser	0.558	11	9.371	<0.001	Yes
1&2 vs. PLL Serum	0.523	11	8.783	<0.001	Yes
1&2 vs. TCP	0.478	11	8.038	<0.001	Yes
1&2 vs. 3&4	0.0911	11	1.530	0.988	Do Not Test
1&2 vs. Collagen 1	0.00718	11	0.108	1.000	Do Not Test
1&2 vs. 11&12	0.00117	11	0.0196	1.000	Do Not Test
11&12 vs. PLL NO ser	0.557	11	9.352	<0.001	Yes
11&12 vs. PLL Serum	0.522	11	8.764	<0.001	Yes
11&12 vs. TCP	0.477	11	8.019	<0.001	Yes
11&12 vs. 3&4	0.0899	11	1.511	0.989	Do Not Test
11&12 vs. Collagen 1	0.00601	11	0.0903	1.000	Do Not Test
Collagen 1 vs. PLL NO ser	0.551	11	8.274	<0.001	Yes
Collagen 1 vs. PLL Serum	0.516	11	7.748	<0.001	Yes
Collagen 1 vs. TCP	0.471	11	7.082	0.002	Yes
Collagen 1 vs. 3&4	0.0839	11	1.261	0.997	Do Not Test
3&4 vs. PLL NO ser	0.467	11	7.841	<0.001	Yes
3&4 vs. PLL Serum	0.432	11	7.253	0.002	Yes
3&4 vs. TCP	0.387	11	6.508	0.006	Yes
TCP vs. PLL NO ser	0.0793	11	1.333	0.996	No
TCP vs. PLL Serum	0.0444	11	0.745	1.000	Do Not Test
PLL Serum vs. PLL NO ser	0.0350	11	0.588	1.000	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

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e. % Proliferating Cells (Tube 2)

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
PLL NO ser vs. PLL Serum	0.00426	11	4.543	0.107	No
PLL NO ser vs. 3&4	0.00317	11	3.378	0.417	Do Not Test
PLL NO ser vs. TCP	0.00294	11	3.134	0.518	Do Not Test
PLL NO ser vs. 9&10	0.00190	11	2.025	0.926	Do Not Test
PLL NO ser vs. Stock	0.00185	11	1.971	0.937	Do Not Test
PLL NO ser vs. 7&8	0.00181	11	1.934	0.944	Do Not Test
PLL NO ser vs. 11&12	0.00180	11	1.916	0.947	Do Not Test
PLL NO ser vs. 5&6	0.00140	11	1.497	0.990	Do Not Test
PLL NO ser vs. 1&2	0.00112	11	1.199	0.998	Do Not Test
PLL NO ser vs. Collagen 1	0.000379	11	0.361	1.000	Do Not Test
Collagen 1 vs. PLL Serum	0.00388	11	3.702	0.300	Do Not Test
Collagen 1 vs. 3&4	0.00279	11	2.660	0.722	Do Not Test
Collagen 1 vs. TCP	0.00256	11	2.442	0.807	Do Not Test
Collagen 1 vs. 9&10	0.00152	11	1.450	0.992	Do Not Test
Collagen 1 vs. Stock	0.00147	11	1.402	0.994	Do Not Test
Collagen 1 vs. 7&8	0.00143	11	1.368	0.995	Do Not Test
Collagen 1 vs. 11&12	0.00142	11	1.352	0.995	Do Not Test
Collagen 1 vs. 5&6	0.00102	11	0.978	1.000	Do Not Test
Collagen 1 vs. 1&2	0.000745	11	0.711	1.000	Do Not Test
1&2 vs. PLL Serum	0.00313	11	3.345	0.430	Do Not Test
1&2 vs. 3&4	0.00204	11	2.180	0.889	Do Not Test
1&2 vs. TCP	0.00181	11	1.935	0.943	Do Not Test
1&2 vs. 9&10	0.000775	11	0.827	1.000	Do Not Test
1&2 vs. Stock	0.000724	11	0.773	1.000	Do Not Test
1&2 vs. 7&8	0.000689	11	0.735	1.000	Do Not Test
1&2 vs. 11&12	0.000672	11	0.717	1.000	Do Not Test
1&2 vs. 5&6	0.000280	11	0.299	1.000	Do Not Test
5&6 vs. PLL Serum	0.00285	11	3.046	0.556	Do Not Test
5&6 vs. 3&4	0.00176	11	1.881	0.952	Do Not Test
5&6 vs. TCP	0.00153	11	1.637	0.981	Do Not Test
5&6 vs. 9&10	0.000495	11	0.528	1.000	Do Not Test
5&6 vs. Stock	0.000445	11	0.474	1.000	Do Not Test
5&6 vs. 7&8	0.000409	11	0.436	1.000	Do Not Test
5&6 vs. 11&12	0.000393	11	0.419	1.000	Do Not Test
11&12 vs. PLL Serum	0.00246	11	2.627	0.736	Do Not Test
11&12 vs. 3&4	0.00137	11	1.462	0.991	Do Not Test
11&12 vs. TCP	0.00114	11	1.218	0.998	Do Not Test
11&12 vs. 9&10	0.000102	11	0.109	1.000	Do Not Test
11&12 vs. Stock	0.0000519	11	0.0554	1.000	Do Not Test
11&12 vs. 7&8	0.0000164	11	0.0175	1.000	Do Not Test
7&8 vs. PLL Serum	0.00245	11	2.610	0.743	Do Not Test
7&8 vs. 3&4	0.00135	11	1.445	0.992	Do Not Test
7&8 vs. TCP	0.00113	11	1.201	0.998	Do Not Test
7&8 vs. 9&10	0.0000860	11	0.0918	1.000	Do Not Test
7&8 vs. Stock	0.0000356	11	0.0379	1.000	Do Not Test
Stock vs. PLL Serum	0.00241	11	2.572	0.758	Do Not Test
Stock vs. 3&4	0.00132	11	1.407	0.994	Do Not Test
Stock vs. TCP	0.00109	11	1.163	0.999	Do Not Test
Stock vs. 9&10	0.0000504	11	0.0538	1.000	Do Not Test
9&10 vs. PLL Serum	0.00236	11	2.518	0.778	Do Not Test
9&10 vs. 3&4	0.00127	11	1.353	0.995	Do Not Test
9&10 vs. TCP	0.00104	11	1.109	0.999	Do Not Test

TCP vs. PLL Serum	0.00132	11	1.409	0.994	Do Not Test
TCP vs. 3&4	0.000229	11	0.244	1.000	Do Not Test
3&4 vs. PLL Serum	0.00109	11	1.165	0.999	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

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