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Synthesis and Characterization of Multivalent Conjugates

By

Felicia Lynn Svedlund

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Engineering – Materials Science and Engineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Kevin Healy, Chair

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Spring 2016

Synthesis and Characterization of Multivalent Conjugates

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By

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Abstract

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Doctor of Philosophy in Materials Science and Engineering

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Professor Kevin Healy, Chair

The work described in this dissertation presents the synthesis and characterization of a novel multivalent conjugate of mechano-growth factor. Mechano-growth factor is a peptide derived from a splice variant of insulin-like growth factor-1 protein, which has shown promising cardioprotective effects. Multivalent conjugate technology provides a platform in which growth factors of interest are multivalently conjugated to a long, soluble polymer chain. This multivalent conjugation can result in improved pharmacokinetics and decreased degradation of the growth factor, as well as potentially increasing the bioactivity of the growth factor compared to its unconjugated form. Since the benefits of multivalent conjugate technology depend heavily upon the final valency of the conjugated growth factor, as well as the size and distribution of the multivalent conjugate molecules, it was necessary to have a characterization technique that could provide this information. This work focused on using multi-angle light scattering to thoroughly characterize the mechano-growth factor conjugates, as well as other conjugate molecules and macromolecules of interest in the field of tissue engineering. Additionally, this work focused on the development of *in vitro* cell-based assays for use in studying the bioactivity of the mechano-growth factor conjugates.

Chapter 3 presents the development of a reaction method to allow for the multivalent conjugation of MGF peptide to a HyA backbone chain. The conjugation reaction required first the synthesis of a HyA intermediate by the addition of acrylate groups, which were characterized through gelation and NMR spectroscopy. Then the conjugation was achieved by a Michael addition reaction between the acrylate groups on the HyA and the c-terminal cysteine on the MGF peptide. The success of this conjugation reaction was verified through a BCA assay, which also provided an estimate of the final conjugation ratios. The characterization using NMR spectroscopy, acrylated HyA gelation, and BCA was able to confirm the success of conjugation and provide estimates of the peptide concentration and final conjugation ratio.

Chapter 4 provides a more thorough characterization of the conjugate molecules through the application of multi-angle light scattering. This analysis utilized a SEC-MALS-UV-RI method, where the inline use of two concentration detectors allowed for the determination of the relative compositions of the MVCs. Using the measured specific refractive index increment and UV extinction coefficient values measured for the two MVC components, it was possible to

determine the weight fractions of the MGF and HyA in the total MVC molecule. This analysis confirmed that the bioconjugate chemistry technique utilized in this work was successful, and that it was possible to determine the total molecular weight, polydispersity, conjugation efficiency, and valency of the MVCs.

Chapter 5 presents an alternative SEC-MALS methodology for characterizing MVC molecules using branching analysis. It was first demonstrated that the MVC molecules behave as branched molecules, and then it was shown that branching analysis methods could be successfully applied to the MVCs. Although a linear hyaluronic acid was used as the linear counterpart for the calculations instead of a chemically identical linear counterpart, it was still possible to achieve good agreement between the values calculated by branching analysis and multivalent conjugate analysis.

Chapter 6 further demonstrates the power of SEC-MALS as a characterization technique for macromolecules and MVCs in dilute solutions. SEC-MALS was applied to a variety of different projects in order to provide detailed information about the molecular weight, radius of gyration, polydispersity, and valency of these different macromolecular systems.

Chapter 7 focuses on the development of two different assays for assessing the bioactivity of mvMGF. The first assay used hypoxia to mimic the ischemic environment of the cardiac tissue during an MI, while the second assay stressed CMs through cryopreservation and subsequent thawing. Both assays resulted in functional impairment of the CMs, which will allow for the *in vitro* assessment of the cardioprotective ability of the mvMGF.

Dedicated to my family

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List of Abbreviations

A ₂	Second virial coefficient
Ac-HyA	Acrylated hyaluronic acid
ADH	Adipic acid dihydrazide
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
bpm	Beats per minute
BSA	Bovine serum albumin
c	Concentration
CHF	Congestive heart failure
CM	Cardiomyocyte
Cys-Ac-HyA	Cysteine quenched, acrylated hyaluronic acid
D	Polydispersity index
dn/dc	Specific refractive index increment
DRI	Differential refractive index
ε	UV extinction coefficient
ECM	Extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EMCH	3,3'-N-(ε-maleimidocaproic acid) hydrazide
FDA	Food and Drug Administration
g	Branching ratio
GF	Growth factor
HCR	High conjugation ratio (60:1 molar feed ratio MGF:HyA)
HMWH	High molecular weight heparin
HOBt	1-hydroxybenzotriazole hydrate
HPLC	High performance liquid chromatography
HyA	Hyaluronic acid
I	Intensity
IGF-1	Insulin-like growth factor-1
iPSC	Induced pluripotent stem cells
LCR	Low conjugation ratio (30:1 molar feed ratio MGF:HyA)
LMWH	Low molecular weight heparin
LS	Light scattering
M	Molar mass
MALS	Multi-angle light scattering
MGF	Mechano-growth factor
MGF-Cys	MGF plus c-terminal cysteine
MGF-Trp	MGF plus added tryptophan residue
MI	Myocardial infarction
M _n	Number average molecular weight
M _v	Viscosity average molecular weight
MVC	Multivalent conjugate
mvMGF	Multivalently conjugated mechano-growth factor
M _w	Weight average molecular weight
MW	Molecular weight

MWCO	Molecular weight cut-off
M_z	Z average molecular weight
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
RI	Refractive index
RMS	Root mean square
R_g	Radius of gyration
$R_{g,z}$	Z average radius of gyration
R_θ	Rayleigh ratio
SEC	Size exclusion chromatography
SEC-MALS	Size exclusion chromatography – multi-angle light scattering
TEOA	Triethanolamine buffer
UMWH	Unfractionated molecular weight heparin
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
V_h	Hydrodynamic volume
V_R	Retention volume

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CHAPTER 1: MOTIVATION, SPECIFIC AIMS, AND DISSERTATION OUTLINE

1.1 Motivation

Growth factors play a vital role in tissue engineering research and therapeutics due to their ability to direct cell function and fate through signaling pathways. However, few growth factors have been able to be used as effective therapeutics due to their diffusible nature and proteolytic degradation, which results in rapid clearance from the tissue. Multivalent conjugate technology provides a platform in which growth factors of interest are multivalently conjugated to a long, soluble polymer chain. This multivalent conjugation can result in improved pharmacokinetics and decreased degradation of the growth factor, as well as potentially increasing the bioactivity of the growth factor compared to its unconjugated form.

This dissertation focuses on the use of this multivalent conjugate technology to create conjugates of mechano-growth factor peptide and hyaluronic acid. Mechano-growth factor is a peptide derived from a splice variant of insulin-like growth factor-1 protein, which has shown promising cardioprotective effects. Multivalent conjugates of mechano-growth factor could be used to study the bioactivity and cardioprotective abilities of the peptide *in vitro*, which could lead to its eventual development as a cardiac therapeutic. Since the benefits of multivalent conjugate technology depend heavily upon the final valency of the conjugated growth factor, as well as the size and distribution of the multivalent conjugate molecules, it is necessary to have a characterization technique that can provide this information. This work focuses on using multi-angle light scattering to thoroughly characterize the mechano-growth factor conjugates, as well as other conjugate molecules and macromolecules of interest in the field of tissue engineering. Multi-angle light scattering can be used to provide diverse information about these molecules by applying different analysis and characterization methodologies.

1.2 Specific Aims

1. Develop and optimize a synthesis protocol for the multivalent conjugation of mechano-growth factor to hyaluronic acid.
2. Use multi-angle light scattering to thoroughly characterize the multivalent conjugates.
 - a. Characterize the molecular weight, polydispersity, radius of gyration, and valency of the multivalent conjugate molecules using a three-detector multivalent conjugate method.
 - b. Characterize the multivalent conjugates using a branching analysis methodology, and assess its viability as an alternative to the traditional conjugate analysis method.
 - c. Apply the multi-angle light scattering-based characterization to other multivalent conjugate molecules and macromolecules of interest.
3. Develop *in vitro* cell-based assays that can be used to assess the bioactivity of the multivalent conjugates of mechano-growth factor.

1.3 Dissertation Outline

- *Chapter 2* covers the literature review and theoretical background needed to understand the topics covered throughout this dissertation. Summaries of the scientific literature addressing the benefits of multivalent conjugate technology and the desirable properties of hyaluronic acid are included in Chapter 2. There is also a detailed discussion of the characterization of polymers in solution, which covers the commonly used characterization techniques, while going into more detail about size exclusion chromatography and the theory of multi-angle light scattering.
- *Chapter 3* addresses the first specific aim by describing the chemistry used to synthesize the multivalent conjugates of mechano-growth factor. Chapter 3 also provides some preliminary characterization and confirmation of the success of the conjugation reaction.
- *Chapter 4* addresses specific aim 2a, by using size exclusion chromatography – multi-angle light scattering to thoroughly characterize the multivalent conjugates of mechano-growth factor. An established multivalent conjugate analysis method that is dependent upon the use of two inline concentration detectors is used to measure molecular weight, polydispersity, radius of gyration, and valency of the conjugate molecules. Chapter 4 also introduces the branching behavior of multivalent conjugates, which leads into the detailed discussion of branching in Chapter 5.
- *Chapter 5* addresses specific aim 2b. It first describes the theory of branching analysis using multi-angle light scattering and then demonstrates the branching behavior of multivalent conjugates of sFlt-1(3) protein with hyaluronic acid. Branching analysis is applied to these conjugates to yield the branching ratio, branch units per molecule, and long chain branching. These findings are compared to the analysis discussed in Chapter 4 in order to assess the viability of branching analysis as an alternative characterization technique for multivalent conjugates.
- *Chapter 6* addresses specific aim 2c by applying the size exclusion chromatography – multi-angle light scattering characterization to other multivalent conjugates and macromolecules of interest. This chapter characterizes the effect of filtering on molecular weight and polydispersity of hyaluronic acid, assesses an alternative chemistry to synthesize multivalent conjugates, and characterizes the molecular weight and polydispersity of different heparin molecules use to sequester growth factors for tissue engineering applications.
- *Chapter 7* addresses the third specific aim and describes the development of two different *in vitro* assays that can be used to assess the bioactivity of the mechano-growth factor conjugates. These two assays are a hypoxia assay and a cryopreservation assay, which both induce stress and functional impairment in cardiomyocytes. Some preliminary studies were conducted with multivalent mechano-growth factor treatments, which demonstrated possible functional recovery of the stressed cardiac cells. Further studies are needed to fully assess the *in vitro* cardioprotective effects of the conjugates.
- *Chapter 8* provides a summary of the overall conclusions from this work. It also gives insight into questions that still need to be answered and provides a summary of future directions for this research.

CHAPTER 2: BACKGROUND

2.1 Multivalent Conjugate Technology

Growth factors (GFs) are involved in the regulation of cellular processes through their activation of cellular signaling pathways. They play especially important roles in cellular mechanisms of tissue regeneration and wound healing¹⁻⁴, including regeneration of ischemic tissues.⁵ Additionally, GFs are a component of the cellular niche, which combines signals from GFs, the extracellular matrix (ECM), and cell-cell interactions to direct stem cell function and fate.⁶ Due to their key roles in tissue regeneration and directing stem cell behavior, GFs are a central area of research in the field of tissue engineering.

While many GFs could offer promising therapeutics for tissue regeneration, very few have been successfully adopted to clinical applications.^{7,8} This is because most GFs act in a diffusible manner, which limits their long term stability in tissues. *In vivo*, these diffusible GFs undergo proteolytic degradation, and due to their small size, they rapidly diffuse and clear from the tissue. Rapid clearance from the application site limits the clinical use of GFs due to an insufficient duration for therapeutic activity.⁹⁻¹¹ An example of this is a vascular endothelial growth factor (VEGF)-based drug, which was halted in phase II clinical trials¹² due to its short half-life of about 30 minutes¹³ *in vivo*. This meant that the drug required repeated administration, while only providing a slight improvement in neovascularization of the tissue.⁸ Additionally, platelet-derived growth factor (PDGF) has been shown to accelerate wound healing¹⁴, but its half-life is less than two minutes¹⁵ *in vivo*. Due to the need for frequent administration^{16,17}, PDGF has not been adopted by many wound management protocols^{18,19} even though it is approved for this application by the Food and Drug Administration (FDA).

Multivalent conjugate (MVC) technology has the potential to overcome the clinical challenges of GF-based therapeutics by using standard bioconjugate techniques to tether multiple GFs to a single long, soluble polymer backbone chain. Figure 2.1 is two different schematics representing the creation of MVCs. The first schematic shows the polymer chain, in this case hyaluronic acid (HyA), as a stretched out chain for the purposes of visualizing the synthesis. However, the polymer chains will actually behave as hydrated coils in solution, which is represented in the second schematic. Previous studies have shown that these MVCs can improve the pharmacokinetics of the tethered GFs.²⁰⁻²² By tethering the GFs to a high molecular weight polymer chain, their effective size is increased, which slows their diffusion and clearance from tissues.²⁰ Additionally, the use of MVC technology can reduce the proteolytic degradation of the GFs.²⁰ The improved pharmacokinetics and decreased proteolytic degradation provided by MVC technology allow for a longer duration of the GF at the treatment site, which could significantly improve the therapeutic benefits.

Multivalent GFs can also be more potent than the corresponding monovalent GFs. Studies for several different multivalent GFs have demonstrated that the multivalent form of the GF can exhibit a higher bioactivity than the same concentration of unconjugated GF.²³⁻²⁶ This enhanced bioactivity is believed to be due to the effects of receptor clustering, where multiples of the same ligand bind to cell surface receptors in close proximity to one another. This type of receptor

clustering is abundant in cell signaling transduction.^{27,28} Many GFs, such as basic fibroblast growth factor (bFGF) and VEGF will actually bind to natural components of the ECM, such as heparin, *in vivo* to form tethered groups of GFs similar to MVCs.^{29,30} Some cell signaling pathways actually require this type of receptor clustering in order to be activated³¹, and the potency of the activation may be controlled by the degree of multivalency of the interaction.³²

After the first GF-receptor binding event, these MVCs may decrease the entropic cost of subsequent binding events. When the first GF on an MVC binds to a receptor, it pulls the rest of the GFs on that molecule close to the cell surface and other nearby receptors. Binding these GFs that are now held in close proximity would have a lower entropic penalty than binding a free monovalent GF from solution,^{33,34} since the effective local concentration of GF in the vicinity of the cell surface is now increased with the MVC binding compared to monovalent GFs in the surrounding solution.³⁵

Due to the benefits provided by MVC technology, it is desirable to use this technology to design GF therapeutics for numerous different applications in tissue engineering, wound healing, and tissue regeneration. The decreased proteolytic degradation, improved pharmacokinetics, and enhanced bioactivity that can be provided by multivalent conjugation of GFs means that MVCs offer a powerful strategy for the delivery of different GFs to damaged or diseased tissues. Since the benefits of these MVC molecules is so strongly correlated to the overall size of the molecules and the valency of conjugation, it is crucial to have characterization techniques which can accurately provide this information. This leads to a discussion of characterization techniques for polymers in solution in the following section.

2.2 Characterization of Polymers in Solution

2.2.1 Introduction to Basic Concepts

In order to understand the behavior of polymers in solution, it is important to be able to measure and analyze their molecular weight distribution and the conformation and size of the polymer coil. Unlike for small molecular weight chemicals or proteins with a single defined molecular weight, polymers will always have some distribution of chain lengths. This distribution can be narrow or broad depending on how the polymer was synthesized, processed, or chemically modified. This means that for polymer molecules, the focus is not on measuring a single molecular weight but on an average molecular weight for the distribution. First, a molecule with a degree of polymerization i will be called an i -mer and have a molecular weight defined as:

$$M_i = iM_0$$

where M_i is the molecular weight of an i -mer and M_0 is the molecular weight of a repeat unit.³⁶ There are two common averages that are used to define the molecular weight of a polymer sample. The first is the number average molecular weight, M_n , which is defined as:

$$M_n = \frac{\sum_i n_i M_i}{\sum_i n_i}$$

where n_i is the number of i -mers and M_i is the associated molecular weight of the i -mer.³⁶ The second commonly used average is the weight average molecular weight, M_w , which is defined as:

$$M_w = \sum_i w_i M_i$$

where

$$w_i = \frac{in_i}{\sum_i in_i}$$

where w_i is the weight fraction of i -mer in the sample.³⁶ These two averages will define the molecular weight in different ways, so qualitatively, the M_n is used when the number of molecules is the crucial factor and the M_w is used when the weight of each molecule is the most important factor. There are other averages that can be used to describe the distribution of molecular weight in a polymer sample, such as viscosity average molecular weight (M_v) and z average molecular weight (M_z), but M_n and M_w are the most important and most commonly used averages.³⁶⁻³⁸ M_z is defined as:

$$M_z = M_0 \frac{\sum_i i^3 n_i}{\sum_i i^2 n_i}$$

A schematic of the distribution of these different molecular weight averages is shown in Figure 2.2.

The M_n , M_w , and M_z provide values for the different average molecular weights for the polymer sample, but they do not individually provide any information about the breadth of the molecular weight distribution. In order to provide information about the breadth of the distribution, a term called the polydispersity index, $\mathcal{D}$, is defined as the ratio of the M_w to M_n .

$$\mathcal{D} = \frac{M_w}{M_n}$$

This value is always greater than or equal to 1. If the value of $\mathcal{D}$ is equal to 1, that means that the sample is monodisperse and only has one value of molecular weight. As the value of $\mathcal{D}$ becomes greater than 1, this means that the polymer is more polydisperse. Therefore, polymers with a $\mathcal{D}$ value close to 1 can be defined as having narrow molecular weight distributions, while polymers with larger values (i.e. $\mathcal{D} > 2$) can be defined as having broad distributions.³⁶⁻³⁸

Another important concept in characterizing polymers in solution is the size of the polymer coil, which can be described by the radius of gyration, R_g . R_g is defined as the average distance of all monomers from the center of mass of the coil. The center of mass can be defined as the point in space that satisfies:

$$\sum_{i=1}^N m_i \vec{s}_i = 0$$

where $\vec{s}_i$ is the vector from the center of mass to monomer i , m_i is the mass of monomer i , and N is the number of monomers.³⁶ The root mean square (RMS), mass-weighted average distance of the monomers from the center of mass, R_g , is then defined as³⁶:

$$R_g = \langle s^2 \rangle^{1/2} = \left\{ \frac{\sum_{i=1}^N m_i \langle s_i^2 \rangle}{\sum_{i=1}^N m_i} \right\}^{1/2} = \left\{ \frac{1}{N} \sum_{i=1}^N \langle s_i^2 \rangle \right\}^{1/2}$$

where

$$\langle s_i^2 \rangle = \langle \vec{s}_i \cdot \vec{s}_i \rangle$$

The radius of gyration can be useful for understanding the size and conformation of polymer coils in solution. It can be measure directly using multi-angle light scattering (MALS), which is a technique that will be discussed in detail later in this chapter.

2.2.2 Methods for Measuring Polymer Molecular Weight

There are many different methods used for determining the molecular weight of polymers, and each have distinct advantages and disadvantages. The different methods also yield different information, such as the full distribution, M_n , M_w , M_v , or M_z . The different techniques can measure either absolute molecular weight or a relative molecular weight. An absolute measurement means that the molecular weight is determined from an exactly measurable physical quantity. Relative measurements of molecular weight are determined by calibrating with standards of known values. A list of molecular weight characterization techniques, whether they are absolute or relative measurements, and the information they provide is shown in Table 2.1. Some of those methods will be discussed briefly in this section, while others (size exclusion chromatography and multi-angle light scattering) will be discussed in more detail in the following sections.

End Group Analysis. For linear polymer chains, it is possible to use different techniques to count the end groups, which is equivalent to counting the number of molecules. This information can then be used to calculate the M_n . This technique only works for polymers that have end groups with distinct chemistry, such as epoxy resins, polyesters, and polyamides. These end group chemistries can be detected by techniques such as titration, ultraviolet (UV) spectroscopy, or nuclear magnetic resonance (NMR) spectroscopy. End group analysis also requires that the polymers be completely linear, as chain defects and branching will result in erroneous measurements. Since the concentration of end groups in solution has to be high enough for detection, this technique is limited to polymers with molecular weights below about 10,000 g/mol.^{36,37}

Osmotic Pressure. There are two different methods for determining M_n with osmotic pressure measurements. These are vapor pressure osmometry (VPO) and membrane osmometry (MO). These measurements depend on osmotic pressure, which is a colligative property. This means that the osmotic pressure depends only on the number of molecules in solution and not on their properties, such as size or mass. This provides an advantage, since this measurement is completely independent of the level of chemical uniformity of the polymer being measured. This makes it a good technique for measuring the M_n of heterogeneous copolymers. MO provides an absolute measurement of molecular weight, meaning that the molecular weight is related to a directly measurable physical property. However, VPO is not an absolute measurement technique because it requires calibration with standards of known molar mass. Other colligative property-based techniques, such as freezing point depression and boiling point elevation, are also sensitive to the number of polymer molecules in solution, and therefore also provide M_n .^{36,37}

Intrinsic Viscosity. Viscosity is affected by the dimensions of the polymer coils in solution, and is therefore not actually a direct measurement of molecular weight. However, using the Mark-Houwink relation, the M_v can be calculated from the viscometry measurements. This calculation requires that the Mark-Houwink exponent, a , be known for the polymer being measured. The advantages of viscometry are that it is relatively simple, the instrumentation is inexpensive, and that it can be used as a detector in combination with size exclusion chromatography (SEC). Solution viscometry also provides information about polymer size, polymer branching, and chain flexibility, making it a versatile technique.^{36,37}

Analytical Ultracentrifugation. There are two methods of analytical ultracentrifugation. The first is sedimentation velocity, which measures the sedimentation coefficient that can be used to calculate M_w . This method is based on monitoring the movement of the concentration boundary of a dilute polymer solution placed in a high centrifugal field. The second method is sedimentation equilibrium, which provides M_z . This method is based on the fact that when the sedimentation equilibrium is reached in a centrifugal field, the polymer molecules will be distributed based on molecular weight. The limitations of these techniques are the long times required for analysis and the expensive instrumentation needed.³⁷

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). This is a relatively new technique that can achieve very high accuracy and resolution in measuring the full distribution of molecular weight. The polymer sample is embedded in a UV-absorbing matrix that is irradiated with a laser pulse. This ionizes the polymer sample, which is then accelerated by an electric field and travels down the TOF tube. The macromolecules are separated during their flight based on their mass-to-charge ratios so that they reach the detector at different times. Each different molecular weight gives a distinct signal on the mass spectrum. In addition to the resolution and accuracy, this technique has the added benefit of also providing compositional and chemical information about the macromolecules. However, the desorption and ionization process is not always straight forward for large, synthetic polymer molecules. One major issue with this technique is that the amplitude of a signal peak cannot be directly related to the relative abundance of that particular molecular weight in the sample. Not all molecular weights are desorbed and ionized with the same efficiency, so the signals will have unknown sensitivity to molecular weight. This makes MALDI-TOF MS difficult to use in analyzing polydisperse polymer samples.^{36,37}

2.2.3 Size-Exclusion Chromatography

Size exclusion chromatography, also known as gel permeation chromatography (GPC) or gel filtration chromatography (GFC), has become the most commonly used, modern technique for measuring molecular weight and has replaced many of the more classical techniques discussed in the previous section. SEC allows for determination of both average molecular weights and the molecular weight distribution. Therefore, it merits a more detailed discussion.

Separation Mechanism. A schematic of size separation and detection by SEC is shown in Figure 2.3. A solution, or mobile phase, containing the polymer solute molecules flows through an SEC column that is filled with a packing material composed of porous beads with a characteristic pore size. Columns with an appropriate range of pore sizes as well as compatibility with the solvent, polymer sample, and temperature must be selected for the specific application. The largest polymer molecules are unable to penetrate the pores and are therefore eluted from the column first. Progressively smaller polymer molecules are able to penetrate the pores to a greater extent, and are eluted sequentially with the smallest molecules eluting last. The mobile phase is monitored for the presence of solute molecules as it elutes from the column using a detector, which outputs a chromatogram with distinct peaks for the eluted polymers. These peaks will be narrower for less polydisperse polymers and broader for more polydisperse polymers.

SEC separation is actually based on the size of the polymer solute, and not on molecular weight. The size of the polymer coil for SEC separation is the hydrodynamic volume, V_h , which is roughly proportional to R_g cubed.

$$V_h \sim \frac{4\pi}{3} R_g^3$$

In order to relate V_h to molecular weight, a calibration curve is required. The retention volume, V_R , is defined as the volume of solvent that passes through the column before a polymer of a specific molecular weight is eluted. By using a calibration curve relating V_R to molecular weight, it is possible to determine the molecular weight of the polymer solute. Because the molecular weight is not measured directly, but is instead determined from a calibration curve relating molecular weight to V_R , SEC is not an absolute measure of molecular weight.

Calibration Methods. The first, and simplest, calibration method is to run a series of polymer standards with known molecular weights, M , through the column to generate a calibration curve of $\log M$ versus V_R . An example of such a calibration curve is shown in Figure 2.4. All polymers whose size is larger than the packing material pores will be excluded from the pores and elute together at the beginning of the curve, yielding a vertical portion of the curve. Similarly, all polymers below a certain size will be able to penetrate all of the pores and will elute together at the end of the curve. In both of these regimes, there is no separation of the polymer molecules. In the intermediate region of the calibration curve, the polymer solute will be separated by V_h such that a peak at a particular V_R can be related to a corresponding molecular weight. A linear or polynomial fit can be applied to this intermediate region of the plot so that molecular weight can be determined from the retention volume.³⁶

To achieve accurate molecular weight measurements from SEC, it is required to use polymer standards that are the same as the polymer being analyzed. Standards that are exactly the same as the polymer analyte may be difficult to obtain for polymers with heterogeneous composition, such as copolymers, and heterogenous structure, such as branched molecules. Figure 2.5 is a schematic of the elution behavior of two polymers with the same V_h , but different molecular weights due to branching. This means that a branched polymer sample would have an incorrectly measured molecular weight if a linear polymer standard, or even a standard with a different degree of branching, was used for calibration. The SEC elution behavior of branched molecules is discussed in detail in Chapter 5. Additional limitations of this method of calibration are that the molecular weight and polydispersity of a given standard can only be known to a certain level of accuracy. This introduces a level of uncertainty to the molecular weight determination, which is dependent upon the quality of the standards.³⁶

The other method of calibration is called universal calibration because it is able to determine the molecular weight of one polymer based on column calibration with a different polymer. This is possible based on the the concept of intrinsic viscosity and the assumption that:

$$V_h \propto [\eta]M$$

where $[\eta]$ is the intrinsic viscosity. It is assumed that the proportionality constant in this relationship is independent of structure and that V_R depends solely on V_h . The assumption also has to be made that the sample follows the Mark-Houwink equation with known values of the Mark-Houwink parameters, k and a . It is then possible to compare the sample to the reference polymer in order to calculate the molecular weight of the sample.³⁶

SEC Detectors. There are four classes of detectors that are commonly used for SEC. The first of these is a differential refractive index (DRI) detector, which is a concentration detector. This detector measures the refractive index of the eluent. The detector response is proportional to the concentration, with a proportionality constant that includes the specific refractive index increment (dn/dc), which is a constant for the specific polymer. The concentration equation for the DRI detector is defined as:

$$c_{DRI} = \frac{I_{DRI} DRI_{CC}}{dn/dc}$$

where c_{DRI} is the concentration determined from the DRI detector, I_{DRI} is the intensity measured by the DRI detector, and DRI_{CC} is the calibration constant for the instrument. The specific refractive index increment, dn/dc , is an important constant that plays a crucial role in light scattering, which will be discussed in the next section. The specific refractive index increment is defined as:

$$n = n_s + \frac{dn}{dc}c + A_2c^2 + \dots$$

where n is the refractive index of the solution, n_s is the refractive index of the pure solvent, and c is the concentration. The value of the dn/dc is dependent upon the wavelength and temperature at

which it is measured, so it is important to measure the value under the same conditions used for light scattering measurements. The value of dn/dc is independent of molar mass for large molecules, but at lower molecular weights, it becomes dependent on the molar mass. This molecular weight dependence must be taken into consideration for measurement of lower molecular weight polymers. The value for dn/dc can be positive, negative, or zero.

Another concentration-based detector is the UV-vis detector, which uses Beer's law to relate the transmittance of the solution to the concentration of the absorbing species. This relation depends on the UV extinction coefficient, ϵ , which is also a constant for the specific polymer. This relation is given by Beer's Law, which is defined as:

$$\epsilon bc = A = \log \frac{I_0}{I_t}$$

where b is the path length, A is the absorbance, I_t is the intensity of the transmitted light, and I_0 is the intensity of the incident light. The drawback to a UV-vis detector is that many polymers do not absorb at sufficiently long wavelengths to avoid solvent absorption. This fact can be used in the case of mixtures, such as copolymers and MVCs, because a wavelength can be chosen for the UV-vis detector that favors absorption by only one component. Using a UV-vis detector in this way coupled inline with an RI detector can be used to determine the relative composition of the sample at each V_R .³⁶ This type of analysis is discussed in detail in Chapter 4.

The final two types of detectors are light scattering detectors and viscometers. The light scattering detector is advantageous because it allows for absolute measurement of molar mass at each slice of the chromatogram. With the incorporation of multiple detection angles, it can also be used to measure RMS radius. This type of detector will be discussed in much more detail in the next section. Finally, a viscometer takes advantage of the direct relationship between V_h , V_R , and $[\eta]$. By estimating $[\eta]$ at each slice of the chromatogram, it is possible to calculate the molecular weight.³⁶

2.2.4 Multi-Angle Light Scattering

Multi-angle light scattering is a powerful characterization technique for polymers in solution because it allows for absolute measurement of the M_w along with two other important polymer properties, R_g and the second virial coefficient (A_2). The fundamental principle of light scattering characterization is that the amount of light scattered is directly proportional to the molar mass and concentration.³⁹ This can be expressed as:

$$I(\theta) \propto Mc \left(\frac{dn}{dc} \right)^2$$

where $I(\theta)$ is the light scattering intensity measured at a given detector angle, M is the molar mass, and c is the concentration. Additionally, with the use of multiple detection angles, it is possible to measure the radius of gyration of particles larger than approximately 10 nm based on the angular dependence of light scattering.^{36,37} It is a classic technique that is described in

numerous publications and texts for applications such as proteins, biopolymers, and other small particles in solution.³⁹⁻⁴⁵

Introduction to Scattering. Scattering of light is defined as the reradiation of a traveling wave due to a change in the character of the medium in which the wave is propagating. This can be simplified to define scattering as the interaction between light and matter, such as polymer solute molecules in a solvent. The ability of a molecule to scatter light is dependent upon its polarizability, which is directly proportional the refractive index (n) and the dn/dc. Therefore, scattering occurs when there are local changes in refractive index or polarizability, which can be caused by fluctuations in the concentration of polymer in the solvent. Light scattering can be categorized as elastic (MALS), quasielastic (dynamic light scattering), or inelastic (Raman and Brillouin scattering). MALS only uses elastic scattering, where no energy is exchanged between the light wave and the medium. This means that the incident and scattered frequencies are the same.^{36,37}

Scattered light can either have an intensity independent of the scattering angle, which is termed incoherent scattering, or have intensity dependent on the scattering angle, which is termed coherent scattering. Incoherent scattering arises due to random fluctuations in an otherwise homogenous medium. Because these fluctuations are random, on average there is no phase relation between the scattered waves, which means that there is no angular dependence of scattering. However, large polymer molecules have monomers with partially correlated spatial separations, which are a significant fraction of the wavelength of the incident light. The partial spatial correlation means that some of the scattered waves will have phase relations leading to interference, which can be constructive or destructive. This type of scattering is called coherent scattering, and means that the intensity of scattered light is dependent upon the scattering angle. This angular dependence of the scattering allows for measurement of R_g .^{36,37}

Incoherent Scattering. In a dilute polymer solution, there are two different sources of scattering. The first is the random fluctuations in concentration that give rise to incoherent scattering. The second is due to large polymers with monomers with partially correlated spatial separations that are a significant fraction of the wavelength, which leads to coherent scattering (discussed in the next section). Incoherent scattering from random fluctuations in concentration leads to the development of a scattering relationship in which the scattering intensity is proportional to the product of concentration and molecular weight. With incoherent scattering, it is possible to measure the M_w , but R_g can not be measured.^{36,37} To develop the mathematical equation for this incoherent scattering relation it is necessary to first define the excess scattered intensity, I_{ex} :

$$I_{ex} = I_s^{solution} - I_s^{solvent}$$

where I_s is the scattered intensity for the sample solution or for the pure solvent. The excess scattered intensity can then be divided by the incident intensity, I_0 , to yield:

$$\frac{I_{ex}}{I_0} = \frac{4\pi^2 n^2 \left(\frac{dn}{dc} \right)^2}{r^2 \lambda_0^4 N_{av}} \frac{c}{\left(\frac{1}{M} + 2A_2c + \dots \right)}$$

where r is a purely geometric quantity, λ_0 is the incident wavelength, and N_{av} is Avagadro's number. This equation can then be rearranged to define the Rayleigh Ratio, R_θ :

$$R_\theta \equiv \frac{I_{ex} r^2}{I_0}$$

Defined in this way, the Rayleigh ratio depends only on the incident wavelength and the solution. The purely optical factors in this equation can be grouped to define a factor called K :

$$K \equiv \frac{4\pi^2 n^2 \left(\frac{dn}{dc} \right)^2}{\lambda_0^4 N_{av}}$$

which then gives:

$$R_\theta = \frac{Kc}{\frac{1}{M} + 2A_2c + \dots}$$

which can be rearranged to:

$$\frac{Kc}{R_\theta} = \frac{1}{M} + 2A_2c + \dots$$

This is the Zimm equation⁴⁰ for incoherent scattering, which provides the relationship between the scattering intensity, concentration, molecular weight, and the second virial coefficient.³⁶

Coherent Scattering. The second source of scattering in a dilute polymer solution is due to large polymers with monomers with partially correlated spatial separations that are a significant fraction of the wavelength. This type of scattering causes interference due to the phase relations between the scattered waves and is called coherent scattering. Coherent scattering is similar to Bragg diffraction from atomic crystals. Bragg diffraction gives sharp scattering peaks at certain angles, because the spatial correlation between planes of atoms in a crystal is very high. The spatial correlation in polymers is much lower, so scattering from polymers produces a smooth function with the scattering angle, instead of sharp peaks.^{36,37} Spatial correlation in polymers results from the fact that polymer chains are made up of some n -number of bonds between one or several different monomers. These monomers have defined lengths and the bonds between them have constrained bond angles, torsion angles, and bond lengths, which yields some amount of periodic spacing in polymer chains.

Figure 2.6a shows an illustration of Bragg's law. In this figure, $\vec{k}_i$ and $\vec{k}_s$ are the incident and scattered light wave vectors respectively. The planes of atoms are defined as j , $j+1$, and $j+2$, while the spacing between the planes of atoms is defined as D . The angle between the incident wave and the scattering planes is $\theta/2$. In order for constructive interference to occur between scattered waves at the detector, the distance traveled by waves scattered from the $j+1$ plane of atoms must be $m\lambda$ greater than the distance traveled by light scattered from the j plane, where m

is an integer. This condition is the basis for Bragg's law. Figure 2.6b illustrates that the extra distance that a wave must travel when it is scattered by the $j+1$ plane is equal to $2D\sin(\theta/2)$.³⁶ Therefore, Bragg's law can be defined as:

$$m\lambda = 2D \sin\left(\frac{\theta}{2}\right)$$

Another important quantity in coherent scattering is the scattering vector, $\vec{q}$, defined as:

$$\vec{q} = \vec{k}_i - \vec{k}_s$$

By using the geometry of the scattering vector, which is shown in Figure 2.6c, its magnitude can be defined as:

$$|\vec{q}| \equiv q = 2 \left(\frac{2\pi}{\lambda} \right) \sin\left(\frac{\theta}{2}\right) = \frac{4\pi}{\lambda} \sin\left(\frac{\theta}{2}\right)$$

Since the magnitude of q has units of inverse length, scattering will be sensitive to structure in the solution on the length scale of $1/q$. This means that by defining q , which depends on the incident wavelength and the scattering angle, it can be determined what kind of structural information can be provided by the experiment.³⁶ This will be discussed in more detail in the next section.

Bragg's law is satisfied when $\vec{q}$ (determined by the instrument) coincides with the reciprocal lattice vector (determined by the material). The reciprocal lattice vector is defined as a vector pointing in the direction of $\vec{q}$ with an amplitude of $2\pi/D$, so Bragg's law is satisfied when:

$$\frac{2\pi}{D} = \frac{4\pi}{\lambda} \sin\left(\frac{\theta}{2}\right)$$

Since coherent scattering results in interference between scattered waves, which is illustrated in Figure 2.7a, the actual excess scattering intensity measured at a given angle will differ from the Rayleigh excess intensity defined for incoherent scattering. This can be used to define the form factor, $P(\theta)$, as:

$$P(\theta) = \frac{\text{Actual } I_{ex}(\theta)}{\text{Rayleigh } I_{ex}(\theta)}$$

where $0 \leq P(\theta) \leq 1$. The form factor introduces the angular dependence of scattering, shown in Figure 2.7b, and allows for the determination of R_g . For coherent scattering, the Zimm equation incorporates this form factor to become:

$$\frac{Kc}{R_\theta} = \frac{1}{M_w} \frac{1}{P(\theta)} + 2A_2c + \dots$$

For very dilute polymer solutions, as the limit of concentration approaches zero, $P(\theta)$ can be defined as equal to $P(q)$, where:

$$P(q) = 1 - \frac{q^2}{3} R_g^2 + \dots$$

Plugging this into the Zimm equation for coherent scattering gives:

$$\frac{Kc}{R_\theta} = \frac{1}{M_w} \left(1 + \frac{q^2}{3} R_g^2 + \dots \right) + 2A_2c + \dots$$

This is the fundamental Zimm equation⁴⁰ for light scattering from dilute polymer solutions and provides the relationship between scattering intensity, concentration, M_w , R_g , and the second virial coefficient.³⁶

Scattering Regimes. As was mentioned in the previous section on coherent scattering, the magnitude of the scattering vector, q , has units of inverse length and can be used to determine the kind of structural information can be determined by the experiment. Based on q and R_g , four different regimes of scattering can be defined. For each of these, it is possible to determine different structural information about the solute molecule.³⁶ Figure 2.8 illustrates these four different scattering regimes.

The first regime is when $qR_g \ll 1$ and is called the Rayleigh regime. For this regime, the solute molecules behave as point scatterers and $P(q)$ is approximately equal to 1. No information on chain dimensions (i.e. R_g) can be obtained in this regime. The second regime is called the Guinier regime and is when $qR_g \leq 1$. In this regime $P(q)$ becomes:

$$P(q) = 1 - \left(\frac{q^2}{3} \right) R_g^2$$

In the Guinier regime, it is possible to obtain the value of R_g without any knowledge of the shape of the molecules. In this regime, the Zimm equation defined in the section above for coherent scattering can be used without expanding to any higher order terms. This provides a simple mathematical relationship to determine R_g , which is independent of shape. The third regime is defined as $1 \leq qR_g \leq 10$. In this regime, more terms in the power expansion of $P(q)$ become important and the determination of R_g depends on the shape of the molecule. The final regime is defined as $qR_g \gg 1$. In this regime, the scattering is dominated by the internal structure of the molecules, and it is not possible to determine R_g .³⁶

Instrumentation and Analysis. Light scattering data can be obtained using either batch or online methods. In batch analysis, different concentrations of the solute can be injected directly into the light scattering detector to generate a Zimm plot, which is shown in Figure 2.9a. To analyze a Zimm plot, two extrapolations must be made: one to zero scattering angle and one to zero concentration. The intersection of these two extrapolated lines will be equal to $1/M_w$. Additionally, the slope of the zero scattering angle line is equal to R_g , and the slope of the zero

concentration line is equal to A_2 . Since the sample is not separated before injection for batch analysis, this type of analysis only provides average information for the whole sample and does not provide any information about the distribution.^{36,37}

As an alternative approach, light scattering data can be obtained by an online analysis method where a single concentration of solute is injected into a system including a separation technique (e.g. SEC) and at least one concentration detector (e.g. RI and UV detectors). This type of analysis provides information in the form of a Debye plot, which is shown in Figure 2.9b. Debye plots can be used to determine the M_w and R_g values for each slice of the chromatogram. Due to the addition of a separation technique such as SEC, the online analysis method is able to provide information about the distribution for the sample.^{36,37} Figure 2.10a is a schematic of this type of online system, while Figure 2.10b is a picture of the actual system used for all of the SEC-MALS analysis in this dissertation. The system includes SEC separation, a UV detector, an 18-angle MALS detector, and an RI detector. The use of two separate concentration detectors allows for the characterization of the valency of MVCs as discussed in detail in Chapter 4. Figure 2.11 shows a schematic for the flow cell for the MALS instrument used in this dissertation, which has 18 detection angles.

2.3 Physical, Biological, and Chemical Properties of Hyaluronic Acid

The majority of the different MVC molecules discussed earlier are synthesized by conjugating the GF of interest to a HyA chain.^{20,23-26} HyA was chosen for those studies because of its many desirable properties that make it amenable for conjugation and use in tissue engineering applications. HyA will be used as the backbone for all of the MVC molecules studied in this dissertation, so a brief introduction to its physical, biological, and chemical properties will be discussed here.

HyA is a naturally occurring biopolymer that is a glycosaminoglycan (GAG)-type polysaccharide. It is a copolymer of D-glucuronic acid and N-acetyl-D-glucosamine linked with alternating β -1,4 and β -1,3 glycosidic bonds. The chemical structure of HyA is shown in Figure 2.12, which shows two repeat units. Each repeat unit has a molecular weight of 379 g/mol, and the total molar mass of HyA can be as high as 10 MDa. HyA is a non-sulfated, anionic GAG, which is strongly hydrophilic. The ionic nature of HyA means that in aqueous solution, HyA will form a random coil that is highly hydrated. This swelling behavior impacts the diffusion of HyA through tissues and means that crosslinked networks of HyA will behave as hydrated hydrogel materials. This gives HyA its viscoelastic properties and makes it suited for many biological applications such as lubrication and formation of connective tissues.⁴⁶⁻⁵⁰

HyA is a naturally occurring biomacromolecule that is found in almost every tissue of the body. The largest amounts of HyA are found in the ECM⁵¹⁻⁵⁴ and the soft connective tissues.⁵⁴ Half of the HyA in the body is found in the skin^{55,56} and it is also very prevalent in cartilage and the synovial fluid due to its lubrication properties.⁵⁷⁻⁵⁹ HyA serves numerous diverse roles in directing cell function^{60,61}, including promoting cell adhesion^{62,63}, promoting cell migration^{54,64,65}, and promoting angiogenesis^{62,66,67}. HyA can also serve as an immunosuppressive molecule^{68,69} and plays a crucial role in tissue repair and wound healing.⁷⁰

HyA is able to direct these cell behaviors through signal transduction by binding to the CD44 and RHAMM cell surface receptors.⁷⁰⁻⁷³

In terms of physical properties, HyA is a flexible molecule due to the mix of β -1,4 and β -1,3 glycosidic bonds. However, hydrogen bonding results in stiffening of the HyA backbone. HyA molecules in solution have high viscosity. HyA exhibits shear thinning behavior. High shear stress results in breakage of the hydrogen bonds, allowing the HyA to flow and behave as a flexible chain. This shear thinning behavior allows for HyA to be injected using a syringe, which makes it an ideal polymer for injectable therapeutic applications. Additionally, HyA behaves as a viscoelastic material, which means it exhibits both viscous and elastic behaviors.^{46,74} The viscoelastic and mechanical properties of HyA can be determined using rheology. Extensive studies of the mechanical properties of HyA using rheology have shown that the modulus of the HyA is dependent upon the temperature, pH, concentration of HyA in solution, and concentration of crosslinks.⁷⁵ This allows for the mechanical properties of HyA to be easily and precisely tuned, which makes it an ideal polymer for tissue engineering scaffolds with tunable mechanical properties.

HyA is FDA approved and is widely used in cosmetics and medical applications^{62,76}, due to its biocompatibility, biodegradability, and non-immunogenicity.⁷⁰ The fields of medicine that commonly use HyA in therapeutics include dermatology and plastic surgery^{77,78}, orthopedic surgery and rheumatology^{79,80}, pharmacology and drug delivery^{81,82}, and surgery and wound healing.^{83,84} HyA is also commonly used for scientific studies, especially in the area of cell scaffold materials for tissue engineering.⁸⁵⁻⁸⁹ The ease of chemical modification of the carboxyl and hydroxyl groups on the HyA chain also make it an ideal polymer for bioconjugate techniques, including drug delivery strategies and MVC technology.^{20,23,50,85} HyA for experimental use can be isolated from many different natural sources, such as *Streptococcus zooepidemicus*, rooster comb, bovine vitreous humor, and human umbilical cord.⁹⁰⁻⁹⁵ HyA can also be synthetically manufactured to yield higher purity and greater control over molecular weight and polydispersity.

2.4 References

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2.5 Tables

Table 2.1

Summary of molecular weight characterization techniques, whether they are absolute or relative measurements, and the information they provide.

Characterization Method	Absolute or Relative	Information Provided
Size exclusion chromatography	Relative	Full distribution
Intrinsic viscosity	Relative	M_v
Osmotic pressure	Absolute for membrane osmometry; Relative for vapor pressure osmometry	M_n
Other colligative properties	Absolute	M_n
End group analysis	Absolute	M_n
MALDI mass spectrometry	Absolute	Full distribution
Light scattering	Absolute	M_w
Sedimentation velocity	Absolute	M_w
Sedimentation equilibrium	Absolute	M_z

2.6 Figures

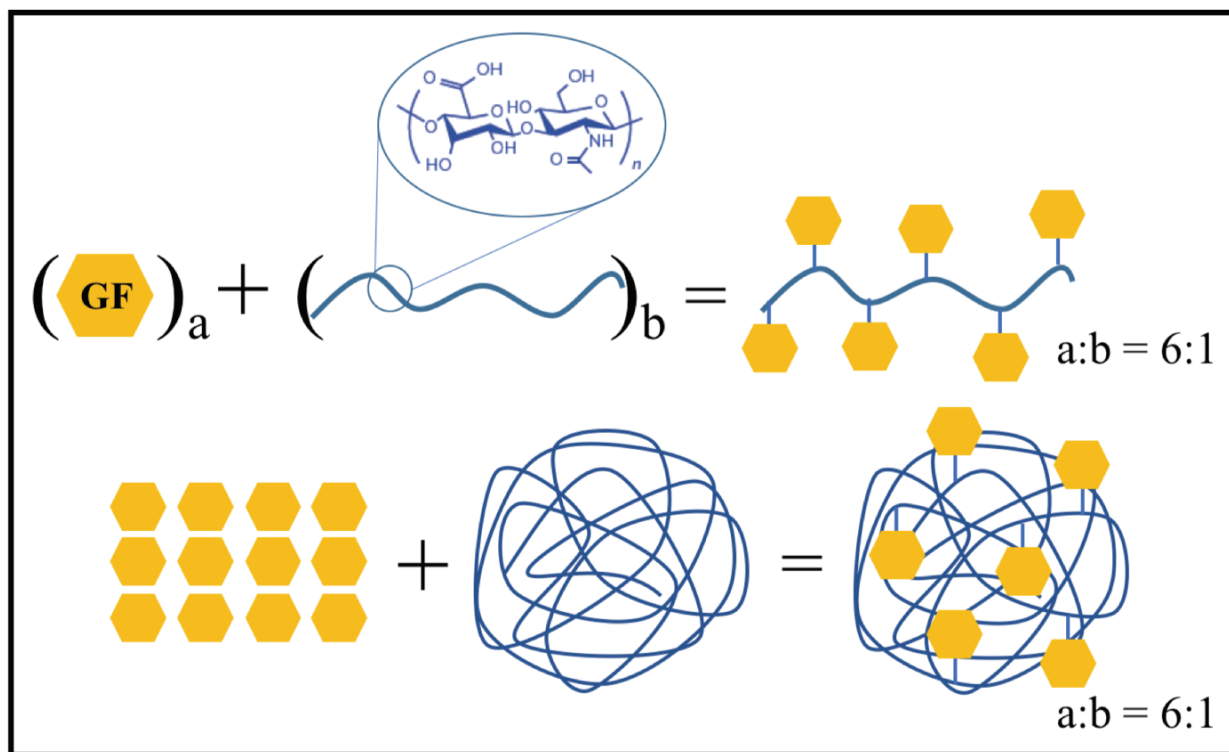


Figure 2.1

Basic schematic of MVC synthesis. The yellow hexagons represent a generic GF, while the blue coiled lines represent the soluble polymer backbone (e.g. HyA).

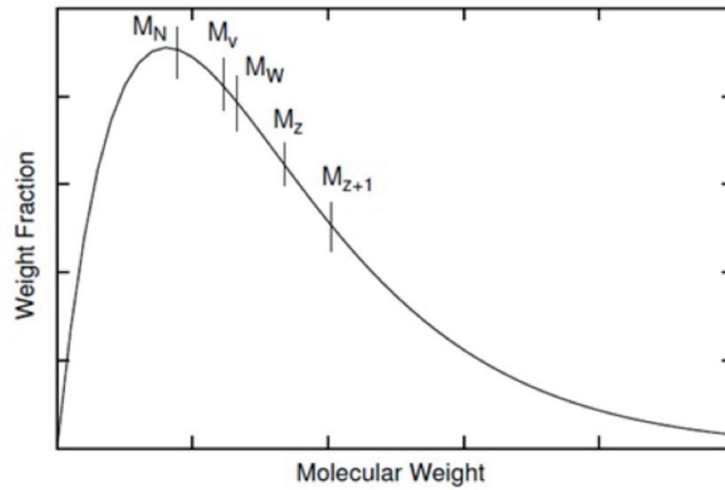


Figure 2.2

Schematic of the distribution of the different average molecular weights.⁹⁶ $M_Z > M_W > M_V > M_N$

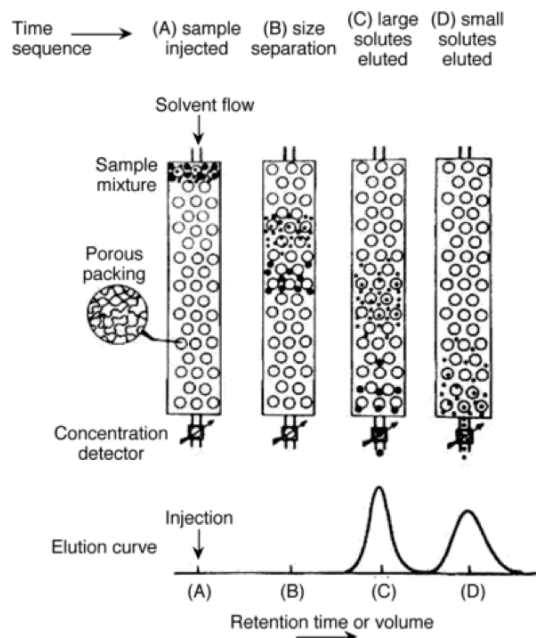


Figure 2.3

Schematic of size separation and detection by size exclusion chromatography (SEC).⁹⁷

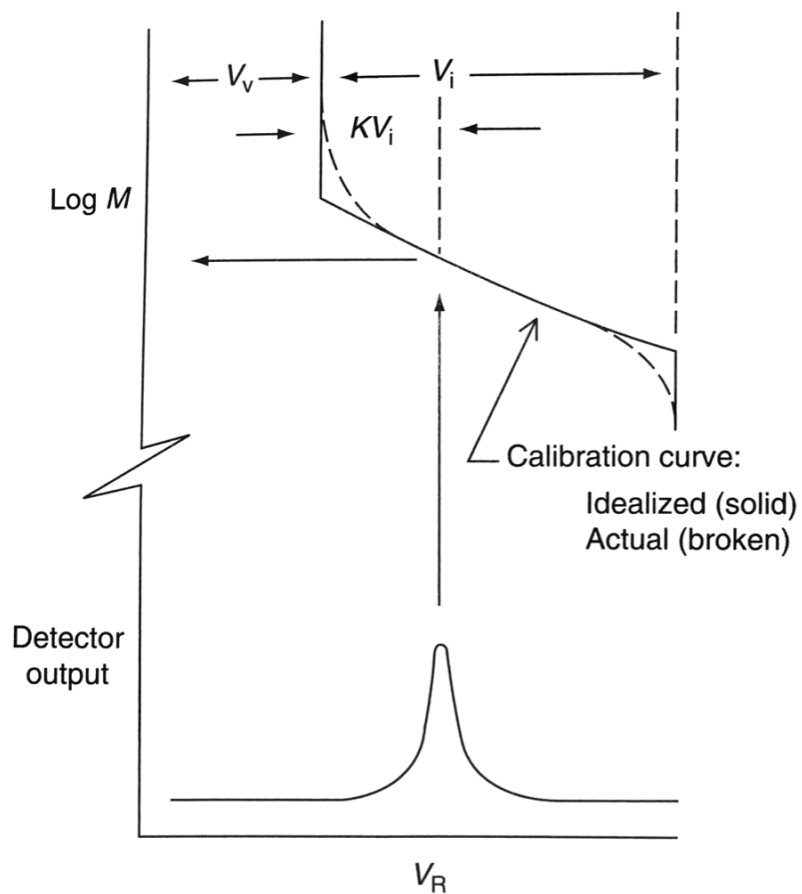


Figure 2.4

Calibration curve for SEC as log of molecular weight versus the retention volume. This shows how the location of the detector signal can be used to determine molecular weight.³⁶

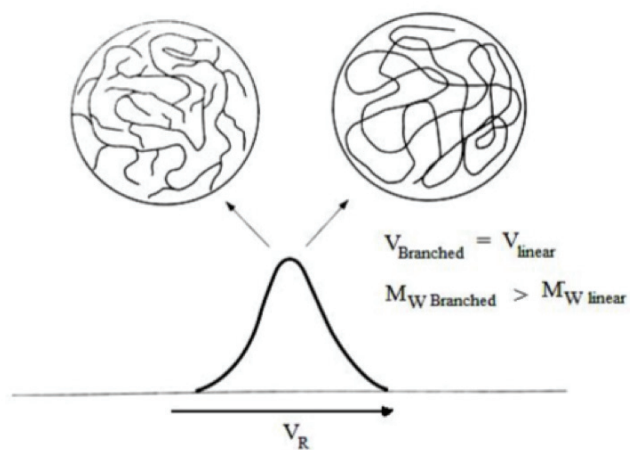


Figure 2.5

SEC elution behavior of a linear and branched molecule with the same hydrodynamic radius but different molecular weights.⁹⁸

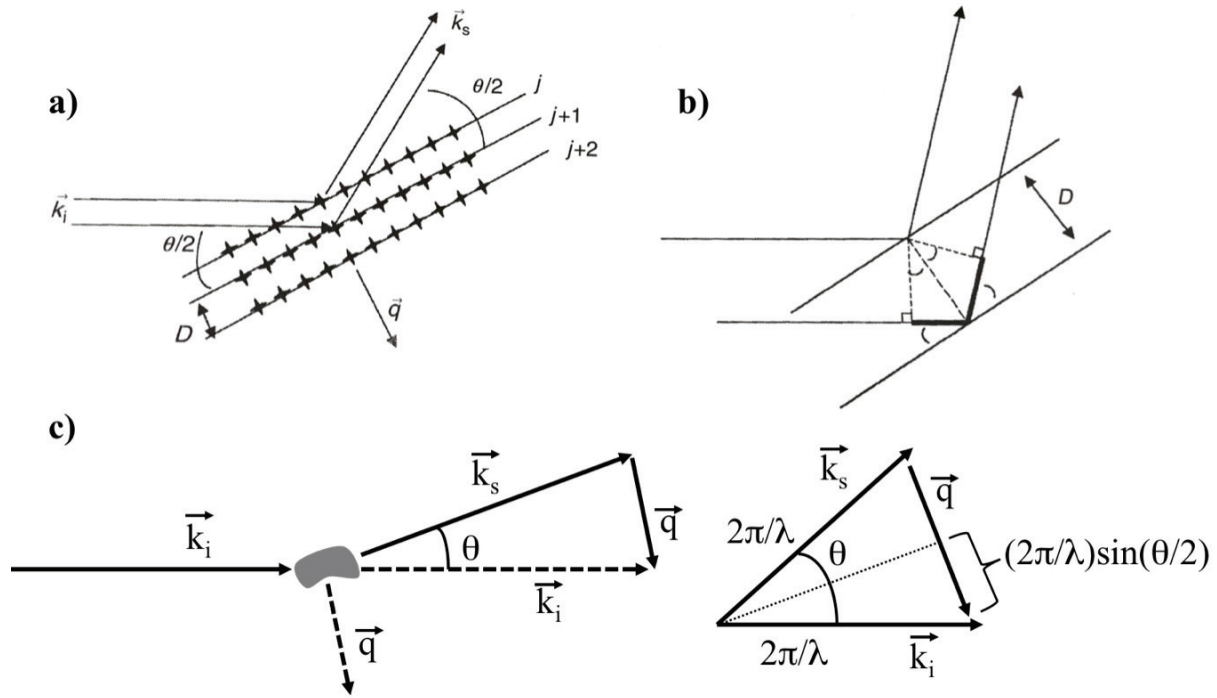


Figure 2.6

Illustration of Bragg's law. (a) The incident light wave is scattered from planes of atoms at an angle of $\theta/2$. The planes are separated by a distance D , and the scattering vector is shown. (b) The extra distance traveled by a wave scattered from the second plane of atoms is shown by the two bold segments, which are each equal to $D \sin(\theta/2)$. (c) Geometric determination of the magnitude of the scattering vector.³⁶

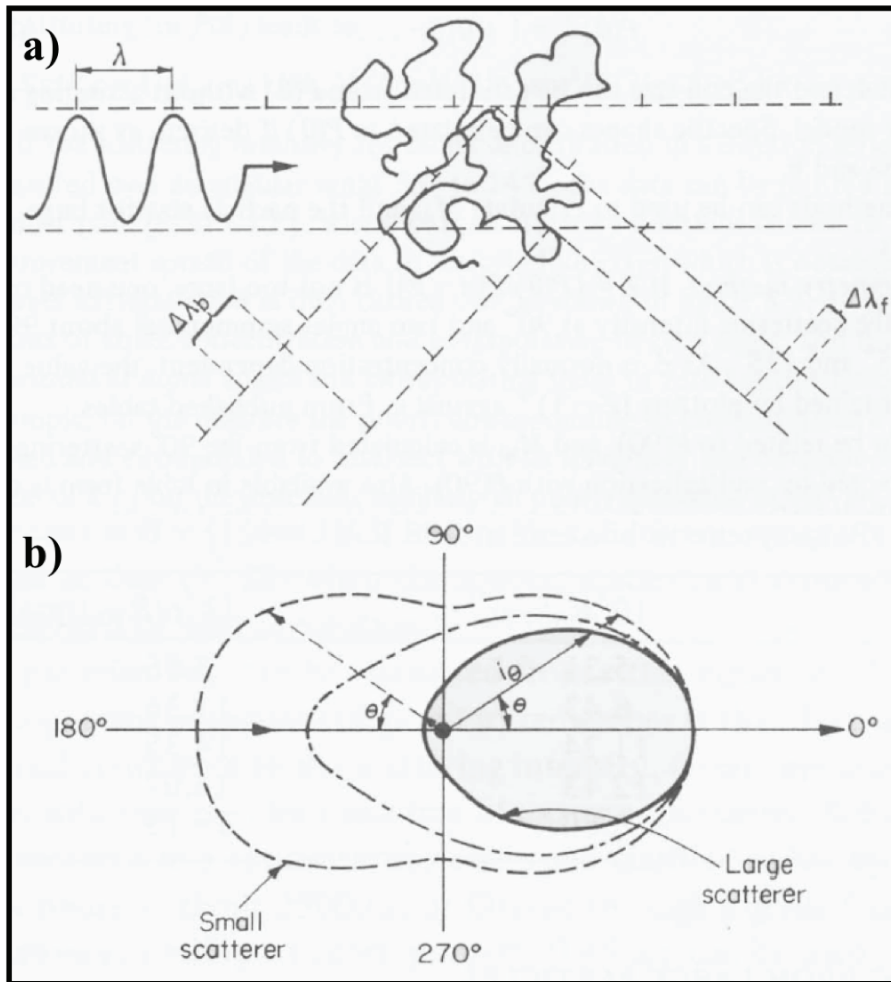


Figure 2.7

(a) Schematic of destructive interference of light scattered from a large polymer particle. $\Delta\lambda$ indicates the amount by which the waves arriving at the detector are out of phase. (b) Angular dependence of light scattering illustrated by the distribution of the intensity of light scattered at different angles.⁹⁹

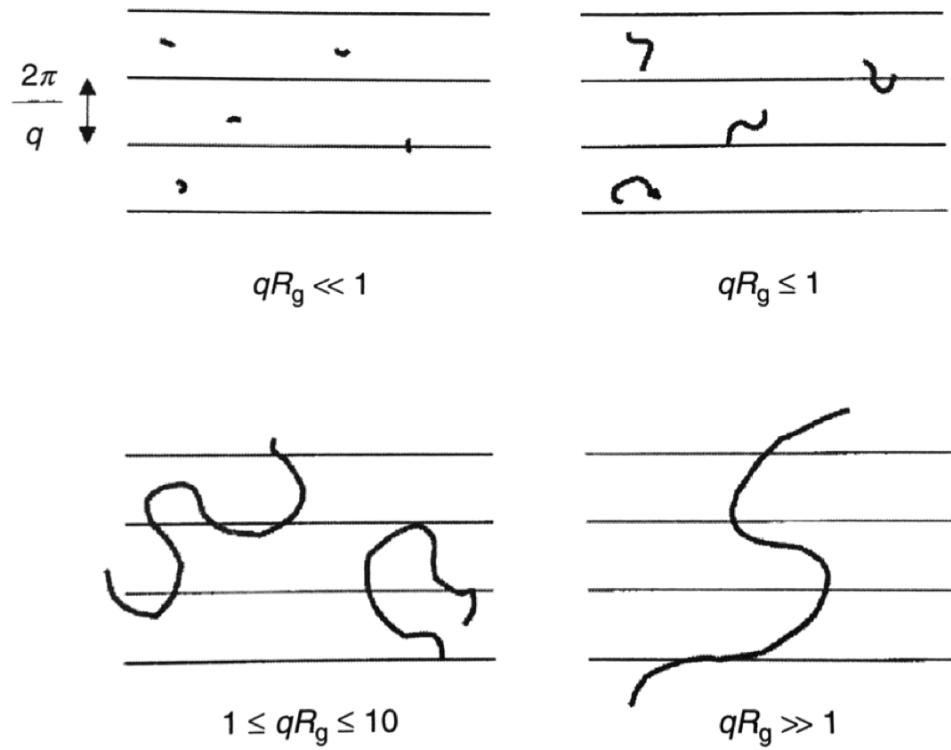


Figure 2.8
Illustration of the four different scattering regimes.³⁶

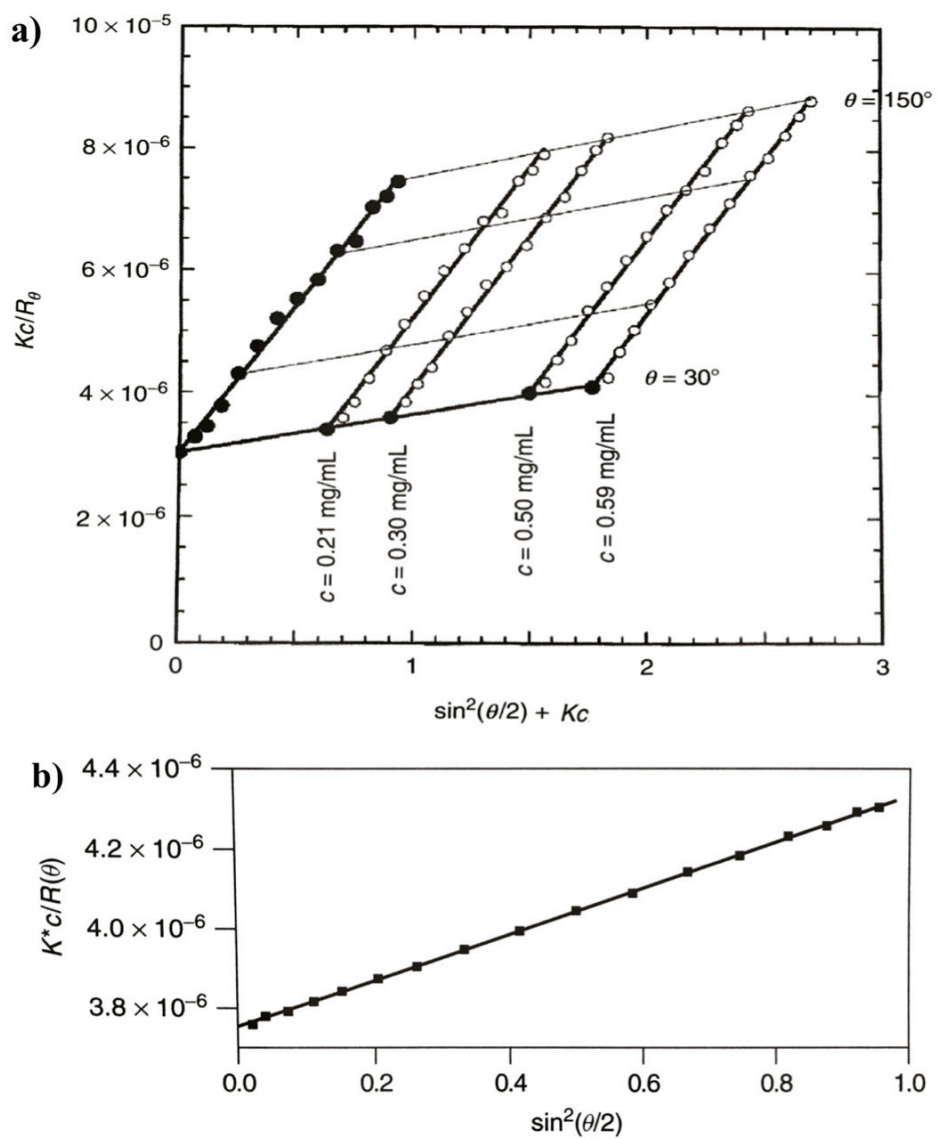


Figure 2.9
Representative (a) Zimm plot and (b) Debye plot used for MALS analysis.^{36,37}

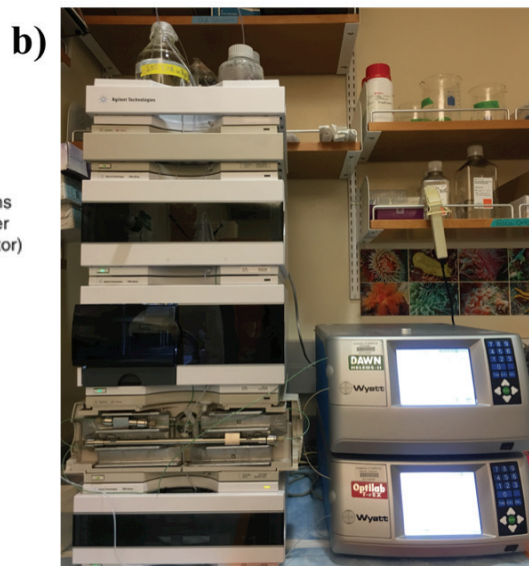
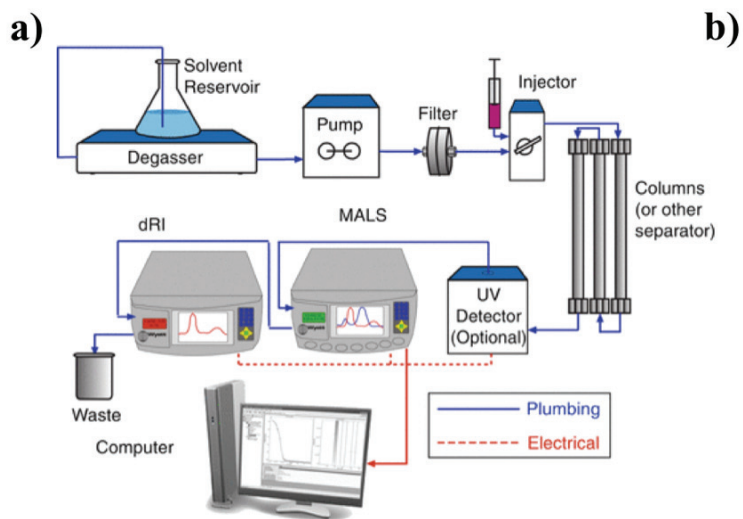


Figure 2.10

(a) Schematic of an online MALS system including a separation technique and two concentration detectors. (b) Picture of the system used for all SEC-MALS analysis in this dissertation.^{36,100}

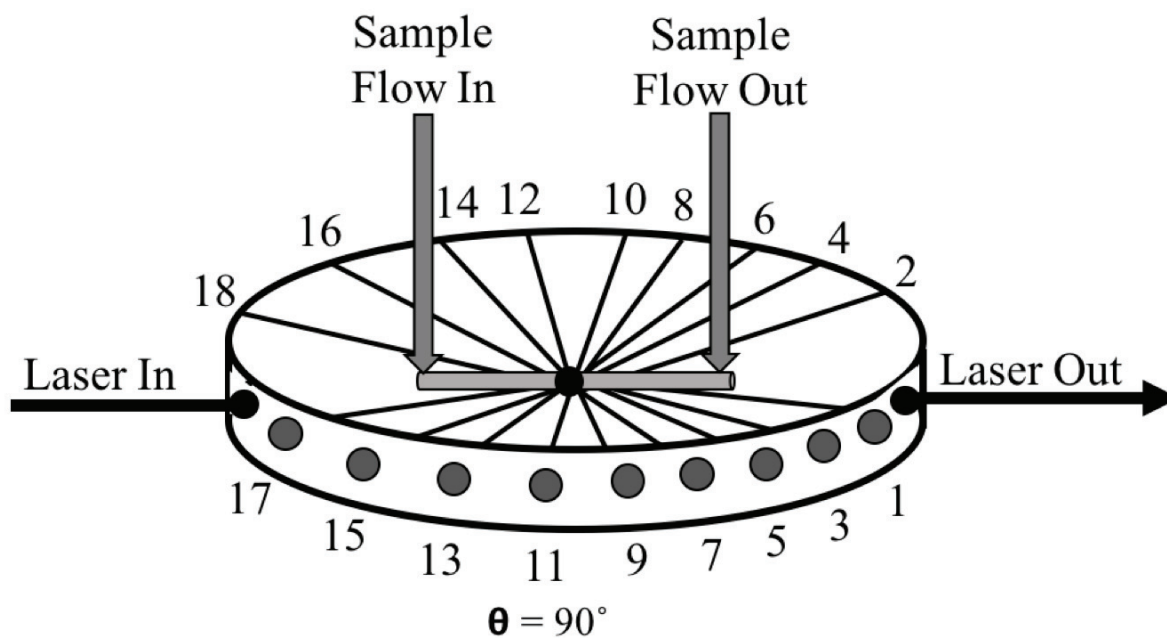


Figure 2.11

Schematic of the flow cell in a multi-angle light scattering detector. This shows an instrument with 18 different detector angles.

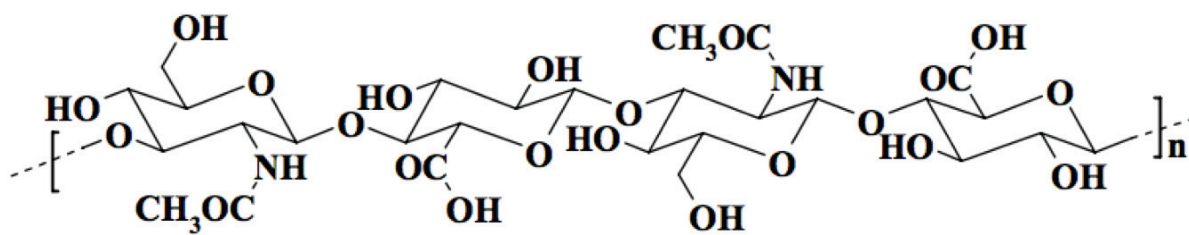


Figure 2.12

Chemical structure of HyA. Two repeat units are shown.⁴⁶

CHAPTER 3: SYNTHESIS AND CHARACTERIZATION OF MULTIVALENT CONJUGATES OF MECHANO-GROWTH FACTOR

3.1 Abstract

Congestive heart failure (CHF) results from damage caused to cardiac muscle tissue during stressful conditions, such as ischemia during a myocardial infarction. CHF affects over 5 million people and results in healthcare costs of around \$32 billion in the US annually, but current treatments only mitigate the symptoms and attempt to prevent progression of the disease. Therefore, current research interest focuses on creating a therapeutic to prevent or repair this cardiac tissue damage. In order to create a potential cardiac therapeutic, mechano-growth factor (MGF) peptide was multivalently conjugated to HyA to combine its cardioprotective properties with the potential for improved bioactivity and pharmacokinetics provided by multivalent conjugate (MVC) technology. This work focuses primarily on the development of the chemical reactions to synthesize the MVCs, and the initial characterization of the conjugates. A two-part conjugation reaction method was developed in which the HyA is first modified with acrylate groups. The second part of the conjugation uses the acrylate groups on the HyA in a Michael addition reaction with a c-terminal cysteine residue added to the MGF peptide. The success of the two different parts of the reaction method are characterized through several techniques including gelation, NMR spectroscopy, and a BCA assay.

3.2 Introduction

Approximately 5.1 million Americans suffer from congestive heart failure (CHF), and about half of those people will die within 5 years of being diagnosed with the disease. In 2009, CHF contributed to 1 in 9 deaths in the United States.¹ According to the American Heart Association, CHF cases result in approximately \$32 billion worth of healthcare costs each year in the United States.²

CHF occurs when the heart muscle has become weakened and can no longer pump enough blood to provide oxygen and nutrients to the body's organs. CHF is caused by a number of different cardiac diseases, which weaken or damage the cardiac muscle tissue. One major cause of CHF is myocardial infarction (MI) in which blood flow to the heart is stopped by the blockage of a coronary artery. This loss of blood flow creates an ischemic environment, resulting in the death of cardiomyocytes (CMs), which comprise the heart muscle tissue. Once blood flow is returned to the heart, this leaves a scarred area of muscle tissue that is unable to heal and no longer functions normally. Over time, this scarred area of the muscle begins to thin and dilate, which reduces the heart's ability to pump blood efficiently.³⁻⁵

Current treatment options for CHF focus on mitigating and controlling the disease, usually with the use of medications, such as ACE inhibitors and beta blockers. Lifelong management of the disease is necessary, and over time it can progress to the point that medications are no longer sufficient. Patients with severe heart failure may need a left ventricular assist device (LVAD) or a heart transplant. LVADs are typically used as a temporary solution for patients awaiting a heart transplant, while permanent LVADs are implanted in terminally ill patients who are ineligible for

a heart transplant. While heart transplantation can replace a severely damaged heart with a healthy donor heart, there are not enough donor hearts available for patients with severe CHF. Additionally, heart transplantation has a high rate of failure and requires lifelong suppression of the patient's immune system, among many other risks.³⁻⁵

Since the majority of current treatments for CHF focus solely on slowing the progression of the disease and mediating the symptoms, there is a significant amount of interest in developing a therapy that will actually reduce or repair the damage to the cardiac muscle tissue. Therefore, it is desirable to design a therapeutic that will provide a cardioprotective effect to cardiac cells and tissues. The term cardioprotective encompasses inhibition of apoptosis and retention of function for cardiac cells undergoing stressful conditions.

Mechano-growth factor (MGF) is a novel peptide that has shown promising cardioprotective characteristics. MGF peptide is derived from a splice variant of insulin-like growth factor I (IGF-1) in which 49 additional base pairs from exon 5 are inserted, which shifts the reading frame to generate a protein splice variant containing a distinct E-domain at the c-terminus. The isolated E-domain is a 24 amino acid sequence in humans that had been named MGF peptide, or simply MGF.⁶⁻⁸ The amino acid sequence of MGF peptide is shown in Table 3.1.

IGF-1 is expressed by skeletal muscle, smooth muscle, and cardiac muscle in humans, with skeletal muscle being a major source of circulating IGF-1. In response to different stimuli, the IGF-1 gene can be spliced differently to yield several different isoforms: IGF-1Ea (liver form) and IGF-1Ec (MGF). The IGF-1Ec splice variant, which contains the MGF peptide E-domain, was discovered and characterized due to its upregulation during skeletal muscle hypertrophy. Since this isoform is expressed in response to mechanical stimulation, it was named mechano-growth factor.⁶⁻⁹ During skeletal muscle injury, the MGF splice variant is rapidly expressed and then decreases over time to be replaced by upregulation of IGF-1Ea. The initial expression of the MGF splice variant serves to activate satellite cells, while the later expression of the IGF-1Ea variant modulates protein synthesis to complete the repair of damaged muscle tissue.^{10,11} Through blocking of the IGF-1 receptor, it was shown that MGF peptide activity is modulated by a different receptor pathway that has yet to be fully defined.¹⁰

More recent studies have shown that MGF expression is also temporally regulated in cardiac tissue in response to ischemia. MGF is upregulated within an hour post-MI and remains upregulated for up to 8 weeks, suggesting it plays an important role in mitigating cardiac tissue damage.^{12,13} Studies of stressed cardiac cells treated with MGF peptide demonstrated that MGF inhibits the intrinsic apoptosis pathway through interference with the caspase cascade. Caspase-3 activity was shown to be significantly reduced in MGF-treated cells compared to controls.¹⁴ Treatment with MGF immediately post-MI in mice and sheep models improved preservation of cardiac function and reduced apoptosis of cardiac cells at the infarct.^{14,15} Additionally, MGF has been shown to stimulate the migration and proliferation of mesenchymal stem cells^{16,17} and to stimulate pro-angiogenic activity of vascular endothelial cells¹⁸, which may play a role in repairing damaged cardiac tissue.

Multivalent conjugate (MVC) technology is a powerful technique for presenting proteins or growth factors of interest to cell-surface receptors. This technology involves the conjugation of

multiple proteins or growth factors to a soluble polymer backbone, such as hyaluronic acid. Previous studies have shown that creation of these high molecular weight bioconjugate molecules can reduce the proteolytic degradation¹⁹ and improve the pharmacokinetics¹⁹⁻²¹ of the growth factor. Additionally, by varying the valency, or ratio of growth factor molecules conjugated to each polymer chain, it is possible to enhance the bioactivity of the growth factor over that of unconjugated growth factor at the same concentration.²²⁻²⁵ In theory, this effect may be due to clustering of receptors on the cell surface. When one growth factor on the MVC is bound by a cell surface receptor, it brings the other growth factors on that MVC into proximity of additional receptors, thereby effectively increasing the concentration of growth factors at the cell surface.²⁶ Additionally, there is an entropic penalty associated with a cell surface receptor binding a growth factor. MVC molecules can lower the total entropic penalty for multiple binding events because when the first growth factor is bound, it brings the MVC molecule into close proximity of the cell surface, thereby lowering the entropic penalty for further binding events.^{27,28}

In this work, multivalent conjugates of MGF peptide with HyA were synthesized in order to combine the cardioprotective effects of the peptide with the potential for improved bioactivity and pharmacokinetics of MVC technology. This chapter focuses on the chemistry of synthesizing these conjugates and the basic characterization to confirm the success of the conjugation reaction.

3.3 Materials and Methods

3.3.1 Acrylation of Hyaluronic Acid

Hyaluronic acid (HyA) with a molecular weight of ~ 600 kDa as determined by the manufacturer through viscosity measurements was obtained from LifeCore Biomedical. First, the HyA was modified to have hydrazide groups (ADH-HyA) by following previously reported chemistry.²⁹⁻³³ Briefly, the hyaluronic acid was dissolved in 100 mL of deionized (DI) water overnight to form a 3 mg/mL solution. Adipic acid dihydrazide (ADH) (Sigma-Aldrich) was then added in 30 molar excess to the HyA, and the pH of the reaction was adjusted to 6.8. 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Fisher Scientific) and 1-hydroxybenzotriazole hydrate (HOBt) (Sigma-Aldrich) were dissolved separately at 3 mmol in DMSO/DI water (1:1 volume ratio, 3 mL) and then added to the reaction solution sequentially. The solution pH was again adjusted to 6.8 and was maintained using 0.1 M NaOH and 0.1 M HCl for at least 4 hours, before letting the reaction proceed overnight. Following this reaction, the pH was adjusted to 7 and the HyA solution was dialyzed exhaustively in 10,000 Da molecular weight cut-off (MWCO) dialysis tubes (Spectrum Labs) against first 1 g/L NaCl in DI water for two days and then against pure DI water for one day. After dialysis, 5 mg/mL of NaCl was added to the HyA solution, and then this solution was precipitated in 100% ethanol. The precipitated ADH-HyA was pelleted by centrifugation and then redissolved in DI water. The solution was dialyzed again following the same method. In order to generate acrylate groups on the HyA (Ac-HyA), the solution was subsequently reacted with N-acryloxysuccinimide (Acros Organics) (700 mg per 300 mg HyA in 100 mL DI water).^{29,32-35} The final Ac-HyA product was lyophilized for at least 3 days to remove all of the water and then stored at -20 °C. The degree of

available carboxyl groups that were conjugated with acrylate groups was confirmed to be ~30% by proton (^1H) NMR as previously described.^{32,33,36}

3.3.2 Multivalent Conjugation of Mechano-Growth Factor to Hyaluronic Acid

MGF peptides with a cysteine residue added to the c-terminal (MGF-Cys) were obtained from American Peptide Company. The peptide sequences are shown in Table 4.1. The cysteine residue was added to allow the conjugation of the peptide to Ac-HyA through a Michael-type addition reaction between the thiol on the peptide and the acrylate group on the modified HyA. Briefly, Ac-HyA was dissolved overnight at 1 mg/mL in phosphate-buffered saline (PBS). MGF peptide was dissolved in PBS at 1 mg/mL and added to the Ac-HyA solution in different molar feed ratios, such as 15:1, 30:1, and 60:1 (MGF:HyA). These reactions were allowed to proceed overnight and the following day, L-cysteine (Sigma-Aldrich) was dissolved at 10 mg/mL in PBS and added in 2-fold excess relative to the acrylate groups on the HyA in order to quench any unreacted acrylate groups. This reaction was allowed to proceed overnight, and then the solutions were transferred to 100 kDa MWCO Float-A-Lyzer dialysis tubes (Spectrum Labs) and dialyzed exhaustively against PBS.

3.3.3 Gelation of Ac-HyA to Confirm Successful Acrylation

Ac-HyA was dissolved overnight in triethanolamine buffer (TEOA; 0.3M, pH 8) at a concentration of 10 mg/mL. Thiolated 5 kDa poly(ethylene glycol) (PEG) crosslinker (Laysan Bio, Inc.) was dissolved in TEOA buffer at 173 mg/mL, and then 1.25 mg of crosslinker was added per mg of Ac-HyA. This solution was allowed to gel overnight at 37 °C to confirm that the acrylation reaction was successful.

3.3.4 Bicinchoninic Acid Assay

A bicinchoninic acid (BCA) assay kit (Thermo Scientific Pierce) was used to quantify the concentration of peptide in solution after conjugation. This kit uses a colorimetric two-component assay reagent to measure total protein concentration compared to a protein standard. The first step of the two step detection reaction depends on the biuret reaction, in which copper (Cu^{2+}) ions chelate with the amino acid residues in the protein. This reduces the copper to Cu^{1+} and forms a light blue complex. The second step involves the reaction of BCA with the reduced copper ions to form a water soluble complex with an intense purple color. This BCA/copper complex exhibits linear absorbance with changing protein concentration at 562 nm. This assay has a linear detection range from 20-2,000 $\mu\text{g/mL}$ of protein.

Multivalent conjugate solutions, along with bovine serum albumin (BSA) protein (Thermo Scientific Pierce) standards, were plated in a standard 96-well cell culture plate for the assay. A volume of 25 μL of each sample and standard were added per well, with three repeats per sample and standard. The BSA standards were created by serial dilution from 8 to 250 $\mu\text{g/mL}$. 200 μL of the BCA assay reagents was added to each well and then the plate was incubated at 37 °C for 15-30 minutes. The plate was allowed to cool to room temperature for 5 minutes and then the absorbance at 562 nm was read on a Molecular Devices SpectraMax i3x microplate reader. The absorbance versus concentration was plotted for the BSA standards, and a linear fit was applied

to obtain an equation for relative absorbance to protein concentration. This equation was used to calculate the concentration of each of the mvMGF samples from their absorbance measurements.

3.4 Results and Discussion

3.4.1 Conjugation Reaction

Multivalent conjugates of MGF peptide were synthesized following the reaction schematic shown in Figure 3.1. The first part of the reaction shows the activation of hyaluronic acid by converting a percentage of the side groups into acrylate groups, which can react with the thiol on a cysteine residue by a Michael addition reaction. This happens by a two-step reaction in which first the HyA is modified with adipic dihydrazide (ADH), and then the hydrazide groups are converted to acrylate groups with the addition of N-acryloxysuccinimide. The second part of the reaction schematic shows the conjugation of the MGF peptide modified with a c-terminal cysteine to the Ac-HyA through Michael addition. A Michael addition reaction was chosen for the conjugation because it can be carried out at room temperature in neutral pH. Also, the reaction does not require the presence of any additional chemicals, which would need to be dialyzed out of the final product. In the final step of the reaction, the unused acrylate groups were quenched with cysteine residues so that they are no longer active in the final product. The reaction schematic in Figure 3.1 illustrates the HyA chains as stretched out chains for ease of showing the reactions, in reality however, the physics of polymer solutions dictates that the HyA chains in a good solvent will instead behave as hydrated spherical particles of polymer. Figure 3.2 is included to better illustrate the structure of the MVC molecules in solution.

MGF peptides were obtained with several different modifications to the original amino acid sequence. Table 3.1 shows the sequence of unmodified MGF peptide, which was used as a control in experimental assays. Table 3.1 also shows the sequence for the Cys-MGF, as well as a version with an added tryptophan residue (MGF-Trp) for use with SEC-MALS characterization (discussed in Chapter 4).

3.4.2 Confirming Successful Acrylation of Hyaluronic Acid

The first step in characterizing the conjugates was first to determine if the acrylation of the HyA was successful. In order to confirm the presence of acrylate groups, and also to quantify the efficiency of the acrylation reaction, the Ac-HyA was characterized by proton (^1H) NMR. Figure 3.4 shows the NMR spectra for unmodified HyA (top) and a batch of the Ac-HyA (bottom). One arrow points out the large peak at about 6 ppm formed by the hydrogen atoms in the CH_3 group for the non-acrylated side group of HyA. Since these three hydrogens all have identical structure, they contribute to one peak. The area under this peak decreases for the Ac-HyA compared to the unmodified HyA because some of these side groups become acrylated. The other arrow points out the two peaks at about 2 ppm formed by the hydrogen atoms on the acrylate groups. Since these hydrogen atoms now have two different bond structures due to the presence of the double bond, they form two separate peaks. These acrylate peaks are not present in the unmodified HyA sample, but appear in our Ac-HyA samples indicating successful acrylation. The efficiency of this acrylation can be calculated by comparing the area under the two peaks at 2 ppm to the area under the peak at 6 ppm. The acrylation efficiency was

determined to be approximately 30%, meaning that 30% of the HyA side groups were converted to acrylate groups.

In order to additionally confirm the presence of acrylate groups, a thiolated-PEG crosslinker was added to a solution of HyA to see if a hydrogel would form. If the HyA had a sufficient number of acrylate groups available for crosslinking, a significant viscosity change could be observed visually after the addition of crosslinker. Figure 3.3 shows a solution of Ac-HyA and thiolated-PEG crosslinker that has formed a viscous hydrogel, thereby confirming that the acrylation reaction was successful.

3.4.3 Bicinchoninic Acid Assay

BCA assay was used to determine the concentration of peptide in the final conjugate solutions after dialysis. Data for a representative set of conjugates is shown in Figure 3.5. For this data set, three different initial molar feed ratio (MGF:HyA) conjugates were measured (15:1, 30:1, and 60:1). The linear fit from the BSA standards was used to determine the concentration of peptide in solution for each of these three conjugates. Then those concentrations were used to calculate an estimated conjugation ratio by assuming that the HyA molecular weight was the same as the unmodified HyA. The final conjugation ratios estimated from the BCA assay are shown in the table in Figure 3.5.

This assay is useful for estimating the concentration of the protein and confirming successful conjugation, but it only provides incomplete information about the conjugates. The accuracy of the peptide concentration measurement is not very high because the BCA assay is made for the characterization of proteins, but instead it is being used with a peptide. The color change from the BCA assay is strongly influenced by the presence of cysteine, tyrosine, and tryptophan residues in the amino acid sequence. However, in a large protein, the universal amino acid backbone also contributes to the color change and minimizes the variability from protein composition differences. In a short peptide, such as MGF, there are not enough amino acids to minimize compositional variability, so the presence a single amino acid could have a significant impact on the BCA measurement. However, the BCA assay is a simple way to confirm the success of conjugation and to get an approximation of the peptide concentration.

Additionally, the BCA assay yields incomplete information since it does not measure the molecular weight of the HyA or its polydispersity. To estimate conjugation ratios, the HyA molecular weight must be assumed to be the same as the unmodified HyA. This is unlikely to be the case since HyA is very sensitive to shear stress and tends to degrade and increase in polydispersity during reactions. Therefore, these conjugation ratios are just rough estimates, but they can confirm the success of the conjugation and provide an estimation of the final conjugation ratios. Chapter 4 will discuss SEC-MALS characterization of these conjugates, which provides much more complete information about the final conjugation ratios.

3.5 Conclusion

In this work, a reaction method was developed to allow for the multivalent conjugation of MGF peptide to a HyA backbone chain. This allows for the combination of the cardioprotective effect

of the MGF peptide and the potential for improved bioactivity and pharmacokinetics provided by multivalent conjugate technology. The conjugation reaction required first the synthesis of a HyA intermediate by the addition of acrylate groups, which were characterized through gelation and NMR spectroscopy. Then the conjugation was achieved by a Michael addition reaction between the acrylate groups on the HyA and the c-terminal cysteine on the MGF peptide. The success of this conjugation reaction was verified through a BCA assay, which also provided an estimate of the final conjugation ratios. The characterization using NMR spectroscopy, Ac-HyA gelation, and BCA discussed in this chapter was able to confirm the success of conjugation and provide estimates of the peptide concentration and final conjugation ratio. However, additional characterization is needed to accurately determined the conjugation ratios, and will be discussed in Chapter 4.

3.6 References

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3.7 Tables

Table 3.1

The amino acid sequence of MGF peptide, plus sequences for the modified forms of MGF peptide used to synthesize multivalent conjugates. The MGF-Trp was used to synthesize conjugates with strong UV absorbance for characterization with the three detector method discussed in Chapter 4.

	Amino Acid Sequence
MGF	Tyr-Gln-Pro-Pro-Ser-Thr-Asn-Lys-Asn-Thr-Lys-Ser-Gln-Arg-Arg-Lys-Gly-Ser-Thr-Phe-Glu-Glu-His-Lys
MGF-Cys	Tyr-Gln-Pro-Pro-Ser-Thr-Asn-Lys-Asn-Thr-Lys-Ser-Gln-Arg-Arg-Lys-Gly-Ser-Thr-Phe-Glu-Glu-His-Lys-Cys
MGF-Trp	Tyr-Gln-Pro-Pro-Ser-Thr-Asn-Lys-Asn-Thr-Lys-Ser-Gln-Arg-Arg-Lys-Gly-Ser-Thr-Phe-Glu-Glu-His-Lys-Trp-Gly-Gly-Cys

3.8 Figures

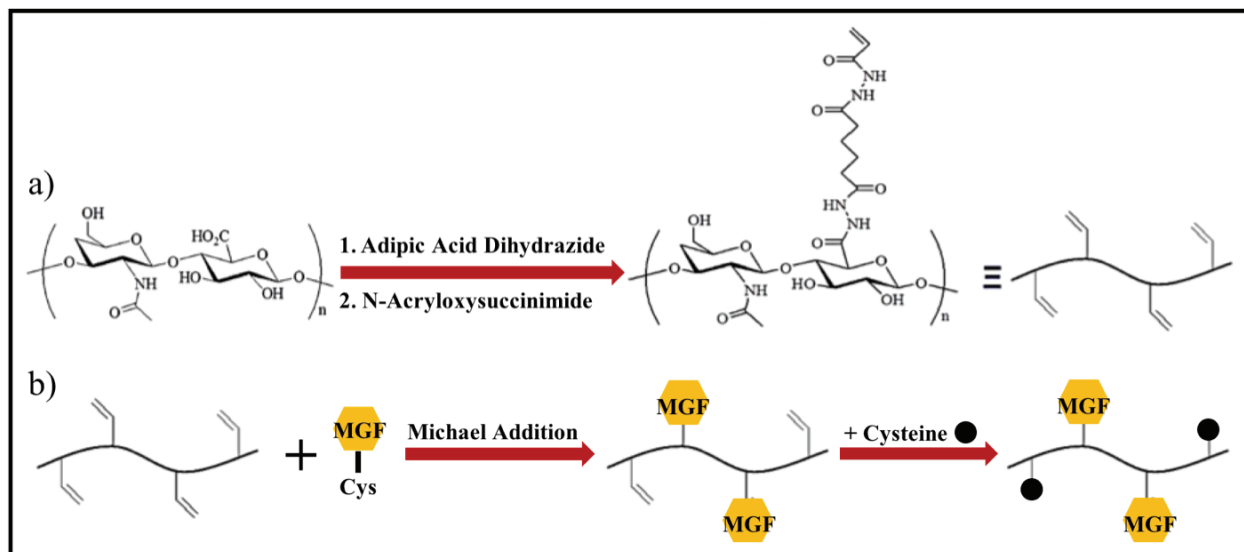


Figure 3.1

Reaction schematic for the conjugation of MGF peptide to HyA. (a) Synthesis of an acrylated HyA precursor and (b) a Michael addition reaction between the acrylate groups on the HyA and the c-terminal cysteine on the MGF peptide.

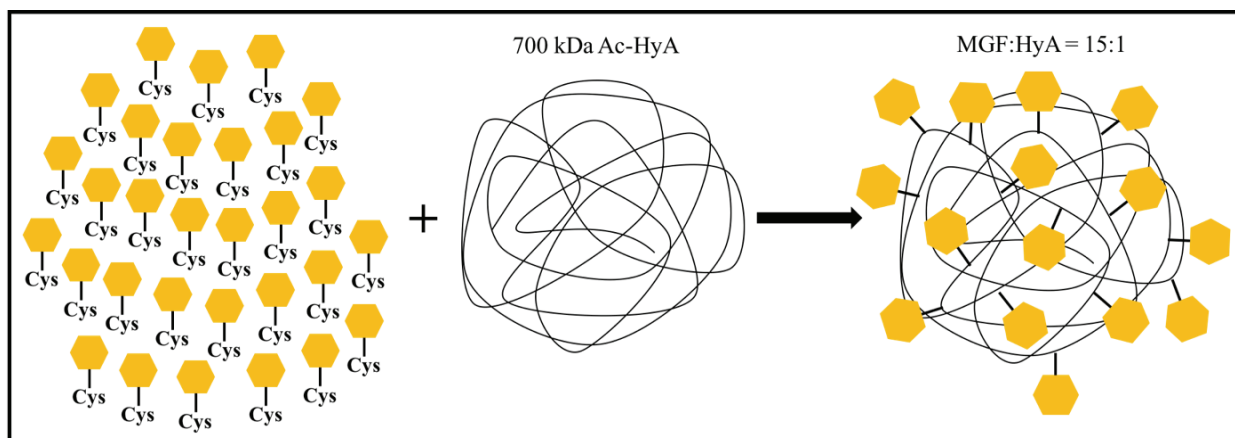


Figure 3.2

Illustration of the conjugates' conformation in a thermodynamically good solvent. This means that the interactions between the polymer molecule and the solvent are energetically favorable, so the polymer coils will expand to form a hydrated spherical particle. In a poor solvent, the interactions between the polymer and solvent would be energetically unfavorable, so the polymer chains would collapse to exclude the solvent.

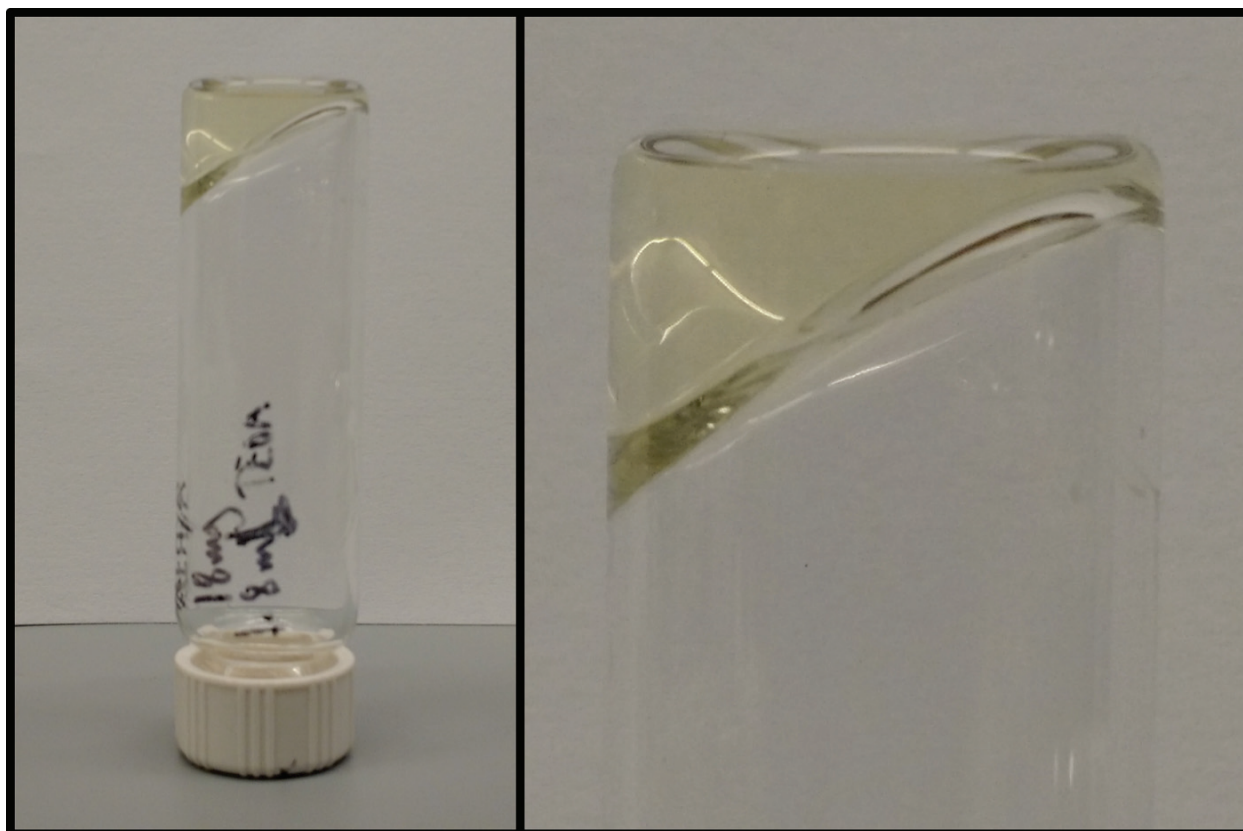


Figure 3.3

Photographs showing the gelation of Ac-HyA with the addition of a thiolated-PEG crosslinker. This confirms the successful acrylation of the HyA.

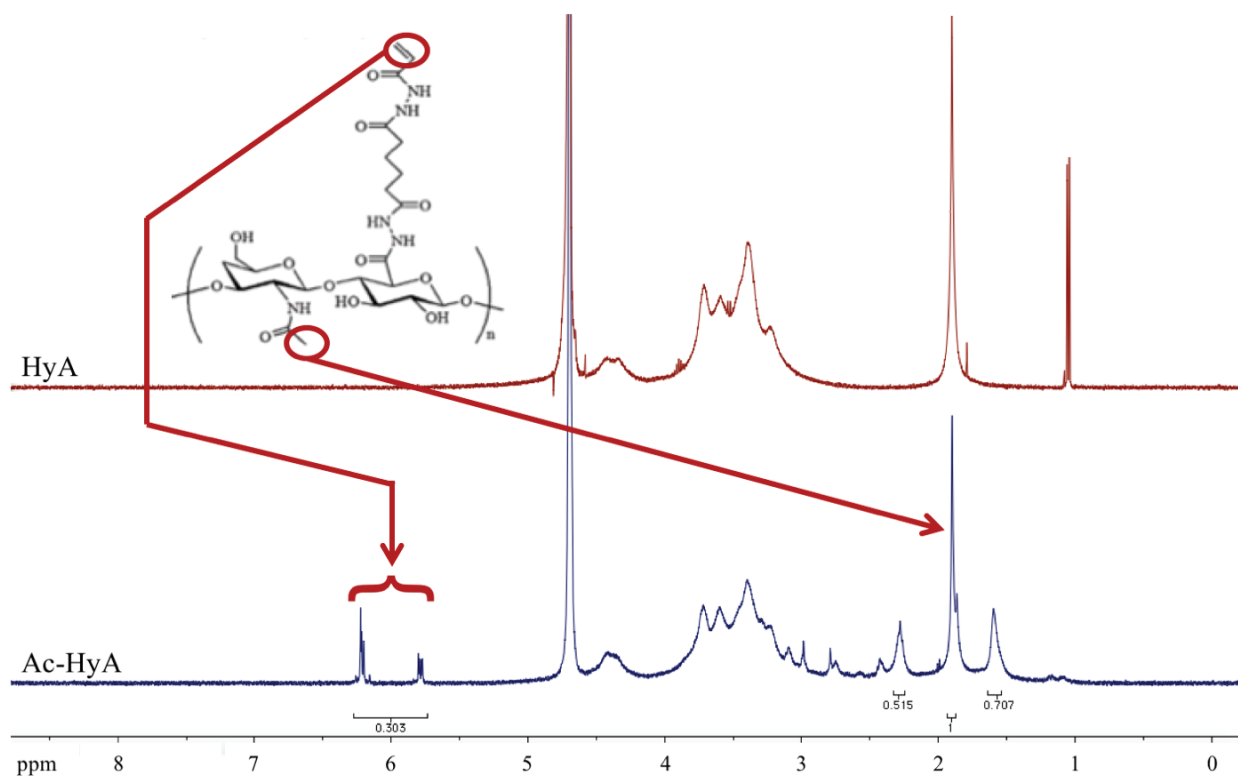
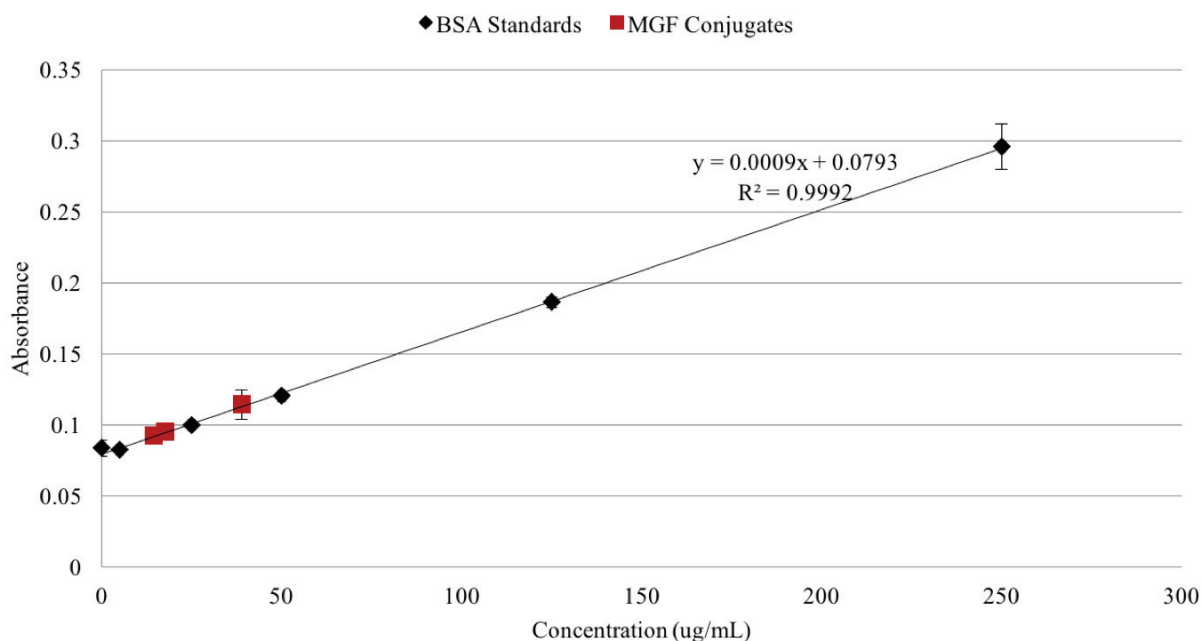


Figure 3.4

NMR spectra for unmodified HyA (top) and the Ac-HyA (bottom). The arrows point out the peaks for the unmodified side group (at ~2 ppm) and the acrylated side group (at ~6 ppm). The efficiency of acrylation was determined to be approximately 30% by comparison of the area under these peaks.



Feed Ratio (MGF:HyA)	15:1	30:1	60:1
Conjugation Ratio	11:1	15:1	23:1

Figure 3.5

BCA assay data used to determine the concentration of peptide in the conjugates and to estimate the final conjugation ratios. The plot (top) shows the absorbance versus concentration for the BSA standards (in black). The equation for the linear fit of the standards was used to calculate the concentration of the MGF in the conjugates based on their absorbance measurements. The conjugates are included on the plot in red. The table (bottom) shows the final conjugation ratios estimated from the measured concentrations of MGF in the conjugates.

CHAPTER 4:

CHARACTERIZATION OF MULTIVALENT CONJUGATES OF MECHANO-GROWTH FACTOR USING SIZE EXCLUSION CHROMATOGRAPHY – MULTI-ANGLE LIGHT SCATTERING

4.1 Abstract

Multivalent conjugation of growth factors to a polymer backbone chain can provide a useful bioconjugate system for controlling bioactivity and pharmacokinetics of the growth factors. Since the valency and overall size of these multivalent conjugate (MVC) molecules are important in controlling their effect in a given biological application, it is vital to have a characterization tool that can provide a complete analysis of these molecules. Multi-angle light scattering (MALS) is able to provide an absolute molecular weight and radius of gyration measurement for the MVCs, while also providing useful information on the distribution and polydispersity of these measurements. This work demonstrates that the use of a size exclusion chromatography (SEC)-MALS system with two inline concentration detectors allows for the determination of the weight fractions of the two different conjugate components. This analysis is based on the measured specific refractive index increment and UV extinction coefficient values for the two different components. This SEC-MALS technique allowed for the thorough characterization of MVCs with two different molar feed ratios of mechano-growth factor peptide to a hyaluronic acid (HyA) backbone chain.

4.2 Introduction

MVC technology is a powerful technique for presenting proteins or growth factors of interest to cell-surface receptors. This technology involves the conjugation of multiple protein or growth factor molecules to a soluble polymer backbone, such as HyA. Previous studies have shown that creation of these high molecular weight bioconjugate molecules can reduce the proteolytic degradation¹ and improve the pharmacokinetics¹⁻³ of the growth factor. Additionally, by varying the valency, or ratio of growth factor molecules conjugated to each polymer chain, it is possible to enhance the bioactivity of the growth factor over that of unconjugated growth factor at the same concentration.⁴⁻⁷ In theory, this effect may be due to clustering of receptors on the cell surface. When one growth factor on the MVC is bound by a cell surface receptor, it brings the other growth factors on that MVC into proximity of additional receptors, thereby effectively increasing the concentration of growth factors at the cell surface.⁸ Additionally, there is an entropic penalty associated with a cell surface receptor binding a growth factor. MVC molecules can lower the total entropic penalty for multiple binding events because when the first growth factor is bound, it brings the MVC molecule into close proximity of the cell surface, thereby lowering the entropic penalty for further binding events.^{9,10}

Since the bioactivity of these MVCs is dependent upon the conjugation ratio of the growth factor molecule to the polymer backbone, it is necessary to have a robust technique for characterizing this conjugation ratio. Additionally, it is important to be able to fully characterize the MVC size and distribution in order to understand the diffusion and residence time *in vivo*. The valency and molecular weight of these conjugates can readily deviate from the expected values due to the degradation of the polymer backbone during the synthesis process. This is especially true in the

case of hyaluronic acid, which is highly susceptible to shear degradation. This means that in order to fully characterize the MVCs, it is necessary to use a technique that measures the overall distribution of size and valency. This thorough characterization of the valency and size becomes crucial in determining the potency of the bioactivity and in optimizing the conjugates for specific applications.¹¹

There are many techniques for characterizing bioconjugate molecules, which only provide incomplete information, so multiple of these have to be used in tandem.¹² For example, protein quantification assays (i.e. bicinchoninic acid assay and Coomassie staining) can be used to measure the final protein concentration in solution, which can be used to estimate the valency. However, this requires an assumption of the average molecular weight of the HyA backbone without any way to actually measure this value or its distribution.^{7,11} Other techniques that yield partial characterization by providing average ligand content, but not complete molecular weight information include Fourier transform infrared spectroscopy (FTIR)¹³, UV-vis spectroscopy¹⁴, NMR¹⁵, and fluorescence assays¹³. Traditional chromatographic techniques can be used to thoroughly characterize the molecular weight of these conjugates^{16,17}, but these do not provide any measurement of the valency. Also, these require very precise standards, since they are relative instead of absolute measures of molecular weight.

Multi-angle light scattering is a powerful tool for characterizing these MVCs because it can measure both absolute molecular weight and radius of gyration (R_g). The fundamental principle of light scattering characterization is that the amount of light scattered is directly proportional to the molar mass and concentration.¹⁸ This can be expressed as:

$$I(\theta) \propto Mc \left(\frac{dn}{dc} \right)^2$$

where $I(\theta)$ is the light scattering intensity measured at a given angle detector, M is the molar mass, c is the concentration, and dn/dc is the specific refractive index increment. Additionally, with the use of multiple detection angles, it is possible to measure the radius of gyration of particles larger than 10 nm.^{19,20} The addition of an inline size exclusion chromatography system allows for the separation of MVC samples by molar mass, so that the MALS measurements include information about the molecular weight and valency distributions instead of just average values. In these studies, we will use an online three-detector method to monitor UV light absorbance, differential refractive index (DRI), and multi-angle light scattering coupled with an SEC system as previously described.²¹⁻²³

4.3 Materials and Methods

4.3.1 Acrylation of Hyaluronic Acid

HyA with a molecular weight of 622 kDa as determined by the manufacturer through viscosity measurements was obtained from LifeCore Biomedical. First, the HyA was modified to have hydrazide groups (ADH-HyA) by following previously reported chemistry.²⁴⁻²⁸ Briefly, the HyA was dissolved in 100 mL of deionized (DI) water overnight to form a 3 mg/mL solution. Adipic acid dihydrazide (ADH) (Sigma-Aldrich) was then added in 30 molar excess to the HyA, and the

pH of the reaction was adjusted to 6.8. 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Fisher Scientific) and 1-hydroxybenzotriazole hydrate (HOBt) (Sigma-Aldrich) were dissolved separately at 3 mmol in DMSO/DI water (1:1 volume ratio, 3 mL) and then added to the reaction solution sequentially. The solution pH was again adjusted to 6.8 and was maintained using 0.1 M NaOH and 0.1 M HCl for at least 4 hours, before letting the reaction proceed overnight. Following this reaction, the pH was adjusted to 7 and the HyA solution was dialyzed exhaustively in 10,000 Da molecular weight cut-off (MWCO) dialysis tubes (Spectrum Labs) against first 1 g/L NaCl in DI water for two days and then against pure DI water for one day. After dialysis, 5 mg/mL of NaCl was added to the HyA solution, and then this solution was precipitated in 100% ethanol. The precipitated ADH-HyA was pelleted by centrifugation and then redissolved in DI water. The solution was dialyzed again following the same method. In order to generate acrylate groups on the HyA (Ac-HyA), the solution was subsequently reacted with N-acryloxysuccinimide (Acros Organics) (700 mg per 300 mg HyA in 100 mL DI water).^{24,27-30} The final Ac-HyA product was lyophilized for at least 3 days to remove all of the water and then stored at -20 °C. The degree of available carboxyl groups that were conjugated with acrylate groups was confirmed to be ~30% by proton (¹H) NMR as previously described.^{27,28,31}

4.3.2 Multivalent Conjugation of Mechano-Growth Factor to Hyaluronic Acid

Mechano-growth factor (MGF) peptides with a cysteine residue added to the c-terminus (MGF-Cys) were obtained from American Peptide Company. The peptide sequences are shown in Table 4.1. The cysteine residue was added to allow the conjugation of the peptide to Ac-HyA through a Michael-type addition reaction between the thiol on the peptide and the acrylate group on the modified HyA. This reaction scheme is described in more detail in Chapter 3. Briefly, Ac-HyA was dissolved overnight at 1 mg/mL in phosphate-buffered saline (PBS). MGF peptide was dissolved in PBS at 1 mg/mL and added to the Ac-HyA solution in two separate molar feed ratios, which we will call the low conjugation ratio (LCR; 30:1 MGF:Ac-HyA) and the high conjugation ratio (HCR; 60:1 MGF:Ac-HyA). These reactions were allowed to proceed overnight and the following day, L-cysteine (Sigma-Aldrich) was dissolved at 10 mg/mL in PBS and added in 2-fold excess relative to the acrylate groups on the HyA in order to quench any unreacted acrylate groups. This reaction was allowed to proceed overnight, and then the solutions were transferred to 100 kDa MWCO Float-A-Lyzer dialysis tubes (Spectrum Labs) and dialyzed exhaustively against PBS.

4.3.3 SEC-MALS Characterization of Multivalent MGF

The multivalent conjugates of MGF and hyaluronic acid (mvMGF) were characterized using size exclusion chromatography – multi-angle light scattering as described previously.²¹ The SEC-MALS system consisted of an Agilent 1100 HPLC system (degasser, quaternary pump, autosampler, column holder/temperature controller, and UV-vis diode array detector) coupled in-line with a Wyatt Technology DAWN HELEOS-II multi-angle laser light scattering detector and a Wyatt Technology Optilab T-rEX refractive index (RI) detector. For the measurements, the wavelength of the diode array detector was set to 280 nm, the wavelength of the MALS detector was 658 nm, and the wavelength of the RI detector was 660 nm. A Shodex OH pak SB-804 HQ column was used for the HPLC separation of HyA and mvMGF, while a Wyatt Technologies

WTC-010N column was used for unconjugated MGF peptide. Agilent ChemStation software was used to control the HPLC system, while Wyatt Technology Astra VI software was used for data collection and analysis. Normalization of the MALS detectors, band broadening correction, and peak alignment was performed using a 100 μ L injection of 2 mg/mL bovine serum albumin (BSA) (Sigma Aldrich) at a flow rate of 0.3 mL/min in PBS and the algorithms in the Astra VI software.

Multivalent conjugate samples were first filtered through 0.22 μ m filters to remove any aggregates or other large particles. The effect of filtering on HyA molecular weight and polydispersity is discussed in detail in Chapter 6. 200 μ L injections at a concentration of 0.25-0.5 mg/mL HyA were performed at a flow rate of 0.3 mL/min with PBS as the mobile phase and the column temperature maintained at 25 $^{\circ}$ C. Analysis was limited to the moderate angle detectors to eliminate high noise levels from the extreme low and high angle detectors. Zimm light scattering formalism was used for processing of all light scattering data.

4.3.4 Determination of Specific Refractive Index Increment and UV Extinction Coefficient Values

Specific refractive index increment (dn/dc , mL g^{-1}) values for the different MGF sequences, the Ac-HyA, and the cysteine quenched Ac-HyA (Cys-Ac-HyA) were determined using batch analysis with the RI detector. Solutions with 6 different concentrations from 0.016 to 0.5 mg/mL were injected directly into the RI detector, and the differential refractive index was measured at each concentration. For each concentration, 3 mL of solution was injected at a rate of 0.3 mL/min using a syringe pump. After each sample was measured, the data was plotted on a graph of differential refractive index versus concentration. A linear fit was applied, and the slope of the fit was the value for the dn/dc . The dn/dc value for unmodified HyA was obtained from a previous publication.²¹ The UV extinction coefficient (ϵ , mL mg^{-1} cm^{-1}) values for the different MGF sequences and the Ac-HyA were obtained using an online 100% mass recovery method in the Astra VI software, which calculated UV extinction coefficient from the RI peak. The UV extinction coefficient for HyA was too low to measure on the SEC-MALS system, so the value measured with a spectrophotometer in a previous publication was used.²¹ The UV extinction coefficient for the Cys-Ac-HyA was also too small to be measured with SEC-MALS, so it was assumed to have the same value as unmodified HyA. These values are reported in Table 4.2.

4.4 Results and Discussion

4.4.1 Online Conjugate Analysis of Multivalent MGF

Online measurement with the SEC-MALS system allows for simultaneous measurements of UV absorbance, light scattering intensity, and differential refractive index. In an online measurement, the weight average molar mass (M_w) and z average radius of gyration ($R_{g,z}$) are determined for each slice of the measured chromatogram (see Figure 4.1a) using the condensed form of the Rayleigh-Gans-Debye equation³²:

$$\frac{K^*c}{R(\theta, c)} = \frac{1}{M_w P(\theta)} + 2Bc$$

$$K^* = \frac{4\pi^2 \left(\frac{dn}{dc}\right)^2 n_0^2}{N_A \lambda_0^4}$$

where $R(\theta, c)$ is the excess Rayleigh ratio^{33,34} as a function of the scattering angle, θ , and the solute concentration, c . This ratio is directly proportional to the intensity of light scattered by the sample in excess of the intensity of light scattered by the pure solvent. M_w is the weight average molecular weight, B is the second virial coefficient, N_A is Avagadro's number, λ_0 is the vacuum wavelength of the laser, n_0 is the refractive index of the solvent, and dn/dc is the specific refractive index increment of the macromolecule in solution. $P(\theta)$ describes the angular dependence of scattered light and how it relates to R_g . $P(\theta)$ can be expanded to¹⁸:

$$P(\theta) \approx 1 - \frac{16\pi^2 n_0^2}{3\lambda_0^2} \langle R_g^2 \rangle \sin^2 \frac{\theta}{2} + O\left(\sin^4 \frac{\theta}{2}\right) - \dots$$

Debye plots [$K^* c/R(\theta)$ vs $\sin^2(\theta/2)$] were created for each slice of the chromatogram, and a representative plot is shown in Figure 4.1b, which shows the fit of the data generated by the different angle detectors.

The use of both the UV and RI detectors, allows us to have two concentration detectors inline during the analysis. Each of these concentration detectors relates the concentration of the solute to the measured intensity, shown by:

$$c_{DRI} = \frac{I_{DRI} DRI_{cc}}{\frac{dn}{dc}}$$

$$c_{UV} = \frac{I_{UV} UV_{RF}}{\epsilon L}$$

where c is the solute concentration, I is the intensity, DRI_{cc} is the DRI calibration constant, UV_{RF} is a constant called the response factor, L is the path length, dn/dc is the specific refractive index increment, and ϵ is the UV extinction coefficient.

The two concentration detectors allow for the determination of the relative compositions of the MVCs, based on the weight fractions of the peptide and HyA components. This is because the total values of the specific refractive index increment and UV extinction coefficient for the MVCs are weight average values based on the individual components. This is represented mathematically by:

$$\frac{dn}{dc} = \left[\left(\frac{M_{MGF}}{M_{Total}} \right) \left(\frac{dn}{dc}_{MGF} \right) \right] + \left[\left(\frac{M_{HyA}}{M_{Total}} \right) \left(\frac{dn}{dc}_{HyA} \right) \right]$$

$$\varepsilon = \left[\left(\frac{M_{MGF}}{M_{Total}} \right) (\varepsilon_{MGF}) \right] + \left[\left(\frac{M_{HyA}}{M_{Total}} \right) (\varepsilon_{HyA}) \right]$$

where M is molar mass, dn/dc is the specific refractive index increment, and ε is the UV extinction coefficient. Since the two concentration detectors are inline, it can be assumed that they will measure the same concentration of solute. By setting the two concentration equations for the two detectors equal, and using the above equation for dn/dc and ε , it is possible to solve for the weight fractions of the two individual MVC components.

4.4.2 Specific Refractive Index Increment and UV Extinction Coefficient Measurements

Since the ability to determine the weight fractions of the two different components of the MVCs is dependent upon the dn/dc and ε values of the individual components, it is imperative to accurately measure these values. Table 4.2 shows the values of these constants that were determined for each component of the MVCs. For the HyA backbone, the dn/dc and ε values were originally measured for the Ac-HyA, which resulted in much higher UV absorption than unmodified HyA due to the presence of the double bonds in the acrylate groups used for conjugation. However, in the final conjugate molecules, those acrylate groups are reacted with the cysteine residues on the MGF peptide, and the unused acrylate groups are quenched with free cysteine residues. Therefore, it was more accurate to measure the dn/dc and ε values for cysteine-quenched Ac-HyA (Cys-Ac-HyA), and use these values for the HyA component of the MVCs. The quenching of the acrylate groups reduced the UV absorption for the Cys-Ac-HyA to such a low value as to be unmeasurable by an online SEC-MALS method. Therefore, it was assumed that the ε value of the Cys-Ac-HyA was reduced back to the same value as the unmodified HyA, which has almost no UV absorption.

The dn/dc and ε values for MGF plus the added cysteine residue for conjugation are shown in Table 4.2. However, it was found during analysis that the ε value for the conjugates was too close to that of the HyA backbone, making it impossible to distinguish between the two components. To address this issue, the MGF amino acid sequence was modified to include a tryptophan residue and a short spacer before the c-terminal cysteine. This significantly increased the ε value of the peptide due to the high UV absorption of the aromatic rings on the tryptophan residue. The new peptide sequence of this modified peptide is shown along with the original sequence in Table 4.1. The measured values of the dn/dc and ε for the tryptophan-modified peptide are included in Table 4.2.

The UV extinction coefficient values were calculated from the RI peak using an online 100% mass recovery method. Figure 4.1a shows a representative chromatogram including the RI peak, which was used to calculate the ε value. Figure 4.2 shows the data used to calculate the dn/dc values of the two components of the MVCs. The points represent the differential refractive index measured for each concentration injected in batch mode. A fit line was applied, and the slope of the line displayed on each chart is the determined dn/dc value.

4.4.3 Analysis of SEC-MALS Data

The conjugates demonstrated a strong signal relative to the noise in all three detectors (UV, LS, and RI), allowing for sound and reliable analysis of the data. An example chromatogram demonstrating the strong signal peaks is shown in Figure 4.1a. The slight offset of the light scattering peak from the two concentration detector peaks (UV and RI), along with the slight difference in their shapes, indicates that the samples are somewhat polydisperse. The measured polydispersity index ($\bar{D}$) is reported in Table 4.3 to be 1.2 for both the LCR and HCR conjugates. Figure 4.3 is plots of the molar mass versus elution volume overlaid on the LS peak for the LCR (a) and HCR (b) conjugates. This plot is broken down into the molar mass contributions over the peak for the MGF and HyA components, as well as the total conjugate molecule. The peptide component shows less variance in molar mass over the LS peak compared to the HyA component, which is confirmed by the lower $\bar{D}$ values for the MGF contribution compared to HyA in Table 4.3. It can be seen that the contribution to the molar mass from the MGF peptide is much smaller than that of the HyA backbone, and that the two components sum to the total molar mass, as was expected. For the LCR molecules, the weight-averaged molar mass for the total conjugate was $7.6\text{E}5$ g/mol, while the HyA component was $7.1\text{E}5$ g/mol and the MGF component was $5.1\text{E}4$ g/mol. For the HCR molecules, the weight-averaged molar mass for the total conjugate was $7.8\text{E}5$ g/mol, while the HyA component was $7.1\text{E}5$ g/mol and the MGF component was $7.1\text{E}4$ g/mol. These values are reported in Table 4.3 along with the calculated number-averaged molecular weights.

This data is further illustrated in Figures 4.4 and 4.5. Figure 4.4 shows plots of the cumulative weight fraction versus molar mass, and Figure 5.5 shows the differential weight fraction versus molar mass for both the LCR (a) and HCR (b) conjugates. The plots are broken down into the MGF component, HyA component, and total conjugate. These plots are just two different ways of displaying the same data, and are able to show that the total molecule can be broken down into the MGF and HyA components, which can then be separately characterized. As is shown in both figures, the variance of the molar mass over the weight fraction is smaller for the MGF than for the HyA, which again accounts for its lower $\bar{D}$ value in Table 4.3. Both of these figures also show that the molar mass of the MGF contribution is higher for the HCR molecule than for the LCR molecule, as was expected. This can be seen by the slight shift in the MGF trace or peak to higher molar mass values and by the larger separation between the HyA trace or peak and that for the total conjugate molecule.

Finally, Figure 4.6 shows the peptide fraction versus elution time overlaid with the LS peak for the LCR (a) and HCR (b) molecules. It can be seen that the MGF contributes to only a small fraction of the total conjugate molar mass, which was expected because the peptide has a very small molar mass compared to the HyA chains. Additionally, it can be seen that the HCR molecule has a greater peptide fraction of between 0.075 and 0.125 as compared to the LCR molecule, which has a peptide fraction between 0.05 and 0.075. This further demonstrates that it was possible to achieve a higher conjugation ratio with a higher initial molar feed ratio of peptide to HyA.

4.4.4 Determination of Final Conjugation Ratios and Comparison to Feed Ratios

Table 4.3 summarizes all the data measured for the LCR and HCR conjugate molecules. The conjugation ratio for each of these conjugates was determined by dividing the molecular weight of the peptide component by the molecular weight of a single MGF molecule. This yielded the valency, or number of MGF molecules per HyA backbone chain. The valency value was calculated using both the number-averaged and weight-averaged molecular weight values. The conjugation efficiency was calculated by comparing these valency values to the original molar feed ratios (30:1 for the LCR and 60:1 for the HCR). For the LCR, the ratio of MGF:HyA determined using M_n was 13.7:1 and using M_w was 15.8:1 with conjugation efficiency values of 45.8% and 52.7% respectively. For the HCR, the ratio determined using M_n was 19.8:1 and using M_w was 21.7:1 with conjugation efficiencies of 33.0% and 36.1% respectively. It can be seen that the conjugation efficiency decreases with a higher molar feed ratio, which is to be expected due to the decreased number of available conjugation sites. This data is summarized in Table 4.3 and is also presented in Figure 4.7, which compares the input molar feed ratios to the output conjugation ratios.

4.4.5 Branching Analysis of Multivalent MGF

Branching analysis is discussed in greater detail in Chapter 5, but it was of interest to see if the mvMGF behaves as a branched molecule. Figure 4.8 shows the conformation plots for three different runs of the LCR (a) and HCR (b) conjugates and demonstrates the uptick at lower molar mass that is expected for branched molecules. This uptick is purely virtual and is caused by abnormal SEC elution behavior due to the branched molecules anchoring in the column packing material and eluting later than expected based on molar mass. This means that some larger molar mass molecules elute with the lower molar mass fraction, causing the artifact in the conformation plots.

The branching behavior of the mvMGF molecules can also be seen in Figure 4.9, which shows the molar mass versus elution time overlaid with the LS chromatogram for the LCR (a) and the HCR (b). For unbranched molecules with normal elution behavior, the molar mass would be expected to decrease linearly over the elution volume. However, with branched molecules, the trace of molar mass versus volume will level out or actually have an uptick in molar mass at larger volumes due to the late elution of larger molar mass molecules. As can be seen in Figure 4.9, the mvMGF molecules demonstrate the leveling off behavior expected of branched molecules.

To further confirm that the mvMGF molecules behave as branched molecules, linear regression fits were applied to the linear portions of the conformation plots in Figure 4.8. The slopes of these linear regression fits can further confirm the presence of branching, because strongly branched molecules demonstrate a decreased slope as compared to a linear molecule. This is because at a given molar mass, branching decreases the size by increasing the compactness of the macromolecule. A purely linear polymer in a good solvent is expected to have a slope of around 0.58, so it would be expected that the mvMGF would have a slope lower than 0.58.^{20,35-42} However, as can be seen in Figure 4.8, the slopes for the mvMGF molecules are actually all larger than that expected for a linear molecule. The values of the slopes are believed to be

incorrect due to the limited linear portions of the conformation plots that were available for generating the linear regression fits. It is likely that the abnormal SEC elution behavior is impacting the data too strongly to achieve an accurate fit, making these conformation plot unreliable for accurate assessment of branching behavior. This means that the radius of gyration values reported in Table 4.3 are most likely erroneous, and are only reported as an estimation of the true values. This is additionally confirmed by the inability to yield any reasonable data by applying branching analysis to these conjugates. To further investigate the branching behavior of these MVCs, it would be necessary to reduce or eliminate the abnormal elution behavior by changing to an SEC column with less of an anchoring effect or by switching to a different separation method such as field flow fractionation, which completely eliminates the SEC column and thereby the abnormal elution behavior. This abnormal elution behavior caused by anchoring in the SEC column proves to be a limitation for branching analysis, which was unable to be applied for mvMGF.

4.5 Conclusions

The mvMGF conjugates synthesized and partially characterized in Chapter 3, were more fully characterized through the application of multi-angle light scattering. While the characterization methods used in Chapter 3 provide useful information, none of them alone provide a complete analysis of the MVCs. The use of multi-angle light scattering provides an absolute measure of the molecular weight and measures the radius of gyration, while also providing information on the distribution of these values. This analysis utilized an SEC-MALS-UV-RI method, where the inline use of two concentration detectors allowed for the determination of the relative compositions of the MVCs. Using the measured specific refractive index increment and UV extinction coefficient values measured for the two MVC components, it was possible to determine the weight fractions of the MGF and HyA in the total MVC molecule. This analysis confirmed that the bioconjugate chemistry technique utilized in this work was successful, and that it was possible to determine the total molecular weight and valency of the MVCs. This analysis also yielded information on the polydispersity of the conjugates, as well as on the efficiency of the conjugation reaction. Since the valency and size of MVC molecules are so important to their behavior in different biological applications, it was vital to have a characterization tool that could provide this type of thorough analysis. The SEC-MALS system can be further utilized to assess branching behavior of MVCs, but more in depth branching analysis was not performed for mvMGF due to the results being so heavily influenced by anchoring in the SEC column. Branching analysis will be the topic of discussion in Chapter 5 and will be covered in more detail. SEC-MALS provides a powerful and vital tool for the characterization of multivalent conjugates.

4.6 References

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4.7 Tables

Table 4.1

Amino acid sequences for two different variations of the MGF peptide.

	Amino Acid Sequence
MGF-Cys	Tyr-Gln-Pro-Pro-Ser-Thr-Asn-Lys-Asn-Thr-Lys-Ser-Gln-Arg-Arg-Lys-Gly-Ser-Thr-Phe-Glu-Glu-His-Lys-Cys
MGF-Trp	Tyr-Gln-Pro-Pro-Ser-Thr-Asn-Lys-Asn-Thr-Lys-Ser-Gln-Arg-Arg-Lys-Gly-Ser-Thr-Phe-Glu-Glu-His-Lys-Trp-Gly-Gly-Cys

Table 4.2

Molecular weights, specific refractive index increments, and UV extinction coefficients determined for the different MVC components and utilized in the SEC-MALS analysis. The molecular weights of the HyA and Ac-HyA were determined with SEC-MALS, while the peptide molecular weights were provided by the manufacturer.

	Molecular Weight (Da)	Specific Refractive Index Increment, dn/dc (mL g^{-1})	UV Extinction Coefficient, ϵ ($\text{mL mg}^{-1} \text{cm}^{-1}$)
HyA	687,750	0.167 ²¹	0.022 ²¹
Ac-HyA	689,300	0.175	0.306
Cys-Ac-HyA	689,300*	0.106	0.022 [†]
MGF-Cys	2,952	0.169	0.360
MGF-Trp	3,253	0.164	1.356

* The molecular weight of the Cys-Ac-HyA was assumed to be the same as was measured for the Ac-HyA.

[†] The UV extinction coefficient was too low to be measured on the SEC-MALS system, so it was assumed to be the same value as previously reported for unmodified HyA.

Table 4.3

Summary of the measured SEC-MALS data including the total, HyA, and MGF component molecular weights along with the polydispersity values. The conjugation ratios were determined by dividing the MGF per HyA chain molecular weight by the molecular weight of a single MGF molecule. The conjugation efficiencies were calculated by dividing the final conjugation ratios by the input molar feed ratios of MGF:HyA, which were 30:1 for the LCR and 60:1 for the HCR.

	Low Conjugation Ratio	High Conjugation Ratio
Total M_n (g/mol)	6.1E5 ($\pm 3.9E4$)	6.7E5 ($\pm 5.7E4$)
Total M_w (g/mol)	7.6E5 ($\pm 5.0E4$)	7.8E5 ($\pm 4.6E4$)
Total $\bar{D}$	1.2 (± 0.03)	1.2 (± 0.04)
$R_{g,z}$ (nm)	69.8 (± 8.4)	63.1 (± 5.8)
HyA M_n (g/mol)	5.7E5 ($\pm 3.7E4$)	6.1E5 ($\pm 5.6E4$)
HyA M_w (g/mol)	7.1E5 ($\pm 4.8E4$)	7.1E5 ($\pm 4.3E4$)
HyA $\bar{D}$	1.3 (± 0.03)	1.2 (± 0.04)
MGF per HyA Chain M_n (g/mol)	4.5E4 ($\pm 2.1E3$)	6.4E4 ($\pm 3.9E3$)
MGF per HyA Chain M_w (g/mol)	5.1E4 ($\pm 2.1E3$)	7.1E4 ($\pm 4.0E3$)
MGF per HyA Chain $\bar{D}$	1.2 (± 0.02)	1.1 (± 0.02)
MGF:HyA (M_n)	13.7 (± 0.6)	19.8 (± 1.2)
Percent Conjugation Efficiency (M_n)	45.8 (± 2.1)	33.0 (± 2.0)
MGF:HyA (M_w)	15.8 (± 0.7)	21.7 (± 1.2)
Percent Conjugation Efficiency (M_w)	52.7 (± 2.2)	36.1 (± 2.0)

4.8 Figures

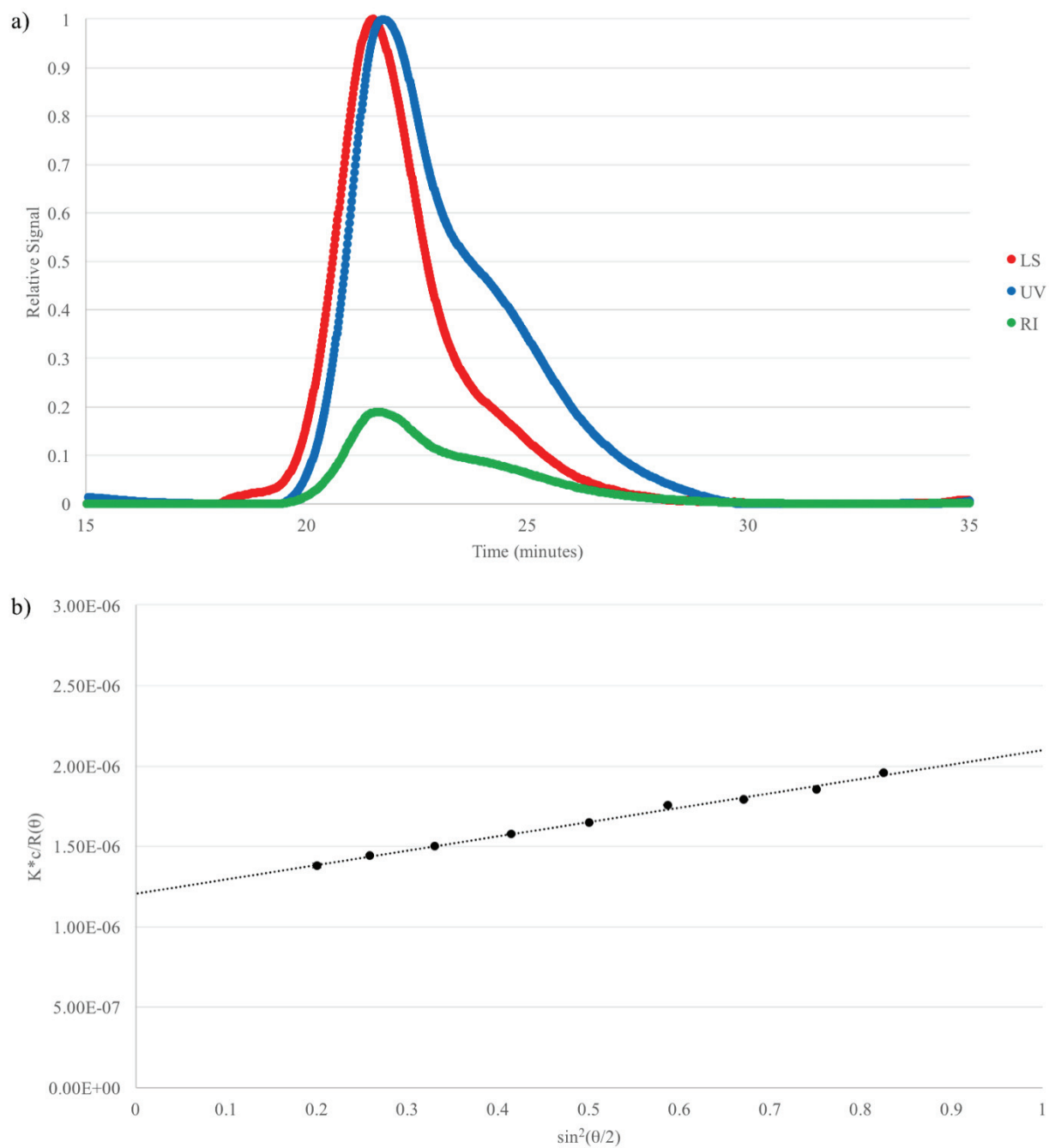


Figure 4.1

(a) Representative chromatogram showing the light scattering (LS), UV, and RI peaks. (b) Representative Debye plot for one slice of the chromatogram showing the fit for the different detector angles.

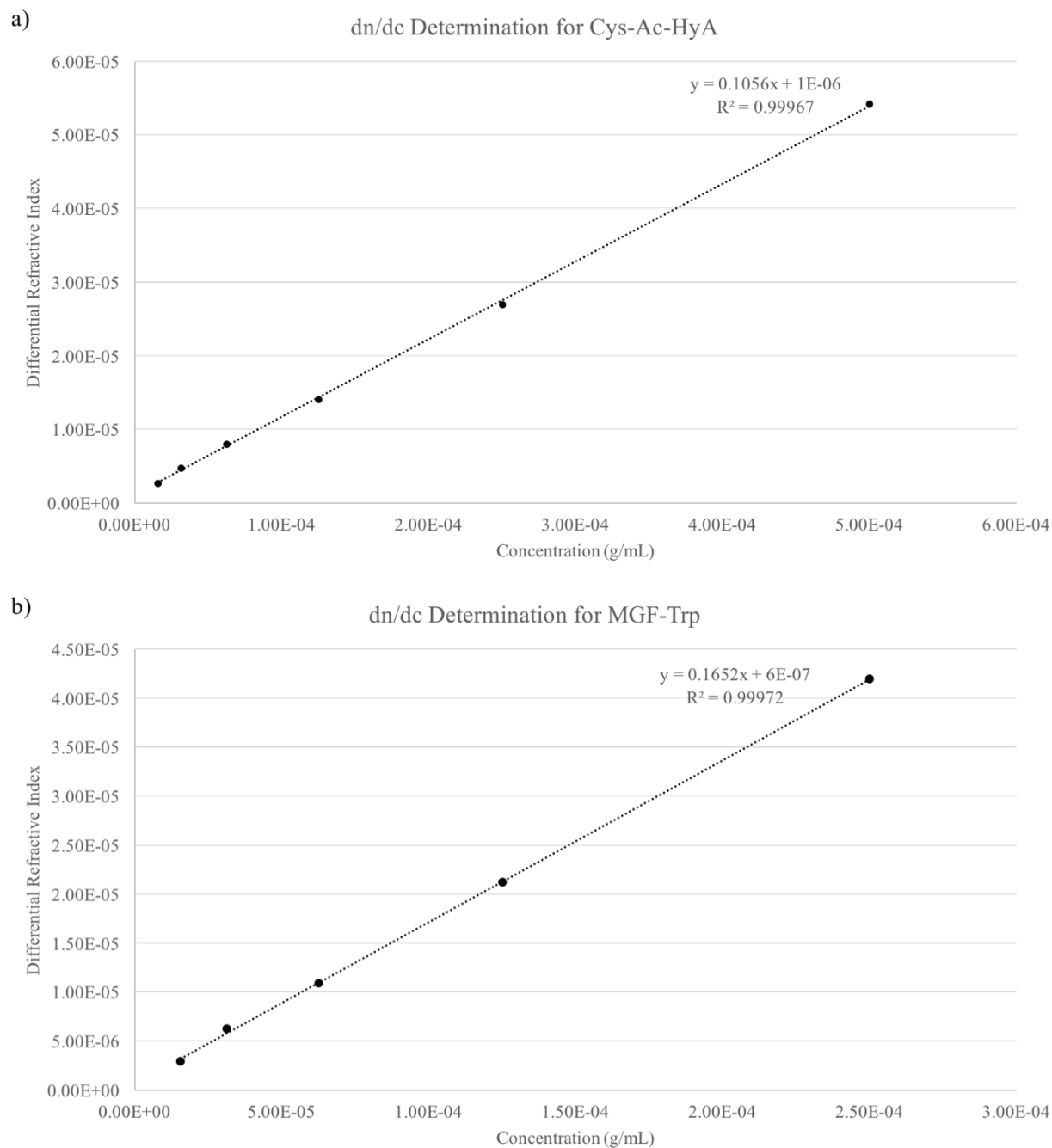


Figure 4.2

Representative plots showing the dn/dc determination for (a) the Cys-Ac-HyA and (b) the MGF-Trp. The slopes of the fit lines are the determined dn/dc values in mL/g.

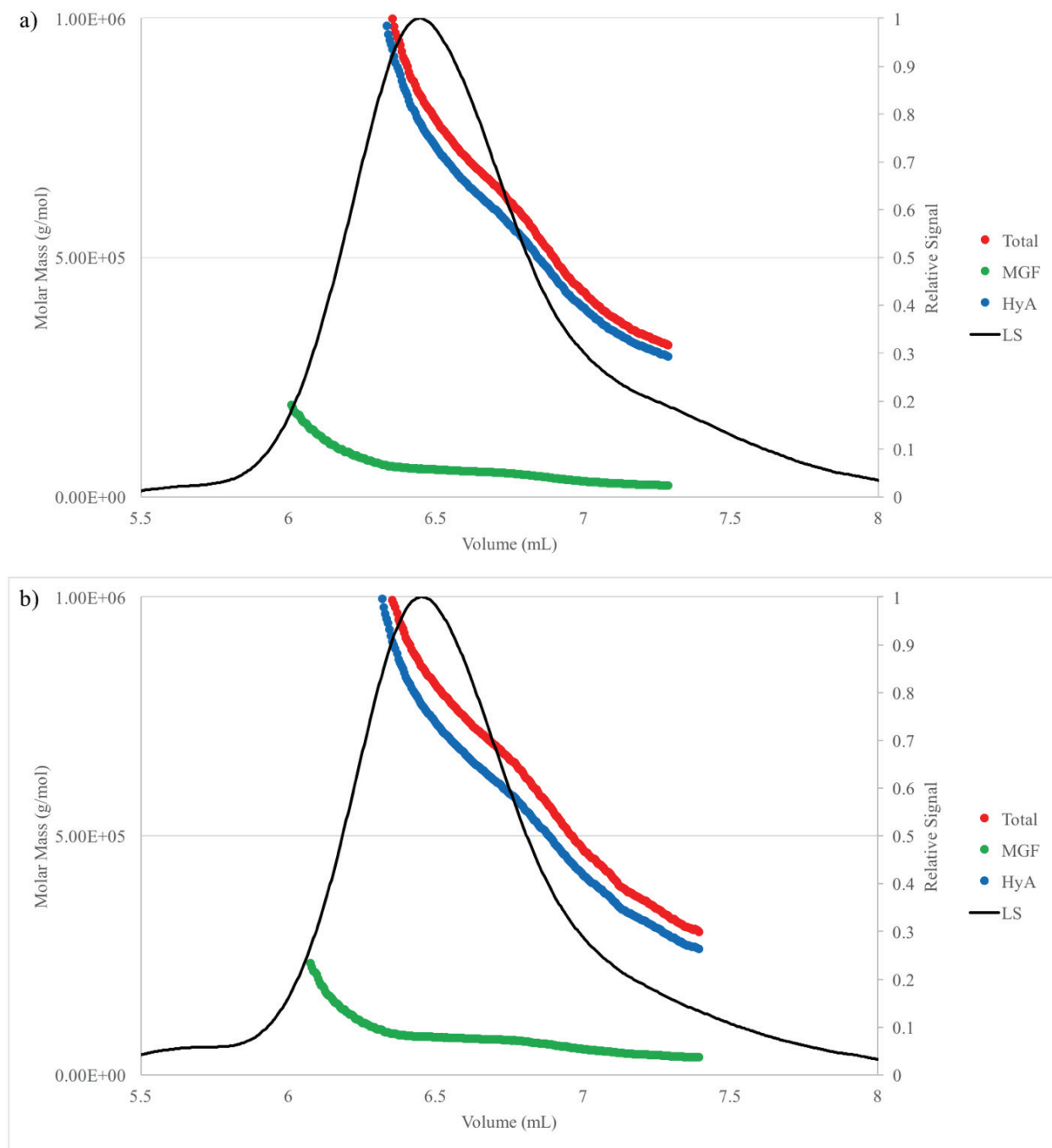


Figure 4.3

Molar mass versus elution volume plots overlaid with the LS chromatogram for (a) the LCR and (b) the HCR conjugates. These plots show the separate contributions from the MGF and HyA components, as well as for the overall conjugate molecules.

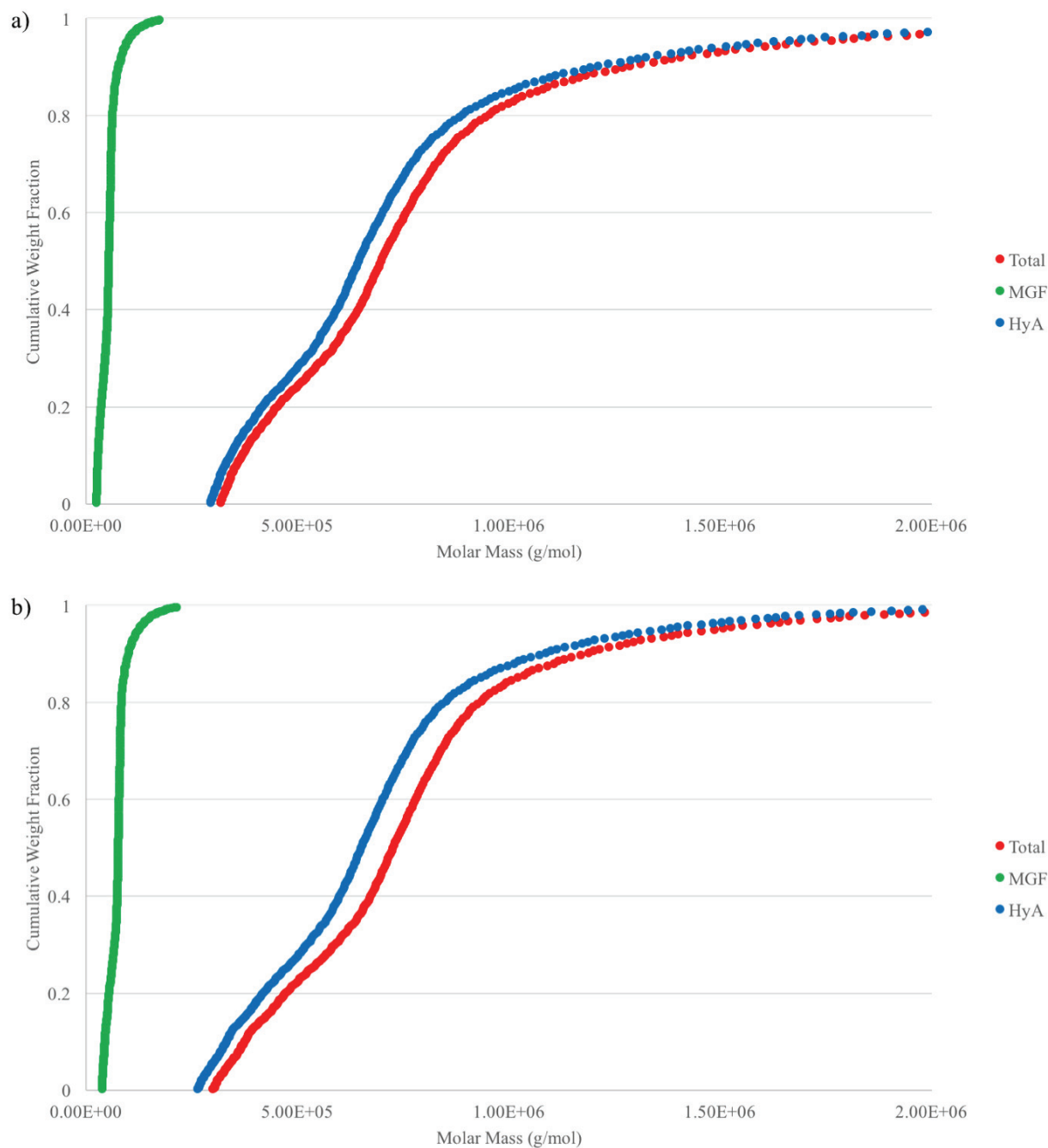


Figure 4.4

Cumulative weight fraction versus molar mass for (a) the LCR and (b) the HCR conjugates. These plots show the separate contributions from the MGF and HyA components, as well as for the overall conjugate molecules.

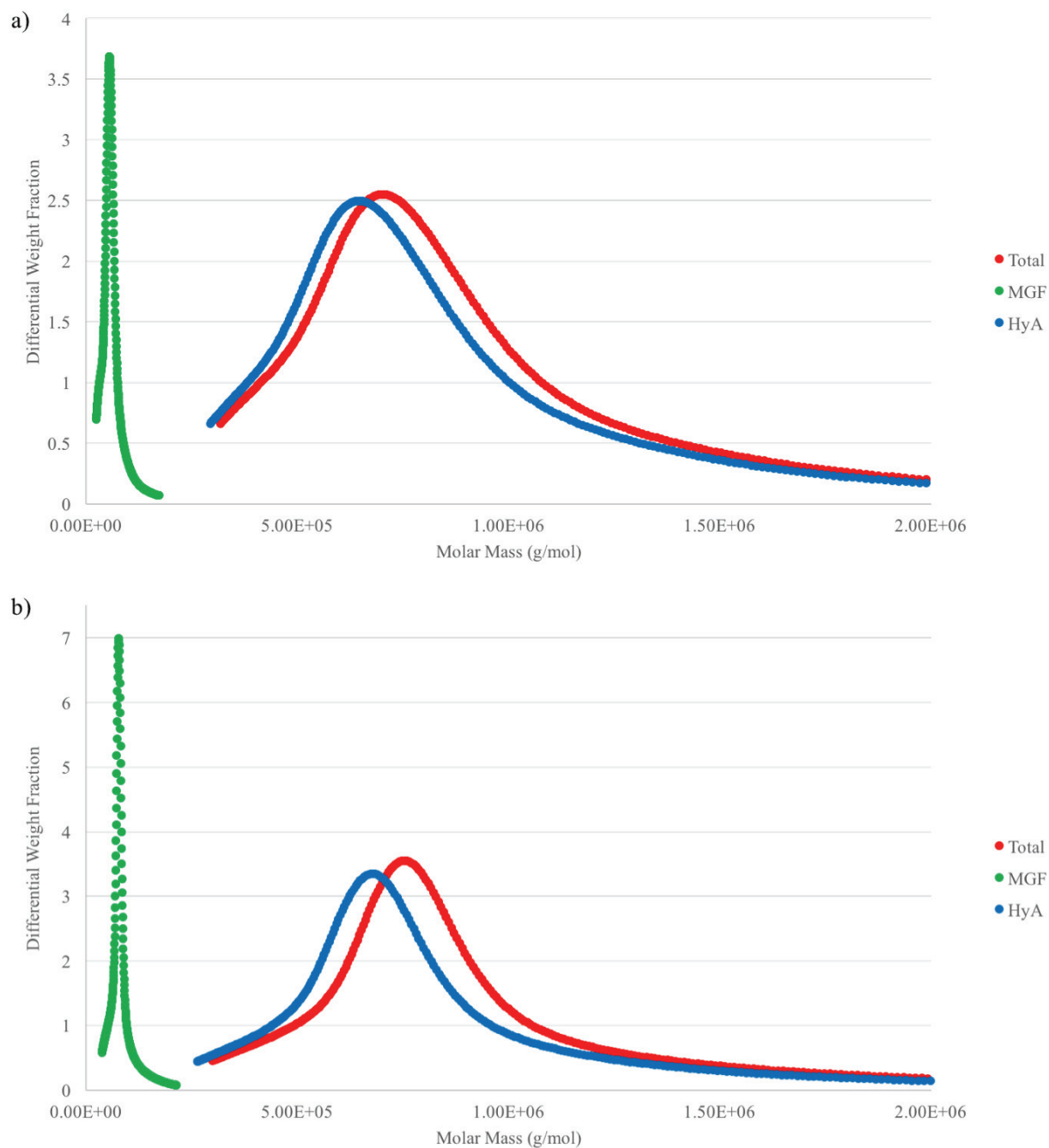


Figure 4.5

Differential weight fraction versus molar mass for (a) the LCR and (b) the HCR conjugates. These plots show the separate contributions from the MGF and HyA components, as well as for the overall conjugate molecules.

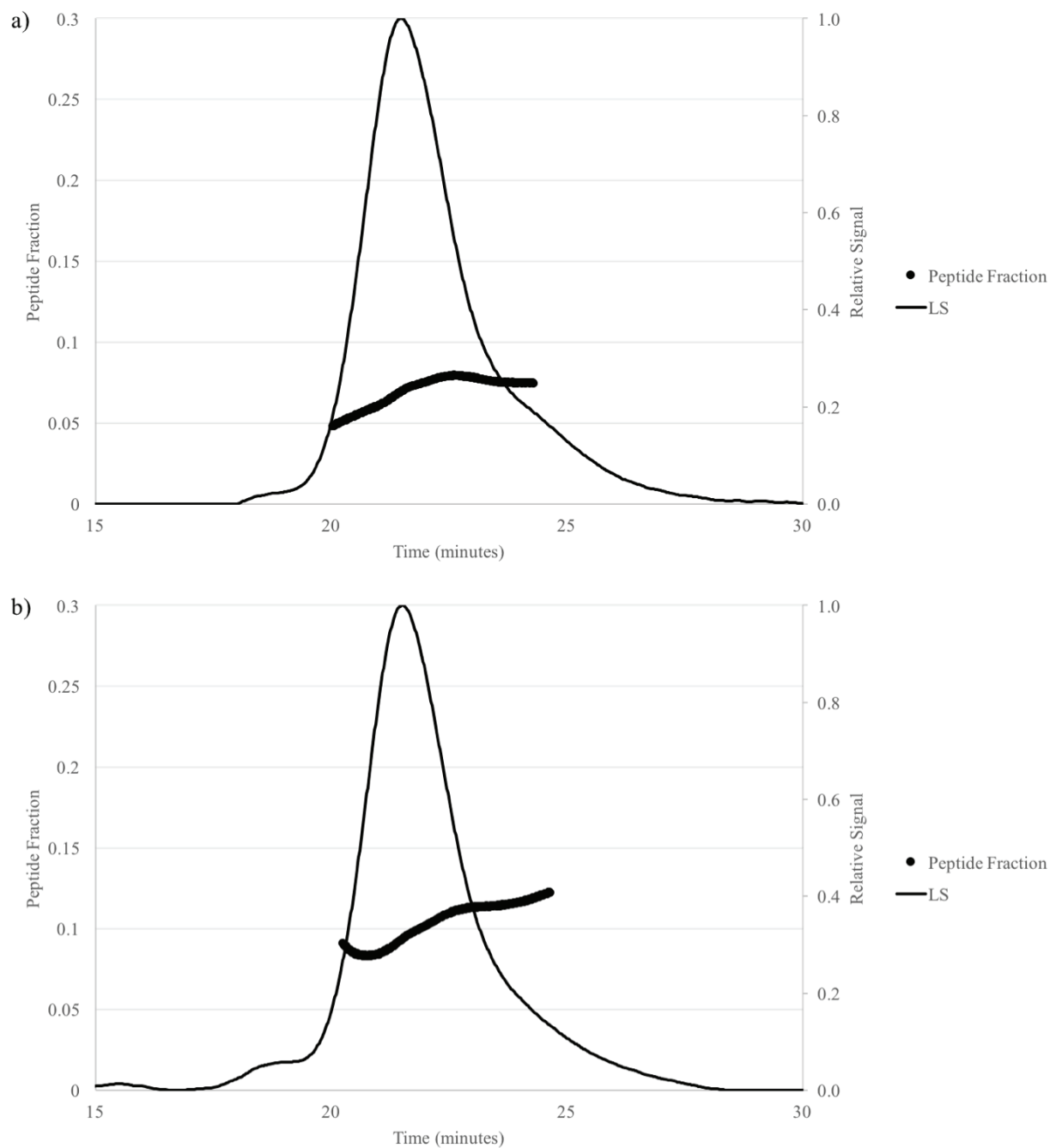


Figure 4.6
Peptide fraction versus elution time overlaid on the LS chromatogram for (a) the LCR and (b) the HCR conjugates.

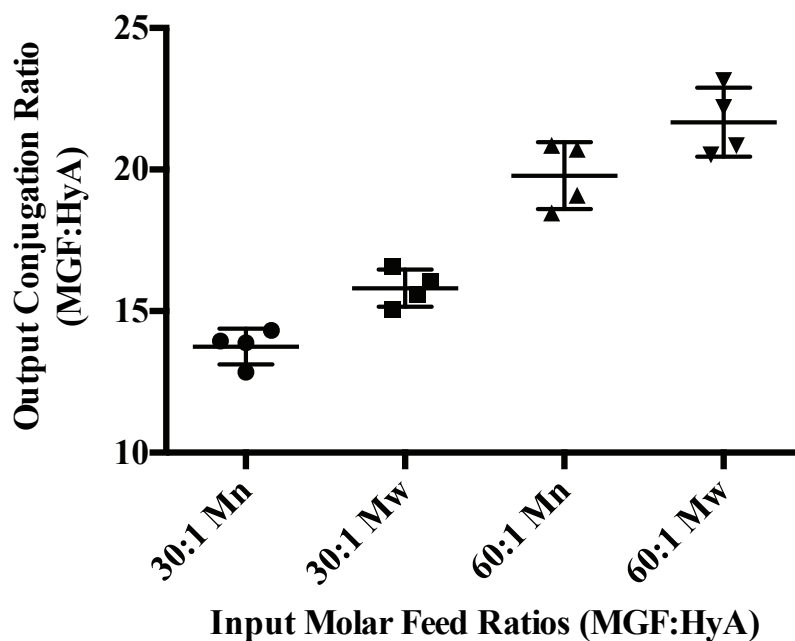


Figure 4.7

Comparison of the input molar feed ratios to the measured conjugation ratios for the LCR and HCR molecules. The ratios were determined for both the weight-averaged molecular weight (M_w) and the number-averaged molecular weight (M_n).

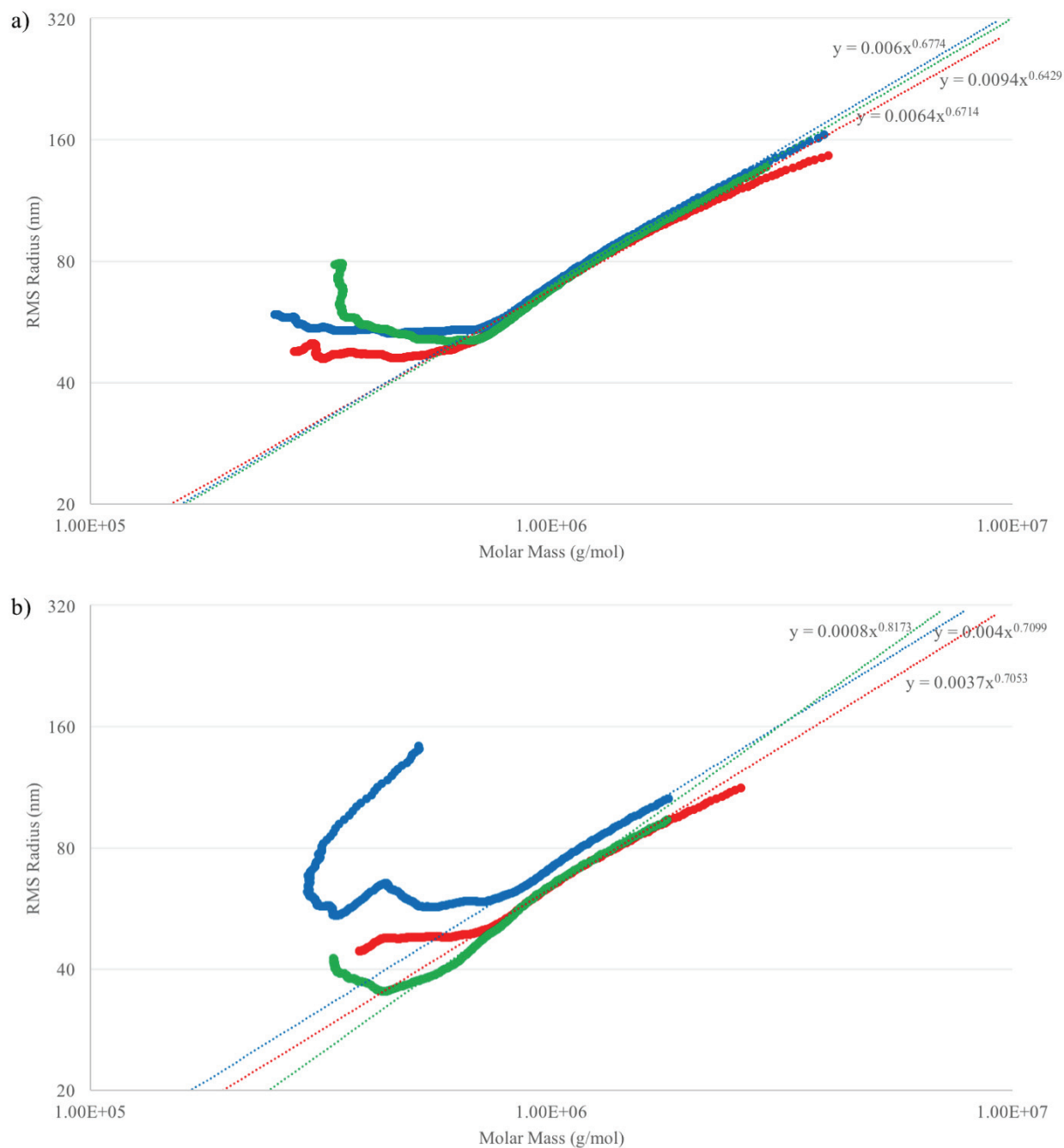


Figure 4.8
Conformation plots for three different runs of the (a) LCR and (b) HCR conjugates demonstrating the virtual uptick indicative of branched molecules.

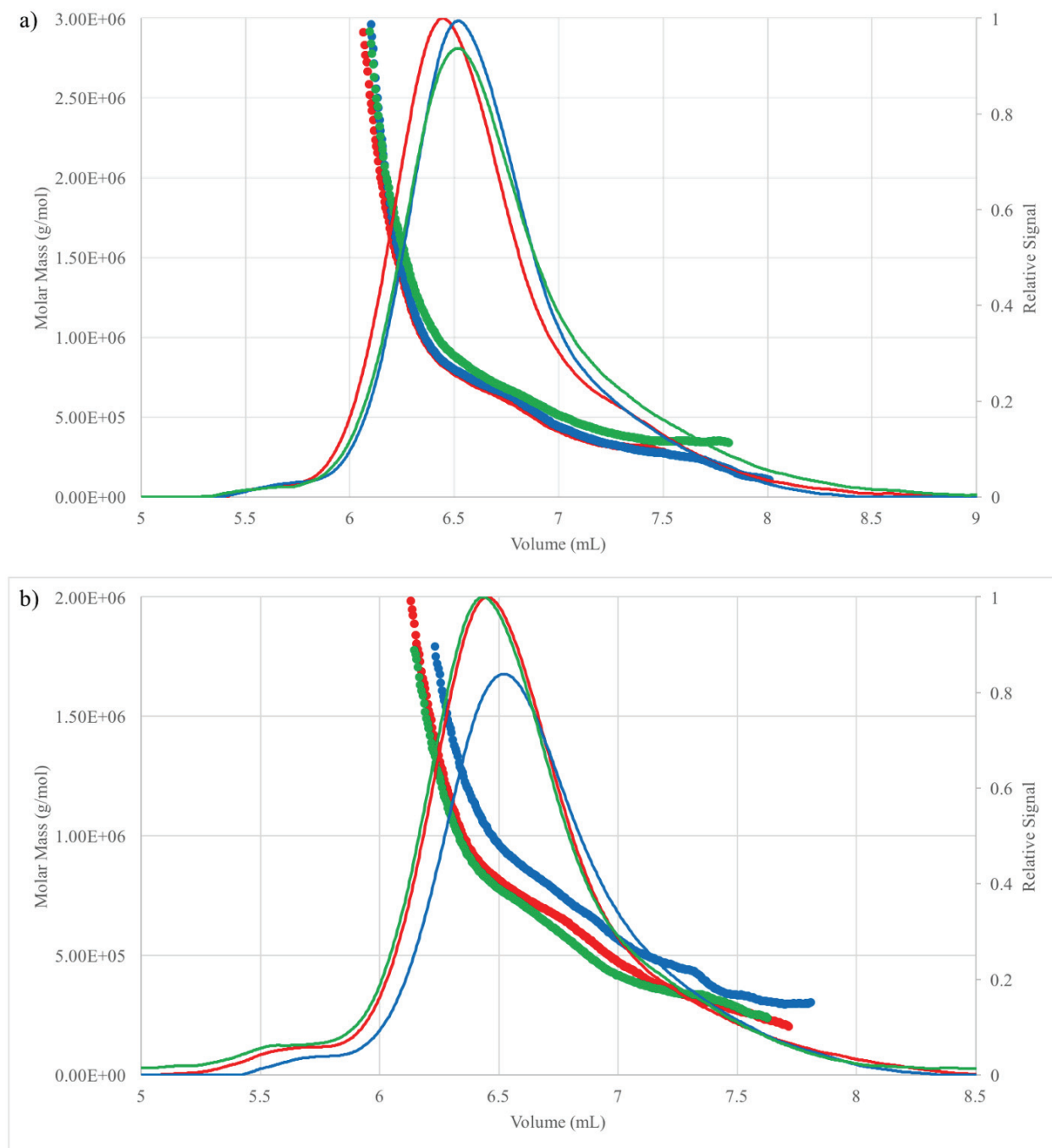


Figure 4.9

Molar mass versus volume plots overlaid on the LS chromatogram for (a) the LCR and (b) the HCR conjugates further demonstrating the abnormal elution behavior that is often observed for branched molecules.

CHAPTER 5: BRANCHING ANALYSIS OF MULTIVALENT CONJUGATES USING SIZE EXCLUSION CHROMATOGRAPHY – MULTI-ANGLE LIGHT SCATTERING

5.1 Abstract

Multivalent conjugate (MVC) technology, which involves the conjugation of multiple proteins or growth factors to a polymer backbone chain, is a powerful technique for improving the bioactivity and pharmacokinetics of these biomacromolecules. Since the bioactivity of these MVCs is highly dependent upon the valency of the conjugated proteins, it is imperative to have a technique for analysis of this conjugation ratio. Previous analysis of MVC molecules has used a size exclusion chromatography – multi-angle light scattering (SEC-MALS) method, which separates the two conjugate components based on their specific refractive index increment and UV extinction coefficient constants to determine a conjugation ratio. However, this SEC-MALS data also demonstrates that the MVC molecules behave as branched polymers, based on their abnormal SEC elution behavior and virtual upswing in their conformation plots. In this work, we have applied traditional branching analysis methods to the SEC-MALS data for the representative MVC molecules to demonstrate that branching analysis can be used to approximate the conjugation ratio. Using the branching analysis with the primary assumption that the linear backbone (e.g. hyaluronic acid) can be used as the linear counterpart for branching analysis, we were able to calculate the branching ratio, branch units per molecule, and long chain branching frequency of the MVCs. We demonstrated good agreement between these calculated values and the values determined through multivalent conjugate analysis, showing that branching analysis can be used as an alternative characterization technique for MVCs.

5.2 Introduction

Multivalent conjugate (MVC) technology is a powerful technique for presenting proteins or growth factors of interest to cell-surface receptors. This technology involves the conjugation of multiple protein or growth factor molecules to a soluble polymer backbone, such as hyaluronic acid. Previous studies have shown that creation of these high molecular weight bioconjugate molecules can reduce the proteolytic degradation¹ and improve the pharmacokinetics¹⁻³ of the growth factor. Additionally, by varying the valency, or ratio of growth factor molecules conjugated to each polymer chain, it is possible to enhance the bioactivity of the growth factor over that of unconjugated growth factor at the same concentration.⁴⁻⁷ In theory, this effect may be due to clustering of receptors on the cell surface. When one growth factor on the MVC is bound by a cell surface receptor, it brings the other growth factors on that MVC into proximity of additional receptors, thereby effectively increasing the concentration of growth factors at the cell surface.⁸ Additionally, there is an entropic penalty associated with a cell surface receptor binding a growth factor. MVC molecules can lower the total entropic penalty for multiple binding events because when the first growth factor is bound, it brings the MVC molecule into close proximity of the cell surface, thereby lowering the entropic penalty for further binding events.^{9,10}

Since the bioactivity of these MVCs is dependent upon the conjugation ratio of the growth factor molecule to the polymer backbone, it is necessary to have a robust technique for characterizing this conjugation ratio. Size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) analysis has been a crucial tool in the characterization of these MVCs. By utilizing the specific refractive index increment and UV extinction coefficient constants for the two MVC components (polymer backbone and protein growth factor) and the fact that these constants sum based on the weight fractions of the components, it is possible to determine the conjugation ratio of the two components. This conjugate analysis methodology requires the use of two inline concentration detectors (such as UV and RI detectors) in order to solve for the weight fractions of the two different MVC components.¹¹

Thorough SEC-MALS characterization of these MVCs has demonstrated that they behave as branched molecules, and therefore we were interested in investigating whether they could be additionally characterized by using branching analysis methodology. Since MALS measures molecular weight and radius of gyration (R_g) of our MVC molecules in dilute solution, it is possible to observe the relationship between these two parameters. At a given molar mass, as the valency or conjugation ratio of our growth factor increases, the size (R_g) decreases. This behavior is the fundamental principle behind the detection and characterization of branching¹²⁻¹⁴, and indicates that the conjugated proteins could be treated as branches off of the polymer backbone chain. At a given molar mass, branching decreases the size by increasing the compactness of the macromolecule. Since the measurement of both molar mass and molecular size are required to obtain information about branching, SEC-MALS is one of the most powerful tools for this analysis.^{13,15,16}

The seminal paper by Zimm and Stockmayer¹⁷ introduced the parameter called the branching ratio, g , which is the only truly measureable branching distribution. This parameter is a numerical descriptor of the degree of branching and is defined as:

$$g = \left(\frac{R_{br}^2}{R_{lin}^2} \right)_M$$

where R is the root mean square radius for a branched (br) and linear (lin) version of a macromolecule at the same molar mass (M). The branching ratio is equal to one for a completely linear polymer and decreases as the degree of branching increases. There are numerous theoretical equations describing the branching ratio for different types of branched molecules such as comb, star, trifunctional, and tetrafunctional.¹⁷⁻²⁴ By using an appropriate theoretical equation, it is possible to exploit the branching ratio to also calculate the branch units per molecule, which should be equivalent to the number of conjugated proteins per molecule. Another parameter called the branching frequency, λ , which is defined as the branch units per 1,000 repeat units can also be calculated.¹²

In order to assess branching analysis as an alternative method to characterize MVCs, we used a representative MVC molecule of sFlt-1(3) (a protein decoy receptor for vascular endothelial growth factor) conjugated to a hyaluronic acid backbone chain. We used two different molecular weights of hyaluronic acid and a single initial reaction ratio of the protein and polymer to create the MVCs. These two molecules were previously characterized using SEC-MALS multivalent

conjugate method.¹ In this work, we characterized these same MVCs using the SEC-MALS branching method and then compared the branching method to our well established multivalent conjugate method to determine the validity of branching analysis as an alternative characterization method for MVCs.

5.3 Materials and Methods

5.3.1 Multivalent Conjugation of sFlt-1(3) Protein to Hyaluronic Acid

Hyaluronic acid of two different molecular weights (650 kDa and 1,000 kDa) was obtained from LifeCore Biomedical. The manufacturer-provided molecular weights were determined through viscosity measurements. Recombinantly expressed sFlt-1(3) protein (a mutant of sFlt-1 containing the first 3 Ig-like extracellular domains)^{1,25} was conjugated to hyaluronic acid through a process described previously.^{1,4-7} First, to make the activated hyaluronic acid intermediate, 3 mg/mL of hyaluronic acid (LifeCore Biomedical) of the two different molecular weights was dissolved completely in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma Aldrich) buffer (pH 6.5). 3,3'-*N*-(ϵ -maleimidocaproic acid) hydrazide (EMCH, Thermo Fisher Scientific, 1.2 mg/mL), 1-hydroxybenzotriazole hydrate (HOBt, Sigma Aldrich, 0.3 mg/mL) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Thermo Fisher Scientific, 10 mg/mL) were added to the hyaluronic acid solution. This solution was allowed to react at 4 °C for 4 hours before being dialyzed exhaustively against phosphate buffered saline (PBS) (pH 7.0) containing 10% glycerol.¹

The addition of thiol groups to the sFlt-1(3) protein was required to conjugate the protein to the activated hyaluronic acid through a reaction with the maleimide group on EMCH. sFlt-1(3) was treated with a 10-fold molar excess of 2-iminothiolane, and the final degree of thiolation was determined using Ellman's reagent (Thermo Fisher Scientific) to be ~1-1.2 thiols per sFlt-1(3). The thiolated sFlt-1(3) was reacted with the activated hyaluronic acid of two different molecular weights at a 10:1 molar feed ratio (sFlt-1(3):HyA) in PBS plus 10% glycerol overnight at 4 °C. The solution was then dialyzed exhaustively against PBS (pH 7.0) in 100 kDa molecular weight cut-off Float-a-Lyzer G2 dialysis tubes (Spectrum Labs).¹

For simplicity, we will refer to the MVC synthesized using the 650 kDa starting HyA as the "650 kDa MVC". Similarly, the MVC produced from the 1,000 kDa starting HyA will be referred to as the "1,000 kDa MVC".

5.3.2 SEC-MALS Characterization of MVCs

The multivalent conjugates of sFlt-1(3) and hyaluronic acid were characterized using size exclusion chromatography – multi-angle light scattering as described previously.¹ The SEC-MALS system consisted of an Agilent 1100 HPLC system (degasser, quaternary pump, autosampler, column holder/temperature controller, and UV-vis diode array detector) coupled in-line with a Wyatt Technology DAWN HELEOS-II multi-angle laser light scattering detector and a Wyatt Technology Optilab T-rEX refractive index (RI) detector. For the measurements, the wavelength of the diode array detector was set to 280 nm, the wavelength of the MALS detector was 658 nm, and the wavelength of the RI detector was 660 nm. A Shodex OH pak SB-804 HQ

column was used for the HPLC separation. Agilent ChemStation software was used to control the HPLC system, while Wyatt Technology Astra VI software was used for data collection and analysis. Normalization of the MALS detectors, band broadening correction, and peak alignment was performed using a 100 μ L injection of 2 mg/mL bovine serum albumin (BSA) (Sigma Aldrich) at a flow rate of 0.3 mL/min in PBS and the algorithms in the Astra VI software.

Multivalent conjugate samples were first filtered through 0.45 μ m filters to remove any aggregates or other large particles. 200 μ L injections at a concentration of 0.2-0.5 mg/mL HyA were performed at a flow rate of 0.3 mL/min with PBS as the mobile phase and the column temperature maintained at 25 $^{\circ}$ C. Analysis was limited to the moderate angle detectors to eliminate high noise levels from the extreme low and high angle detectors. Zimm light scattering formalism was used for processing of all light scattering data. Specific refractive index increment (dn/dc , mL g^{-1}) values for the sFlt-1(3) and activated HyA precursor were determined using batch analysis with the RI detector. The UV extinction coefficient (ϵ , mL mg^{-1} cm^{-1}) value for sFlt-1(3) was obtained using the ExPASy "ProtParam" tool (Swiss Institute of Bioinformatics).²⁶ The UV extinction coefficient for HyA measured with a spectrophotometer in a previous publication was used.¹¹ See Table 5.1. The multivalent conjugate analysis method used the specific refractive index increments and UV extinction coefficients of each of the components, while the branching analysis method required total dn/dc and UV extinction coefficient values for the MVC molecule. These total values (see Table 5.2) were calculated using the following equations:

$$\frac{dn}{dc} = \left[(Wt. Fraction_{sFlt1-(3)}) \left(\frac{dn}{dc}_{sFlt1-(3)} \right) \right] + \left[(Wt. Fraction_{HyA}) \left(\frac{dn}{dc}_{HyA} \right) \right]$$

$$\epsilon = \left[(Wt. Fraction_{sFlt1-(3)}) (\epsilon_{sFlt1-(3)}) \right] + \left[(Wt. Fraction_{HyA}) (\epsilon_{HyA}) \right]$$

where dn/dc is the specific refractive index increment and ϵ is the UV extinction coefficient.

5.3.3 Branching Analysis of MVCs

All data processing was performed using the Astra VI software (Wyatt Technology). The branching method included in the software was applied to the raw data files to perform the branching analysis. Several unmodified linear hyaluronic acid molecules (Lifecore Biomedical) in the range were characterized using the SEC-MALS method described above. Their conformation plots of RMS radius were plotted against molar mass, and an average linear regression line was determined. The line of best fit has the form of $R = kM^b$, where R is the RMS radius and M is the molar mass. See Figure 5.4. The k and b values determined for linear hyaluronic acid were used in the Astra VI branching method as the model linear polymer to calculate the branching ratio, branching units per molecule, and long chain branching.

The branching units per molecule and long chain branching were determined using a trifunctional model assuming a polydisperse slice. Various theoretical models for branching can be found in the literature¹⁷⁻²⁴, but the trifunctional model gave the best fit and was applicable to our MVCs, because each branch point is trifunctional. Since our MVCs are expected to coelute from the SEC column due to branching, they can be considered polydisperse for the branching

analysis. The assumption of a polydisperse slice is taken into account in the Astra VI algorithm for calculating branch units per molecule and long chain branching. For the long chain branching analysis, the repeat unit weight of hyaluronic acid was input as 379 g/mol. Best fit lines were extrapolated from the linear portions of the conformation plots for the multivalent conjugates, so that the predicted RMS radii of these branched molecules could be calculated. The predicted radii for the same molecular weight molecules of linear hyaluronic acid were calculated using the best fit line equation determined from the linear hyaluronic acid samples.

The multivalent conjugate method in the Astra VI software was also applied to the collected data. The multivalent conjugate analysis as described previously¹¹ was applied as a comparison against the results of the branching analysis. The results of the multivalent conjugate analysis method are summarized in Table 5.2.

5.4 Results and Discussion

5.4.1 Demonstration of Branching Behavior

Figures 5.1 and 5.2 show the molar mass versus elution volume and RMS radius versus elution volume plots overlaid on the light scattering chromatographs for the 650 kDa MVC and the 1,000 kDa MVC respectively. These plots demonstrate the abnormal SEC elution behavior that is characteristic of branched molecules. A linear molecule that elutes normally would show a linear decrease in molar mass or RMS radius over the entire light scattering peak. This is due to the fact that large molecules are unable to enter the pores in the column packing material and travel straight through the column to elute earlier, while smaller molecules are able to penetrate the pores and elute later. However, our MVC molecules do not demonstrate the expected SEC elution behavior. The plots begin with a linear decrease in molar mass or RMS radius over the first part of the peak, but then the plots level off or even show an uptick towards larger elution volumes. This behavior is indicative of anchoring in the SEC column, which occurs when part of a large branched molecule is able to penetrate into the pores in the column packing material and thereby anchor the large molecule so that it elutes later than would be expected for its size. This behavior does not occur for all branched molecules, but it will become more pronounced with a higher degree of branching. As can be seen from both sets of plots, the radius measurement is more sensitive than the molecular weight measurement to the polydispersity at higher elution volumes caused by anchoring of the branched molecules.^{12,15,27-30}

Figure 5.3 further demonstrates the abnormal elution behavior indicative of branched molecules. The plots shown in Figure 5.3 are conformation plots (RMS radius versus molar mass) for the two different molecular weight MVCs. Proper elution behavior of a linear molecule would result in a linear conformation plot with the RMS radius increasing as the molar mass increases. However, the conformation plots for both of our MVC molecules demonstrate an upswing in the plot at lower molar masses. It is not physically possible to have two different RMS radii for the same molar mass (e.g. same elution volume/time), so this upswing is completely virtual and is a result of the abnormal elution behavior shown in Figures 5.1 and 5.2. The anchoring effect causes larger molecules to elute later than expected so that molecules with larger radii are mixed in with the smaller molecules that would normally elute in that volume fraction.^{12,15,27-30}

Figures 5.1-5.3 demonstrate that our MVC molecules have abnormal elution behavior that indicates branching. To further prove that our MVCs behave like branched molecules, we can compare the slopes of the MVC conformation plots to the slope of the conformation plot for linear hyaluronic acid. The conformation plot for a linear polymer in a thermodynamically good solvent will have a slope of approximately 0.58. This is shown in the literature for numerous polymers including hyaluronic acid.^{12,31-38} We characterized several of the linear hyaluronic acid molecules used to synthesize our MVCs and generated an average conformation plot with a linear regression line shown in Figure 5.4. As can be seen from this plot, the slope for our linear hyaluronic acid is 0.59, which is very close to the expected slope of around 0.58. The linear portions of the conformation plots for our MVCs shown in Figure 5.3 can be extrapolated with linear regression lines as shown in Figure 5.4. As can be seen from the linear regression equations in Figure 5.4, both of our MVC molecules have conformation plots with slopes around 0.4. This is significantly lower than our measured linear hyaluronic acid slope of 0.59 or the literature value of ~0.58. The slopes of our MVC conformation plots allow us to further detect the presence of branching due to the fact that the fundamental principle behind detection and characterization of branching is that at a given molar mass the radius will decrease for a branched molecule compared to a linear molecule. This means that a change in the value of the slope for a highly branched molecule can be sufficient to detect branching even without a linear counterpart of identical chemical composition. Even though we do not have a linear identical chemical counterpart for our MVCs, we can still demonstrate that the slope of the conformation plots is significantly depressed to ~0.4 as compared to the expected slope of ~0.58 for any linear polymer in a thermodynamically good solvent.

5.4.2 Calculation of Predicted Branched Radii

The linear regression lines shown in Figure 5.4 are expressed in the form of:

$$R = kM^b$$

where R is the RMS radius, M is the molar mass, and b is the slope of the line. By using the equations from Figure 5.4 and the molar mass measured from light scattering for each MVC molecule (see Table 5.2), we can calculate the expected radius values for the portions of the conformation plot that were affected by the abnormal SEC elution behavior. These calculated values along with the calculated radius values for linear hyaluronic acid of the same molar mass are shown in Table 5.3.

5.4.3 Calculation of Branching Ratio, Branch Units per Molecule, and Long Chain Branching

In order to calculate the branching ratio, g, a chemically identical linear counterpart to the branched molecule should be used. This is because g is calculated using the ratio of the root mean square radius of the branched molecule to the root mean square radius of its linear counterpart. We do not however, have a chemically identical linear counterpart to our MVC molecules, so we used the linear hyaluronic acid molecule used to make our MVCs. While this is not the most accurate way to calculate the branching ratio, and makes the assumption that our linear HyA behaves the same as a linear molecule of HyA plus sFlt-1(3), this turns out to be sufficient to obtain branching analysis data that is in good agreement with our data determined

using conjugate analysis. Additionally, several examples in the literature that discuss the branching analysis of copolymers show that it is often sufficient to use a linear counterpart of just one of the copolymer components instead of a chemically identical linear copolymer to estimate branching.^{37,39} Based on this assumption, we inputted the k and b values for our linear HyA regression line shown in Figure 5.4 as the linear counterpart in the Astra VI branching analysis method. Using the equation for branching ratio, the software was able to calculate the g versus molar mass data for our MVCs, which is shown in Figure 5.5.

In order to calculate the average branch units per molecule we used the trifunctional branching model equation developed by Zimm and Stockmayer¹⁷ because each branch point on our multivalent conjugates is trifunctional. The equation for the branching ratio of a polymer with trifunctional branch units is:

$$g_3 = \left[\left(1 + \frac{m}{7} \right)^{\frac{1}{2}} + \frac{4m}{9\pi} \right]^{-\frac{1}{2}}$$

where m is the average number of branch units per molecule and the subscript 3 refers to trifunctional branching.¹⁷ For the trifunctional model in the Astra VI software (Wyatt Technology), we assumed that our molecules are polydisperse because they are expected to coelute from the SEC column due to the branching behavior shown in Figures 5.1-5.3. Figure 5.6 shows the plots for branch units per molecule versus molar mass for the 650 kDa and 1,000 kDa MVCs. The thin, solid line on each of the plots is the cumulative weight fraction, so that at a given molar mass, the branch units per molecule as well as the percent of the sample with that number of branch units per molecule can be determined from the plot. Using the total weight average molecular weight (M_w) measured by SEC-MALS for each of the two conjugate molecules (see Table 5.2), the plots in Figure 5.6 can be used to find the branch units per molecule for each MVC. For the 650 kDa MVC, there are 4.1 branch units per molecule and about 65% of the sample has this number of branch units. For the 1,000 kDa MVC, there are 5.1 branch units per molecule and about 83% of the sample has this number of branch units.

The plots shown in Figure 5.7 analyze the long chain branching of our MVC molecules. By inputting the molar mass of a repeat unit of hyaluronic acid, the software analysis is able to calculate the number of branch points per 1,000 repeat units. This parameter is also called the branching frequency, λ . Using the total weight average molecular weight (M_w) measured by SEC-MALS for each of the two conjugate molecules (see Table 5.2), the plots in Figure 5.7 can be used to find the branching frequency for each MVC. For the 650 kDa MVC, there are 1.5 branch units per 1,000 repeat units of hyaluronic acid. For the 1,000 kDa MVC there are 1.2 branch units per 1,000 repeat units.

5.4.4 Comparison of Branching Analysis to Traditional Conjugation Ratio Analysis

The branch units per molecule and branching frequency values for each of our two MVCs are summarized in Table 5.3. The sFlt-1(3):HyA conjugation ratio and the sFlt-1(3):1,000 repeat units values calculated using the conjugate analysis technique¹¹ are also shown in Table 5.3 for comparison with the branching analysis values. As you can see, these values are very similar,

demonstrating that branching analysis can be used as an alternative characterization method that provides good approximations of the conjugation ratios for our MVCs.

5.5 Conclusions

We have presented an alternative SEC-MALS methodology for characterizing MVC molecules. First, we demonstrated that our MVC molecules behave as branched molecules, and then we showed that branching analysis methods can be successfully applied to these MVCs. Although we are using linear hyaluronic acid as our linear counterpart for these calculations instead of a chemically identical linear counterpart, we still achieve good agreement between our values calculated by branching analysis as compared to multivalent conjugate analysis. Branching analysis is inherently an approximate analysis tool due to the complexity of branched polymers, which makes it almost impossible to achieve a detailed analysis of branching. Often, an estimation of the degree of branching as a function of molar mass is the only branching distribution that is obtainable. Therefore, the assumption that we can use linear hyaluronic acid instead of a chemically identical linear counterpart does not dramatically effect the accuracy of this analysis. In terms of benefits, using the branching analysis method allows for the use of only one inline concentration detector, as compared to the two concentration detectors required for multivalent conjugate analysis. This decreases the complexity and cost of the equipment required for analysis. Additionally, multivalent conjugate analysis requires the molecular weight of each of the two conjugate components to be known very accurately. The proteins and growth factors used to make the MVC molecules can be difficult and cost prohibitive to obtain in large enough quantities to measure their molar masses using SEC-MALS. The branching analysis methodology, however, only requires the total molecular weight of the MVC molecule and not the molecular weights of the individual components.

5.6 References

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5.7 Tables

Table 5.1

Measured constants for SEC-MALS analysis of the multivalent conjugate components.

	Activated Hyaluronic Acid	sFlt-1(3) Protein
Initial Molecular Weight (kDa)	650 and 1,000	50 ^{1,25}
Specific Refractive Index Increment, dn/dc (mL g ⁻¹)	0.145 ¹	0.185
UV Extinction Coefficient, ϵ (mL mg ⁻¹ cm ⁻¹)	0.022 ¹¹	0.894 ²⁶

Table 5.2

Weight average molecular weights and final conjugation ratios for the 650 and 1,000 kDa conjugates measured using the SEC-MALS multivalent conjugate method.¹ The total specific refractive index increments and UV extinction coefficients for the conjugate molecules were calculated using the weight fractions of the sFlt-1(3) and HyA components.

	650 kDa MVC	1,000 kDa MVC
Hyaluronic Acid M_w (Da)	7.1E5	1.2E6
sFlt-1(3) Protein M_w (Da)	3.2E5	3.1E5
Total MVC M_w (Da)	1.0E6	1.5E6
Total MVC dn/dc (mL g ⁻¹)	0.155	0.152
Total MVC ϵ (mL mg ⁻¹ cm ⁻¹)	0.243	0.189
sFlt-1(3):HyA	6.4:1	6.2:1

Table 5.3

Comparison of branching analysis and multivalent conjugate analysis¹ to determine the number of sFlt-1(3) molecules conjugated per HyA molecule. Also included is a comparison of the RMS radii predicted by the linear HyA best fit line compared to the branched best fit lines on the conformation plot in Figure 5.4.

	650 kDa MVC	1000 kDa MVC
Linear HyA RMS Radius (nm)	111.4	139.5
MVC RMS Radius (nm)	92.1	105.8
sFlt-1(3):HyA	6.4	6.2
Branch Units per Molecule	4.1	5.1
sFlt-1(3):1,000 Repeats HyA	2.3	1.6
Branch Units per 1,000 Repeat Units	1.5	1.2

5.8 Figures

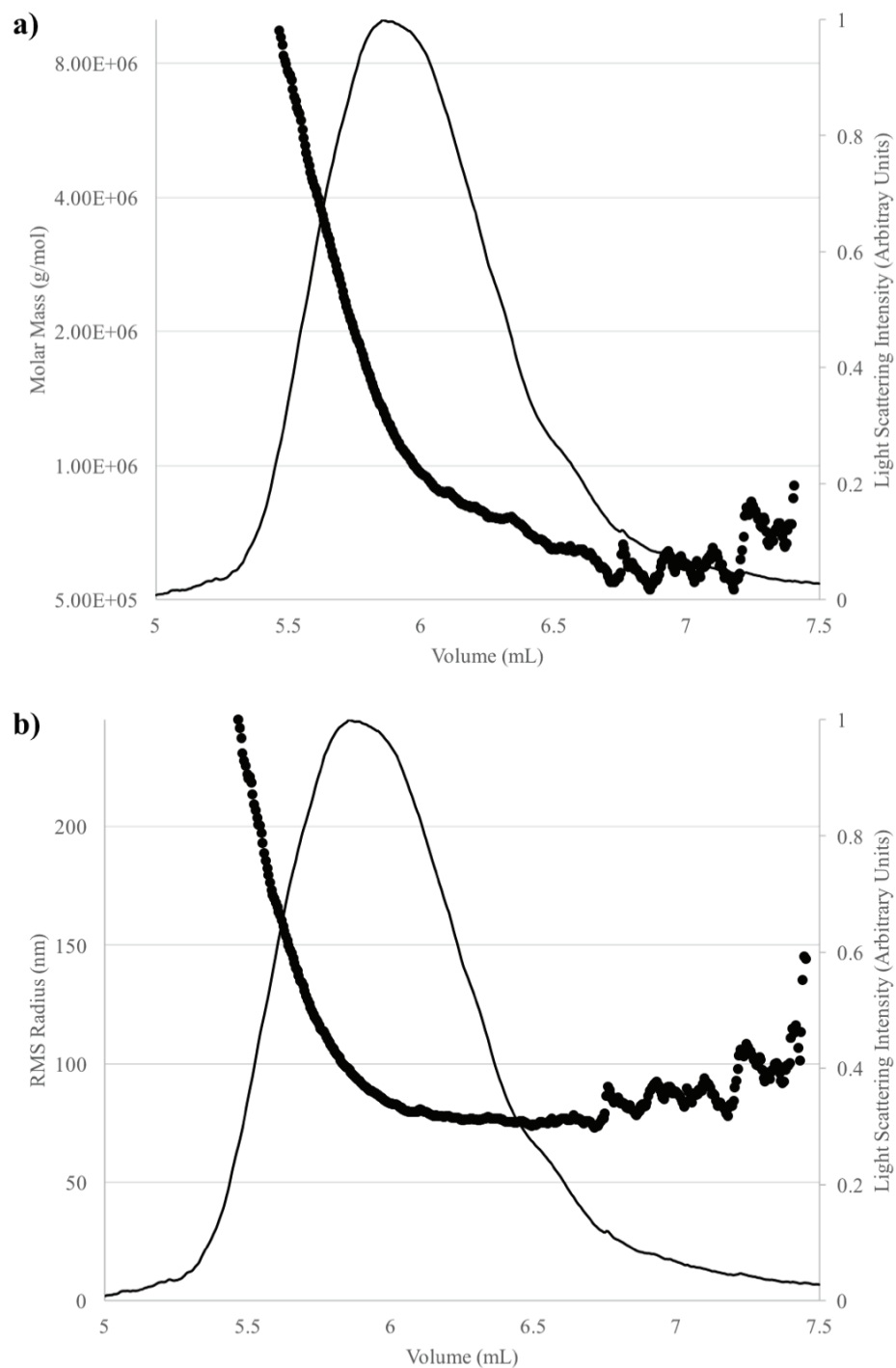


Figure 5.1

650 kDa MVC: Molar mass versus elution volume (a) and RMS radius versus elution volume (b) plots demonstrating the abnormal SEC elution behavior of branched molecules. The thin solid line in each plot is the light scattering chromatograph.

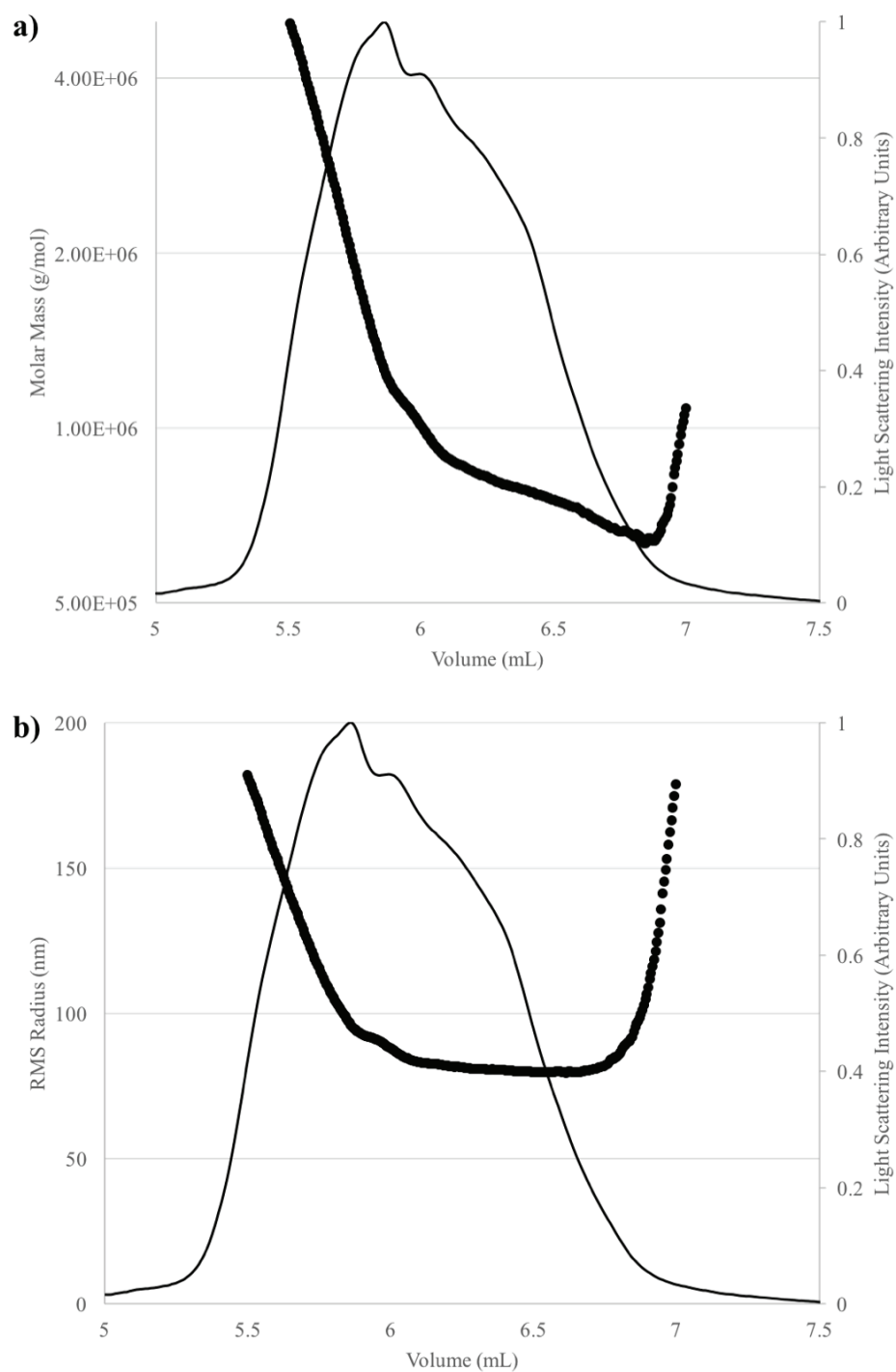


Figure 5.2
1,000 kDa MVC: Molar mass versus elution volume (a) and RMS radius versus elution volume (b) plots demonstrating the abnormal SEC elution behavior of branched molecules. The thin solid line in each plot is the light scattering chromatograph.

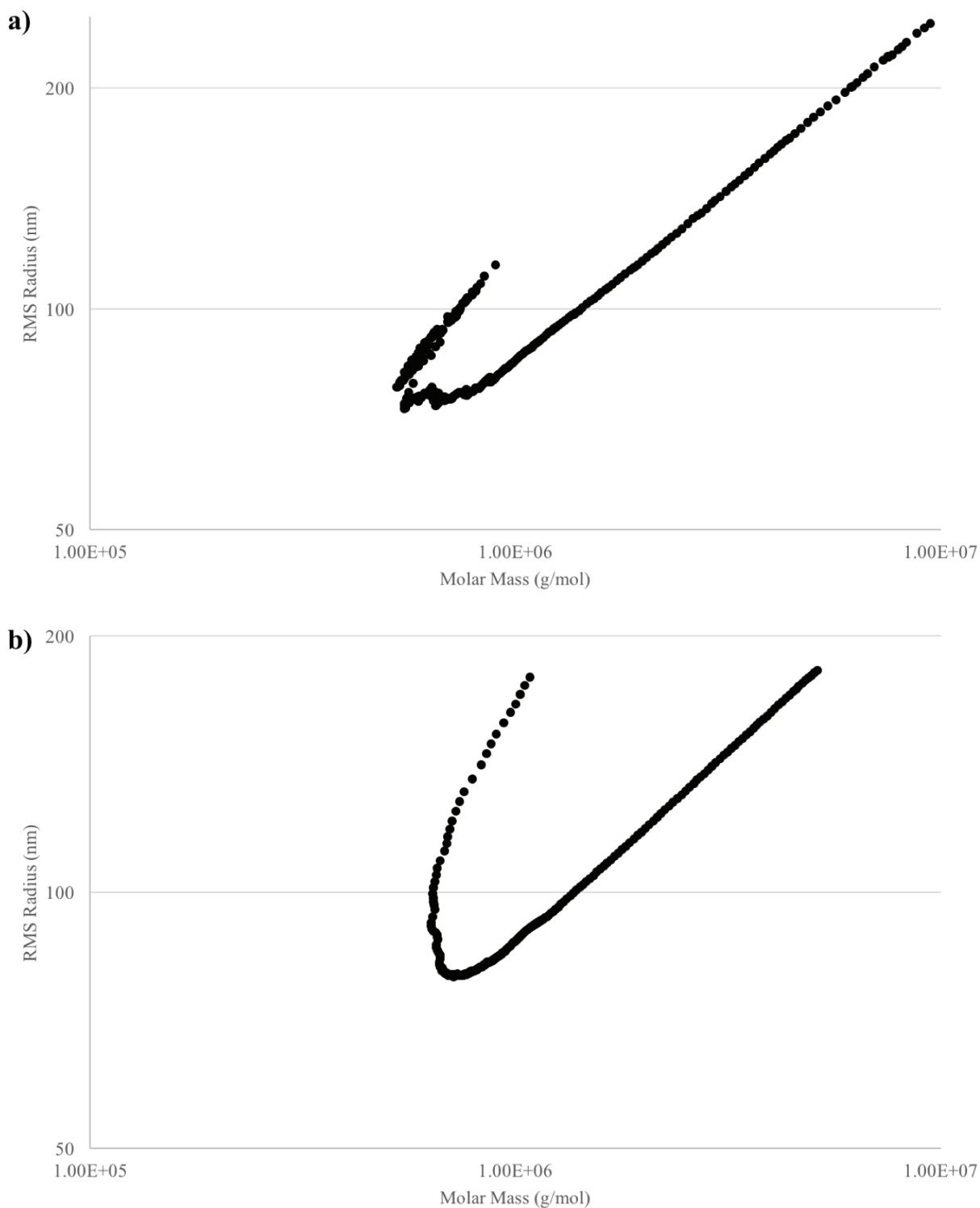


Figure 5.3

Conformation plots for the 650 kDa MVC (a) and the 1,000 kDa MVC (b) demonstrating the virtual upswing caused by anchoring of branched molecules in the SEC column. Only the linear portions of these conformation plots were used to generate the best fit lines in Figure 5.4.

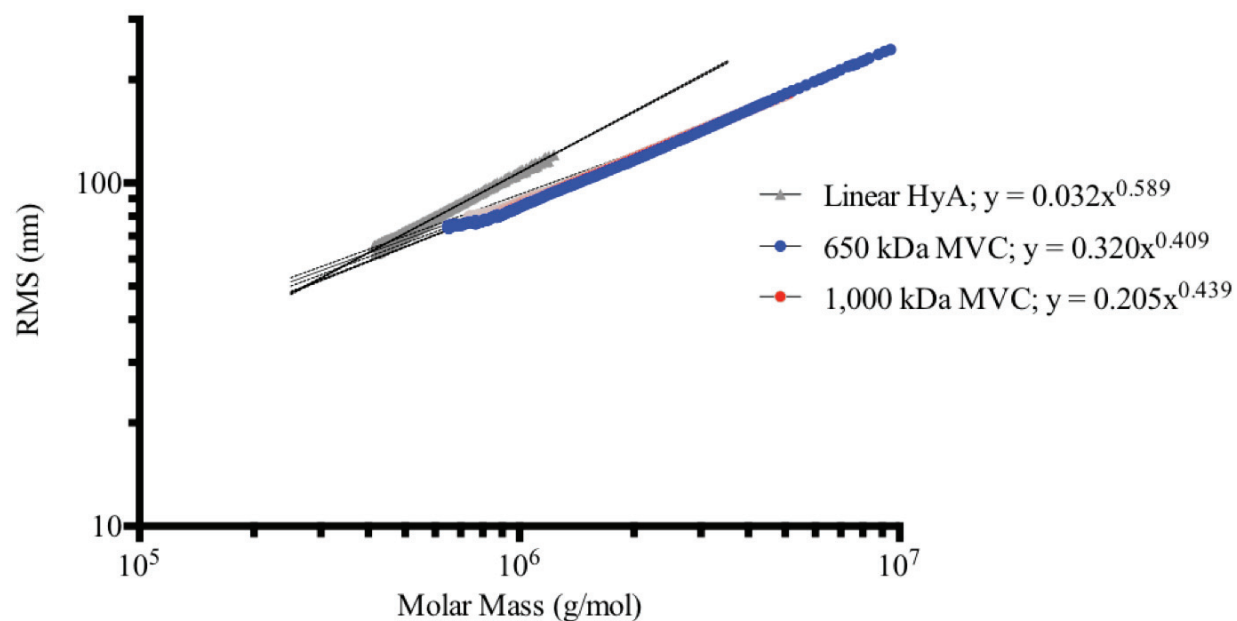


Figure 5.4

Best fit lines for the linear portions of the 650 kDa and 1,000 kDa MVC conformation plots. The slopes of these fit lines show a decrease from the expected value as demonstrated by the solid line, which is the linear regression for the conformation plot of our unmodified linear hyaluronic acid.

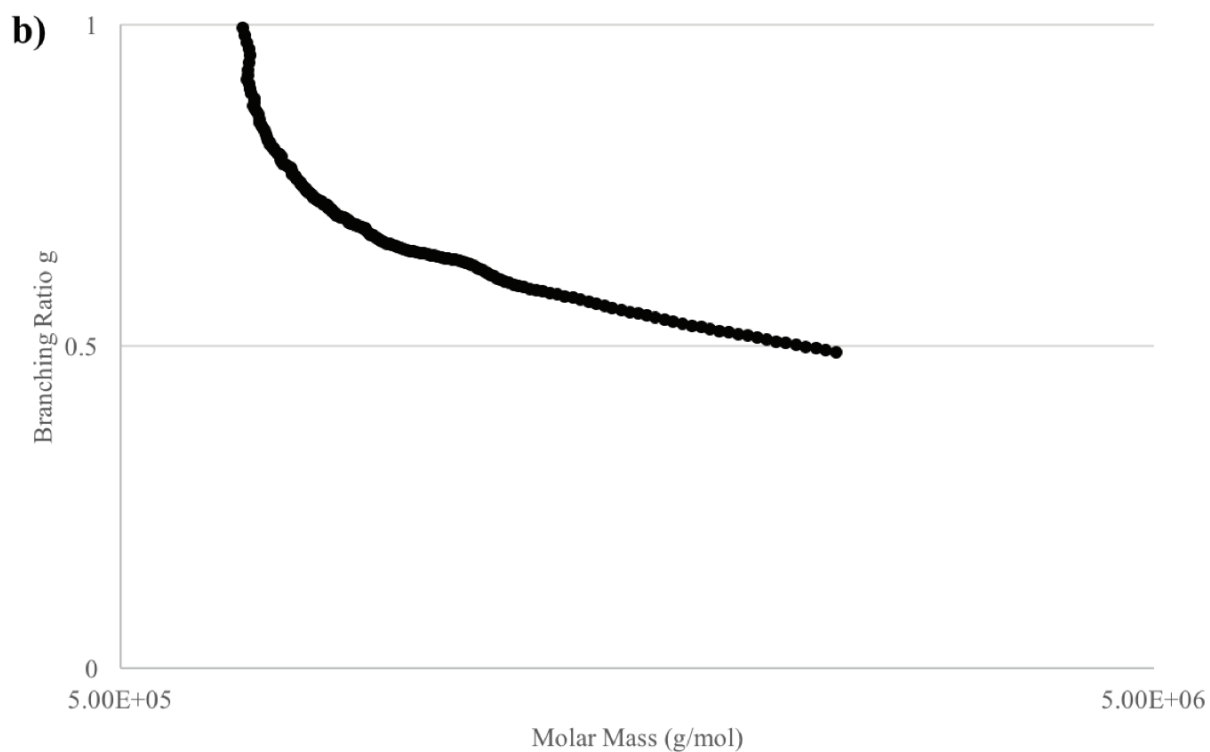
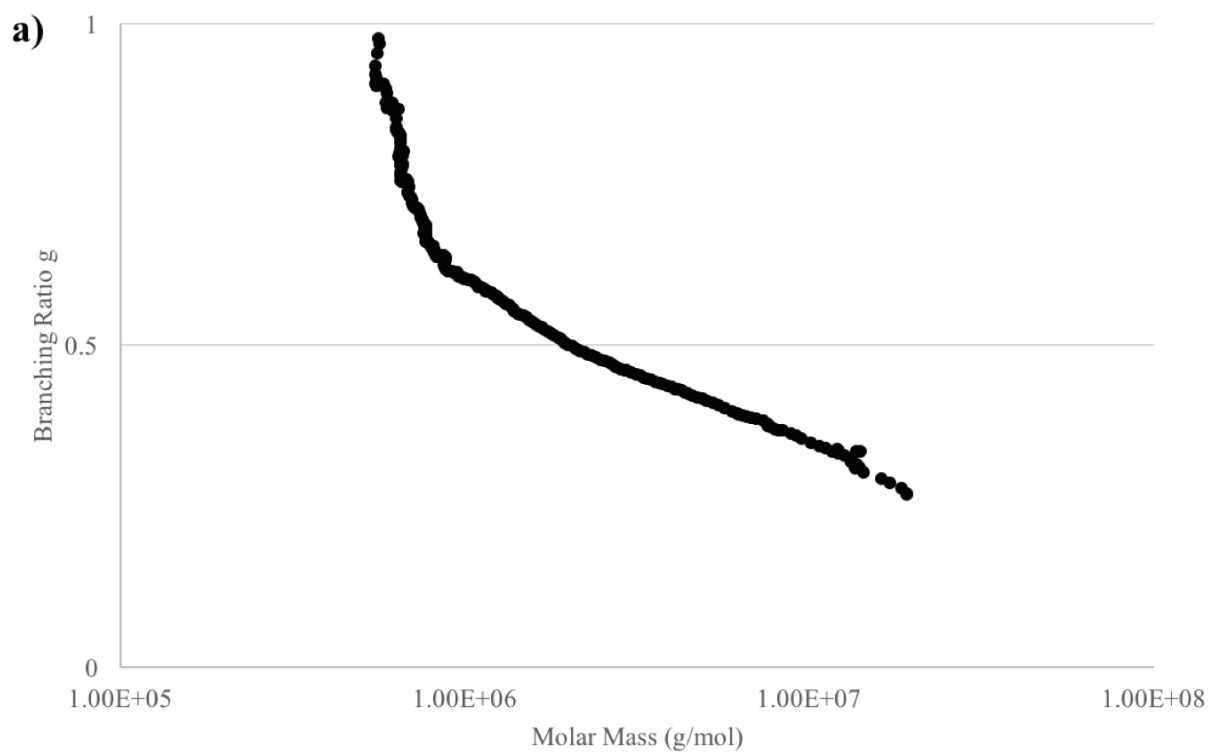


Figure 5.5
Branching ratio, g , versus molar mass for the 650 kDa MVC (a) and the 1,000 kDa MVC (b).

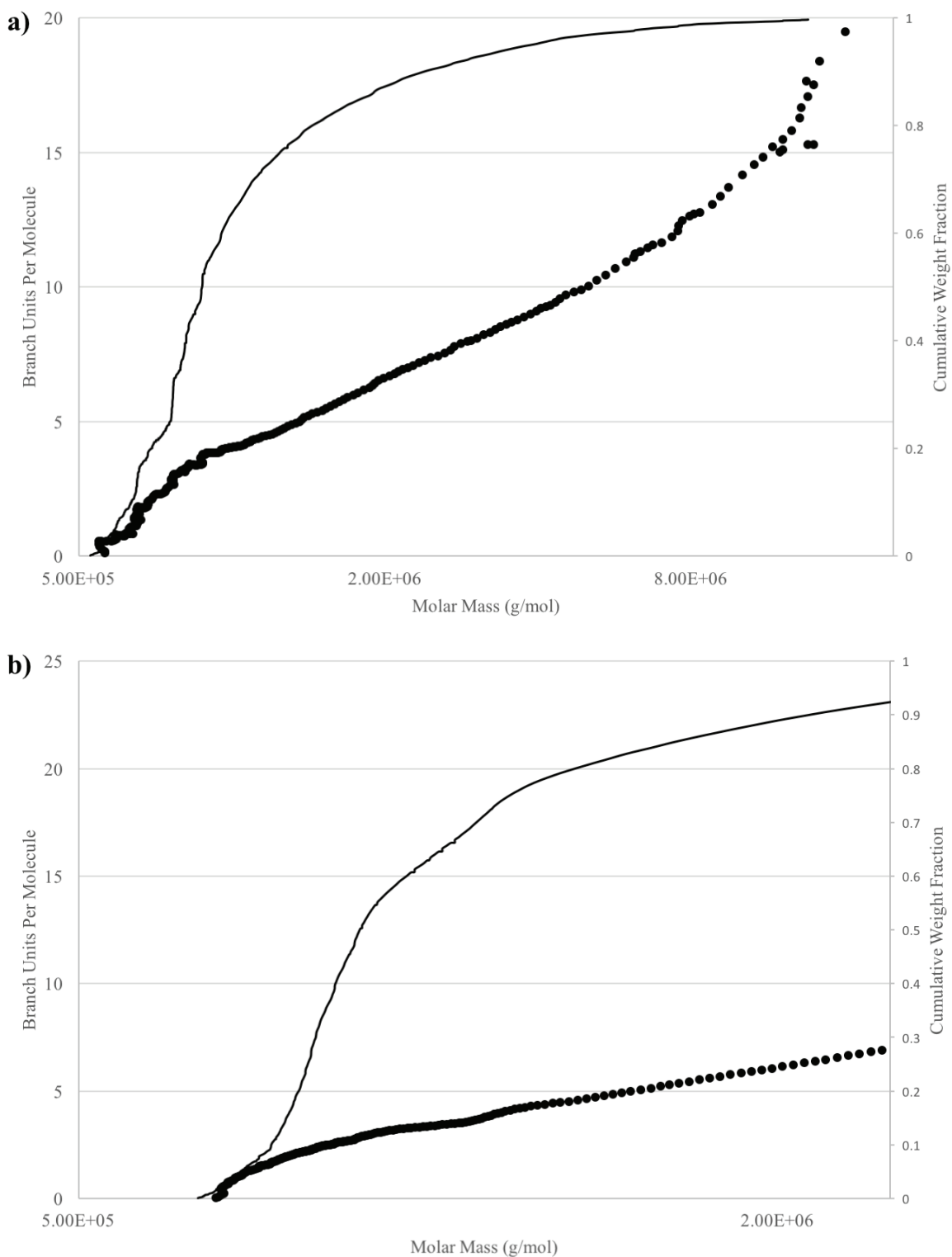


Figure 5.6
Branch units per molecule and cumulative weight fraction versus molar mass for the 650 kDa MVC (a) and 1,000 kDa MVC (b).

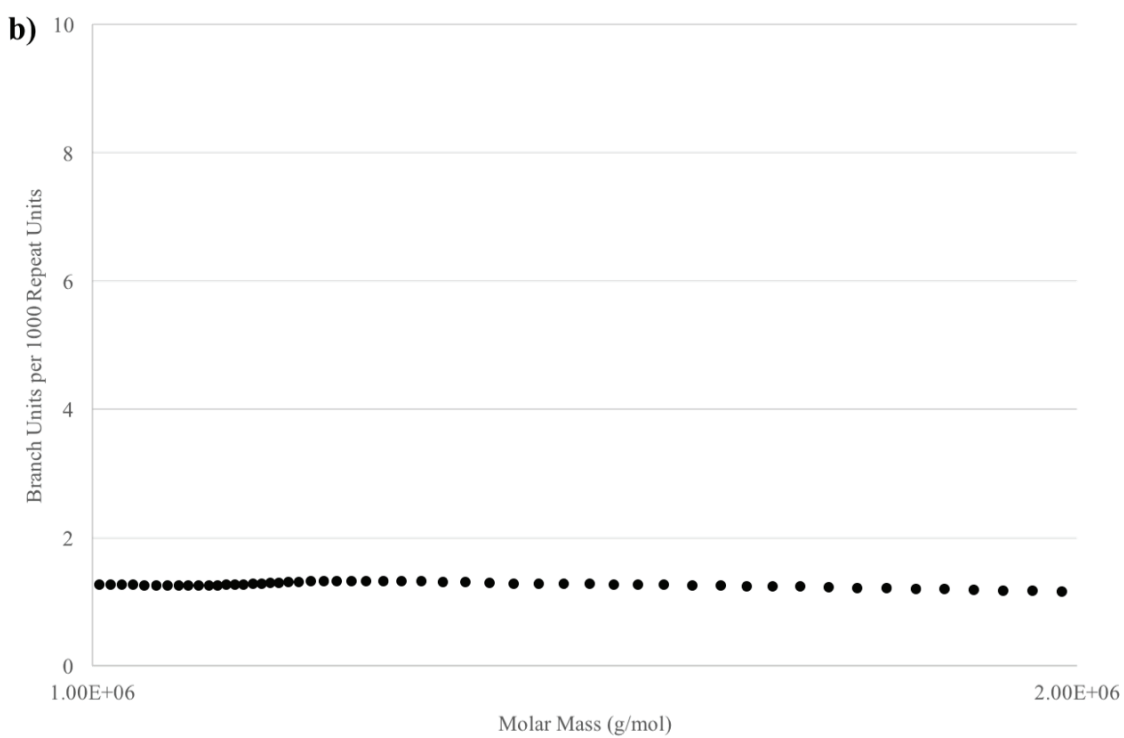
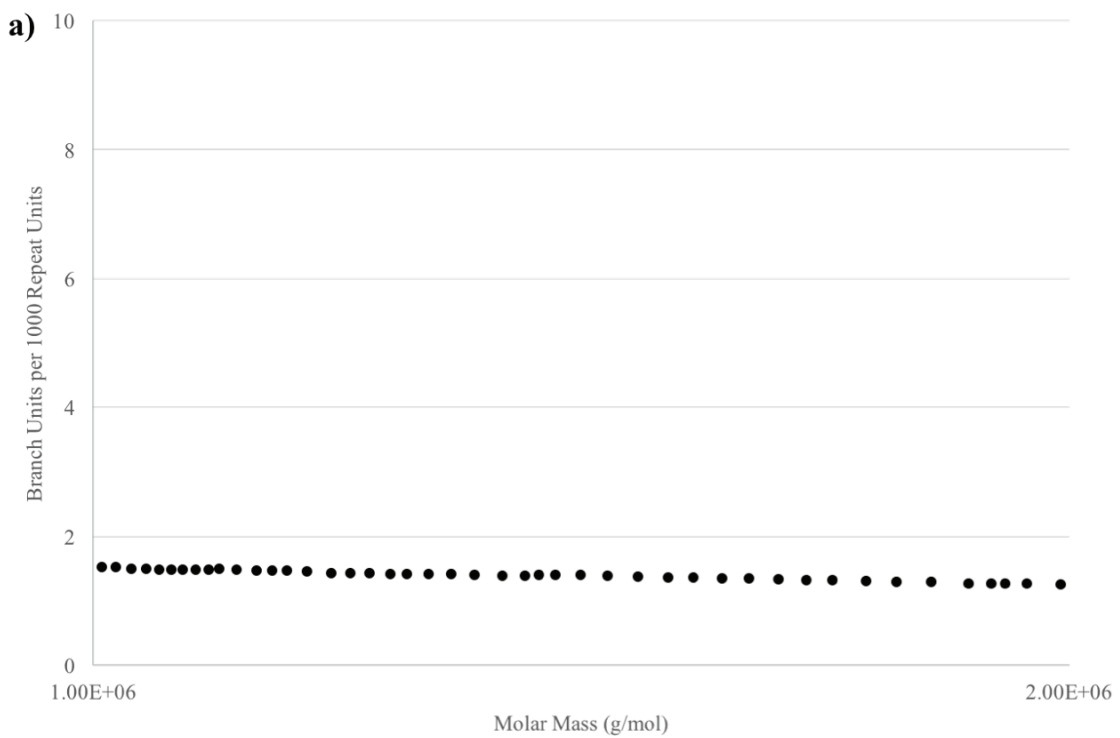


Figure 5.7
Long chain branching analysis: Branch units per 1000 repeat units of HyA versus molar mass for the 650 kDa MVC (a) and the 1,000 kDa MVC (b).

CHAPTER 6: SIZE-EXCLUSION CHROMATOGRAPHY – MULTI-ANGLE LIGHT SCATTERING CHARACTERIZATION OF OTHER MACROMOLECULES

6.1 Abstract

Multi-angle light scattering (MALS) is a powerful tool for characterizing macromolecules in dilute solution, since it can measure both absolute molecular weight and radius of gyration (R_g), while also providing information about the distributions of these values. In these studies, an online three-detector method to monitor UV light absorbance, differential refractive index (DRI), and multi-angle light scattering (MALS) coupled with size exclusion chromatography (SEC) separation was used to characterize several different macromolecules and MVCs for various research projects. SEC-MALS analysis was able to provide detailed information about the molecular weight, radius of gyration, polydispersity ($\mathcal{D}$), and valency of these different macromolecular systems.

6.2 Introduction

As was discussed in Chapters 4 and 5, multi-angle light scattering (MALS) is a powerful tool for characterizing macromolecules in dilute solution, since it can measure both absolute molecular weight and radius of gyration (R_g). The fundamental principle of light scattering characterization is that the amount of light scattered is directly proportional to the molar mass and concentration.¹ This can be expressed as:

$$I(\theta) \propto Mc \left(\frac{dn}{dc} \right)^2$$

where $I(\theta)$ is the light scattering intensity measured at a given angle detector, M is the molar mass, c is the concentration, and dn/dc is the specific refractive index increment. Additionally, with the use of multiple detection angles, it is possible to measure the radius of gyration of particles larger than 10 nm.^{2,3} The addition of an inline size exclusion chromatography (SEC) system allows for the separation of macromolecules by molar mass, so that the MALS measurements include information about the molecular weight distributions instead of just average values. For these studies, the aim was to characterize several additional macromolecule systems utilized in other research projects. A previously described online three-detector method to monitor UV light absorbance, differential refractive index (DRI), and multi-angle light scattering (MALS) coupled with a SEC system⁴⁻⁶ was used in these studies.

Hyaluronic acid, like many other polymers, has been shown to mechanically degrade due to high shear from processes such as stirring and laminar or capillary flow.^{13,14} This raises concerns that HyA solutions forced through syringe filters for sterilization or in order to remove aggregates may be substantially degrading. For many studies, it is crucial that the molecular weight and distribution of the HyA molecules is known, so it was important to investigate the effects of different filtering methods. In order to study the effects of filtering, five different filtering

conditions were used on solutions of unmodified HyA, which were then characterized through SEC-MALS analysis. These methods were (1) hand filtering through a 0.22 μm pore size syringe filter, (2) using a syringe pump to filter through a 0.22 μm pore size syringe filter, (3) hand filtering through a 0.45 μm pore size syringe filter, (4) using a syringe pump to filter through a 0.45 μm pore size syringe filter, and (5) centrifuging.

Multivalent conjugates can be synthesized using different chemistry techniques. In this work, it was desirable to show that the MVCs of MGF discussed in Chapters 3 and 4 could also be synthesized using a previously published alternative EMCH-based chemistry.⁷⁻¹¹ This alternative conjugation chemistry is based on the reaction between a maleimide group on the modified HyA precursor and a thiol on the c-terminal cysteine of the peptide. In order to confirm that mvMGF could be synthesized using this alternative method, the reaction was carried out at an initial molar feed ratio of 30:1 (MGF:HyA) and the final products were characterized using SEC-MALS.

Since the presentation of growth factor molecules can control stem cell fate, it was desirable to design a three-dimensional hydrogel culture system which could sequester and present desired growth factor molecules. In order to utilize the growth factor sequestering ability of heparin, hyaluronic acid-based hydrogels containing conjugated heparin molecules were created. To understand the growth factor affinity and retention of this hydrogel system, thorough characterization of the heparin molecules was required.¹² Therefore, SEC-MALS was employed to measure the molecular weight and polydispersity of different heparin molecules.

6.3 Materials and Methods

6.3.1 SEC-MALS Characterization

The SEC-MALS system used to characterize the different macromolecules consisted of an Agilent 1100 HPLC system (degasser, quaternary pump, autosampler, column holder/temperature controller, and UV-vis diode array detector) coupled in-line with a Wyatt Technology DAWN HELEOS-II multi-angle laser light scattering detector and a Wyatt Technology Optilab T-rEX refractive index (RI) detector. For the measurements, the wavelength of the diode array detector was set to 280 nm, the wavelength of the MALS detector was 658 nm, and the wavelength of the RI detector was 660 nm. Agilent ChemStation software was used to control the HPLC system, while Wyatt Technology Astra VI software was used for data collection and analysis. Normalization of the MALS detectors, band broadening correction, and peak alignment was performed using a 100 μL injection of 2 mg/mL bovine serum albumin (BSA) (Sigma Aldrich) at a flow rate of 0.3 mL/min in PBS and the algorithms in the Astra VI software.

Specific refractive index increment (dn/dc , mL g^{-1}) values for the different macromolecules and multivalent conjugate components were determined using batch analysis with the RI detector. Solutions with six different concentrations from 0.016 to 0.5 mg/mL were injected directly into the RI detector, and the differential refractive index was measured at each concentration. For each concentration, 3 mL of solution was injected at a rate of 0.3 mL/min using a syringe pump. After each sample was measured, the data was plotted on a graph of differential refractive index

versus concentration. A linear fit was applied, and the slope of the fit was the value for the dn/dc . The UV extinction coefficient (ϵ , $\text{mL mg}^{-1} \text{ cm}^{-1}$) values were obtained using an online 100% mass recovery method in the Astra VI software, which calculated UV extinction coefficient from the RI peak. The UV extinction coefficient for HyA was too low to measure on the SEC-MALS system, so the value measured with a spectrophotometer in a previous publication was used.⁴ The UV extinction coefficient for the EMCH-HyA was also too small to be measured with SEC-MALS, so it was assumed to have the same value as unmodified HyA. The measured values of these constants are shown in Table 6.1.

6.3.2 Analysis of the Effect of Different Filtering Methods on HyA Molecular Weight and Polydispersity

In order to study the effects of filtering on molecular weight and polydispersity, HyA with a molecular weight of ~ 600 kDa as determined by the manufacturer through viscosity measurements was obtained from LifeCore Biomedical. The HyA was dissolved overnight at a concentration of 1 mg/mL in PBS. The following day, the HyA solution was diluted to a concentration of 0.1 mg/mL before filtering using several different methods. The following methods were used.

1. The HyA solution was hand filtered through either 0.22 or 0.45 μm pore size EMD Millex 13 mm diameter, PVDF, sterile syringe filters (Fisher Scientific).
2. Other samples of the HyA were filtered through either the 0.22 or 0.45 μm pore size filters by using a syringe pump at a controlled flow rate of 0.3 mL/minute.
3. A final filtering method involved centrifuging the HyA solution at 3,000 rpm for 5 minutes and then collecting only the supernatant solution.

The filtered HyA solutions were run on the SEC-MALS system at 0.3 mL/min with PBS as the mobile phase, and a Shodex OH pak SB-804 HQ column was used for separation with the temperature maintained at 25 °C. Three separate 400 μL injections at a concentration of 0.1 mg/mL HyA were run for each of the different filtering methods. Analysis was limited to the moderate angle detectors to eliminate high noise levels from the extreme low and high angle detectors. Zimm light scattering formalism was used for processing of all light scattering data.

6.3.3 Characterization of Multivalent Conjugates of MGF Synthesized Using Alternative EMCH Chemistry

Hyaluronic acid with a molecular weight of 500 kDa was obtained from LifeCore Biomedical. The manufacturer-provided molecular weight was determined through viscosity measurements. MGF peptide was conjugated to hyaluronic acid through a process described previously.⁷⁻¹¹ First, to make the activated hyaluronic acid intermediate, 3 mg/mL of hyaluronic acid (LifeCore Biomedical) was dissolved completely in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma Aldrich) buffer (pH 6.5). 3,3'-*N*-(ϵ -maleimidocaproic acid) hydrazide (EMCH, Thermo Fisher Scientific, 1.2 mg/mL), 1-hydroxybenzotriazole hydrate (HOBt, Sigma Aldrich, 0.3 mg/mL) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Thermo Fisher Scientific, 10 mg/mL) were added to the hyaluronic acid solution. This solution was allowed to react at 4 °C for 4 hours before being dialyzed exhaustively against phosphate

buffered saline (PBS) (pH 7.0) containing 10% glycerol. This EMCH-modified HyA intermediate will be referred to as EMCH-HyA.

MGF peptide with an added c-terminal cysteine residue (MGF-Cys) was obtained from American Peptide Company. The sequence for this peptide is shown in Table 3.1. The thiol group on the added cysteine residue was required to conjugate the peptide to the activated hyaluronic acid through a reaction with the maleimide group on EMCH. The MGF-Cys was reacted with the EMCH-HyA at a 30:1 molar feed ratio (MGF:HyA) in PBS overnight at 4 °C. The solution was then dialyzed exhaustively against PBS (pH 7.0) at 4 °C in 100 kDa molecular weight cut-off Float-a-Lyzer G2 dialysis tubes (Spectrum Labs).

The mvMGF solutions were first filtered through 0.22 µm pore size filters to remove any aggregates or other large particles. The mvMGF was then run on the SEC-MALS system at 0.3 mL/min with PBS as the mobile phase, and a Shodex OH pak SB-804 HQ column was used for separation with the temperature maintained at 25 °C. Two separate 200 µL injections at a concentration of 0.5 mg/mL HyA were run for analysis. Analysis was carried out using a previously described multivalent conjugate method⁴ and was limited to the moderate angle detectors to eliminate high noise levels from the extreme low and high angle detectors. Zimm light scattering formalism was used for processing of all light scattering data.

6.3.4 Characterization of the Molecular Weight and Polydispersity of Different Heparin Molecules

In order to characterize the molecular weights and polydispersities of different heparin molecules, high molecular weight heparin (HMWH), low molecular weight heparin (LMWH), and unfractionated molecular weight heparin (UMWH) were obtained from Santa Cruz Biotechnology, Inc.¹² The different molecular weights of heparin were run on the SEC-MALS system at 0.3 mL/min with PBS as the mobile phase, and a Shodex OH pak SB-803 HQ column was used for separation with the temperature maintained at 25 °C. Two separate 100 µL injections at a concentration of 5 mg/mL were run for each heparin sample. Analysis was limited to the moderate angle detectors to eliminate high noise levels from the extreme low and high angle detectors. Zimm light scattering formalism was used for processing of all light scattering data.

6.4 Results and Discussion

6.4.1 Analysis of the Effect of Different Filtering Methods on HyA Molecular Weight and Polydispersity

In order to study the effects of filtering, five different filtering conditions were used on solutions of unmodified HyA, which were then characterized through SEC-MALS analysis. These methods were (1) hand filtering through a 0.22 µm pore size syringe filter, (2) using a syringe pump to filter through a 0.22 µm pore size syringe filter, (3) hand filtering through a 0.45 µm pore size syringe filter, (4) using a syringe pump to filter through a 0.45 µm pore size syringe filter, and (5) centrifuging. Figure 6.1 shows curves of molar mass versus elution time for the different filtering conditions. It can be seen that there is very little difference between the different samples, and that they exhibit expected elution behavior over the light scattering peak.

This figure indicates that there isn't much difference between the molecular weight distributions of the HyA samples filtered using the different methods. To further confirm this finding, Figure 6.2 is a plot of the weight-average molecular weight of HyA measured for each of the filtering conditions. This plot shows no significant difference between the different filtering conditions in terms of the average molecular weight of HyA after filtering. However, it can be seen that the samples that were filtered at a controlled rate using a syringe pump exhibit a much tighter distribution than those filtered by hand or with centrifugation. This data is further represented in Table 6.2, which shows the M_n , M_w , and polydispersity index ($\bar{D}$) for each of the filtering conditions. By comparing the values of $\bar{D}$, it can be seen that all of the HyA samples exhibit low polydispersity and that there is very little difference between the polydispersity values for the different filtering methods.

The molecular weight of the original HyA was determined to be 600 kDa by the supplier using a viscosity measurement. Viscosity measured molecular weight (M_v) is predicted to fall between the values for M_n and M_w , such that $M_n < M_v < M_w$. Therefore, if there was no degradation of the HyA due to filtering, it would be expected to measure a M_w above 600 kDa. However, as can be seen from Table 6.2, all of the M_w values for the different filtering conditions are at or slightly below 600 kDa. This indicates that all five of the different filtering techniques result in some slight degradation the HyA, and they all seem to cause a similar extent of degradation. However, even though all the samples have similar final M_w measurements, syringe pump filtering offers an improvement over the other filtering techniques due to the decreased standard deviation of the measurements. Therefore, it is recommended to use a syringe pump at a controlled flow rate for the filtering of HyA solutions for analysis with SEC-MALS.

As a final analysis of these HyA samples, their conformation plots of RMS radius versus molar mass are shown in Figure 6.3. As expected, there is no significant difference between the different filtering conditions. Linear fit lines were applied to each plot to yield equations in the forms of

$$R = kM^b$$

where R is the root mean square (RMS) radius, M is the molar mass, and b is the slope of the line. These equations confirm the validity of our SEC-MALS analysis of HyA because the slopes are all close to 0.58, which is the predicted slope for linear polymers in a thermodynamically good solvent.^{3,15} Additionally, conformation plot slopes for HyA measured in the literature are demonstrated to be approximately 0.58.¹⁶⁻²¹ Figure 6.3 demonstrates that the HyA samples used in these studies behave as linear polymers and yield expected and valid SEC-MALS data. The z-average radius of gyration ($R_{g,z}$) values measured for each of the HyA samples are listed in Table 6.2, and are expected to be accurate due to the normal elution behavior and linear nature of these HyA molecules.

6.4.2 Characterization of Multivalent Conjugates of MGF Synthesized Using Alternative EMCH Chemistry

In order to confirm that mvMGF could be synthesized using the alternative EMCH chemistry, the reaction was carried out at an initial molar feed ratio of 30:1 (MGF:HyA) and the final

product was characterized using SEC-MALS. The conjugates synthesized using this alternative chemistry demonstrated a strong signal relative to the noise in all three detectors (UV, LS, and RI), allowing for reliable analysis of the data. Figure 6.4 is a representative plot of the molar mass versus elution volume overlaid on the LS peak for one run of the mvMGF. This plot is broken down into the molar mass contributions over the peak for the MGF and HyA components, as well as for the total conjugate molecule. It can be seen that the contribution to the molar mass from the MGF peptide is much smaller than that of the HyA backbone, and that the two components sum to the total molar mass, as was expected. The weight-averaged molar mass for the total conjugate was measured to be $5.7\text{E}5$ g/mol, while the HyA component was $4.9\text{E}5$ g/mol and the MGF component was $8.1\text{E}4$ g/mol. These values are reported in Table 6.3 along with the M_n , $\bar{D}$, and $R_{g,z}$ values.

This data is further illustrated in Figure 6.5, which shows the cumulative and differential weight fraction curves. The curves are broken down into the contributions from the MGF and HyA separately, which sum to the curve for the total MVC molecule. These plots are just two different ways of displaying the same data, and are able to show that the total molecule can be broken down into the MGF and HyA components by the conjugate analysis method. This allows for separate characterization of the MVC components, and thereby determination of the MVC valency or final conjugation ratio. Figure 6.6 shows the contribution from just the peptide component by plotting peptide fraction versus elution time. As can be seen from the plot, the peptide fraction is low at around 15%, which is expected since MGF has a much smaller molecular weight (2,952 Da) than the HyA precursor (500,000 Da).

The final conjugation ratio for the conjugates was determined by dividing the molecular weight of the peptide component by the molecular weight of a single MGF molecule (2,952 Da). This yielded the valency, or number of MGF molecules per HyA backbone chain. The valency value was calculated using both M_n and M_w values. The conjugation efficiency was calculated by comparing these valency values to the original molar feed ratio of 30:1 (MGF:HyA). The ratio of MGF:HyA determined using M_n was 21.3:1 and using M_w was 27.3:1 with conjugation efficiency values of 70.9% and 91.1% respectively. This data is summarized in Table 6.3 and demonstrates that mvMGF could be successfully synthesized using the alternative EMCH-based chemistry with a very high efficiency of the conjugation reaction. Although this alternative chemistry was successful and resulted in a high efficiency of conjugation, a Michael addition reaction was chosen as the primary synthesis method for mvMGF. This was because Michael addition reactions can be carried out at room temperature in neutral pH. Also, the reaction does not require the presence of any additional chemicals, which would need to be dialyzed out of the final product.

6.4.3 Characterization of the Molecular Weight and Polydispersity of Different Heparin Molecules

The molecular weight and polydispersity of three different heparin molecules were characterized using SEC-MALS. These heparin molecules were a low molecular weight heparin (LMWH), a high molecular weight heparin (HMWH), and an unfractionated molecular weight heparin (UMWH). The M_n , M_w , and $\bar{D}$ values measured using SEC-MALS are listed in Table 6.4. These values confirm that there is a significant difference between the molecular weights of the high

and low molecular weight heparin molecules. Additionally, it can be seen from the $\bar{D}$ values shown in the table, that the unfractionated heparin has a much higher polydispersity than the fractionated heparin samples. The $R_{g,z}$ values are not reported for these heparin molecules because they are too small to be measured by MALS. MALS can only measure radius of gyration for particles with an $R_{g,z}$ value above about 10 nm, because below about 10 nm the scattering is entirely incoherent. In order to characterize the radius of these heparin molecules, another characterization technique such as dynamic light scattering would have to be employed. The data shown in Table 6.4 confirmed that the heparin obtained from the supplier was indeed representative of low, high, and unfractionated molecular weight heparin. The actual measured molecular weight values, coupled with the distribution information, provided a thorough characterization of these heparin molecules in order to fully understand their growth factor sequestering behavior in the hydrogel cell culture system.

6.5 Conclusions

The analysis in this chapter further demonstrated the power of SEC-MALS as a characterization technique for macromolecules and MVCs in dilute solutions. SEC-MALS was applied to a variety of different projects in order to provide detailed information about the molecular weight, radius of gyration, polydispersity, and valency of these different macromolecular systems. The information provided through this analysis was key to understanding the behavior of these macromolecules in applications such as multivalent conjugate chemistry, bioactivity, and growth factor sequestering behavior.

6.6 References

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6.7 Tables

Table 6.1

Specific refractive index increments and UV extinction coefficients determined for the different macromolecules and utilized in the SEC-MALS analysis

	Specific Refractive Index Increment, dn/dc (mL g^{-1})	UV Extinction Coefficient, ϵ ($\text{mL mg}^{-1} \text{cm}^{-1}$)
HyA	0.160	0.022 [†]
EMCH-HyA	0.145	0.022 [†]
MGF-Cys	0.106	0.360
LMWH	0.125	0.010
HMWH	0.125	0.005
UMWH	0.136	0.012

[†]The UV extinction coefficient for the EMCH-modified HyA was too low to be measured with the SEC-MALS system, so it was assumed to be the same as that reported in a previous publication⁴ for unmodified HyA.

Table 6.2

Molecular weight, polydispersity, and radius of gyration values measured for 600 kDa HyA filtered using five different techniques.

	M_n (kDa)	M_w (kDa)	Đ	R_{g,z} (nm)
0.22 µm Hand Filtered	532.3 (±19.5)	576.2 (±23.8)	1.08	83.3 (±4.9)
0.22 µm Syringe Pump Filtered	566.3 (±6.6)	600.5 (±13.0)	1.06	83.6 (±1.4)
0.45 µm Hand Filtered	556.0 (±28.1)	591.6 (±25.8)	1.06	82.8 (±2.2)
0.45 µm Syringe Pump Filtered	557.1 (±5.9)	590.3 (±5.5)	1.06	80.8 (±1.9)
Centrifuged	561.7 (±17.8)	602.6 (23.0)	1.07	83.6 (±4.9)

Table 6.3

Summary of the measured data for the mvMGF created using EMCH chemistry, including the total, HyA, and MGF component molecular weights along with their polydispersity values. The conjugation ratios were determined by dividing the MGF per HyA chain molecular weight by the molecular weight of a single MGF molecule (2,952 Da). The conjugation efficiencies were calculated by dividing the final conjugation ratios by the input molar feed ratio of 30:1 (MGF:HyA).

	mvMGF
Total M_n (g/mol)	4.6E5
Total M_w (g/mol)	5.7E5
Total Đ	1.2
$R_{g,z}$ (nm)	87.8
HyA M_n (g/mol)	4.0E5
HyA M_w (g/mol)	4.9E5
HyA Đ	1.2
MGF per HyA Chain M_n (g/mol)	6.3E4
MGF per HyA Chain M_w (g/mol)	8.1E4
MGF per HyA Chain Đ	1.3
MGF:HyA (M_n)	21.3:1
Percent Conjugation Efficiency (M_n)	70.9%
MGF:HyA (M_w)	27.3:1
Percent Conjugation Efficiency (M_w)	91.1%

Table 6.4

Molecular weight and polydispersity measured for each of the different heparin molecules.

	M_n (kDa)	M_w (kDa)	Đ
LMWH	5.3	5.9	1.12
HMWH	9.0	10.4	1.15
UMWH	7.6	11.3	1.48

6.8 Figures

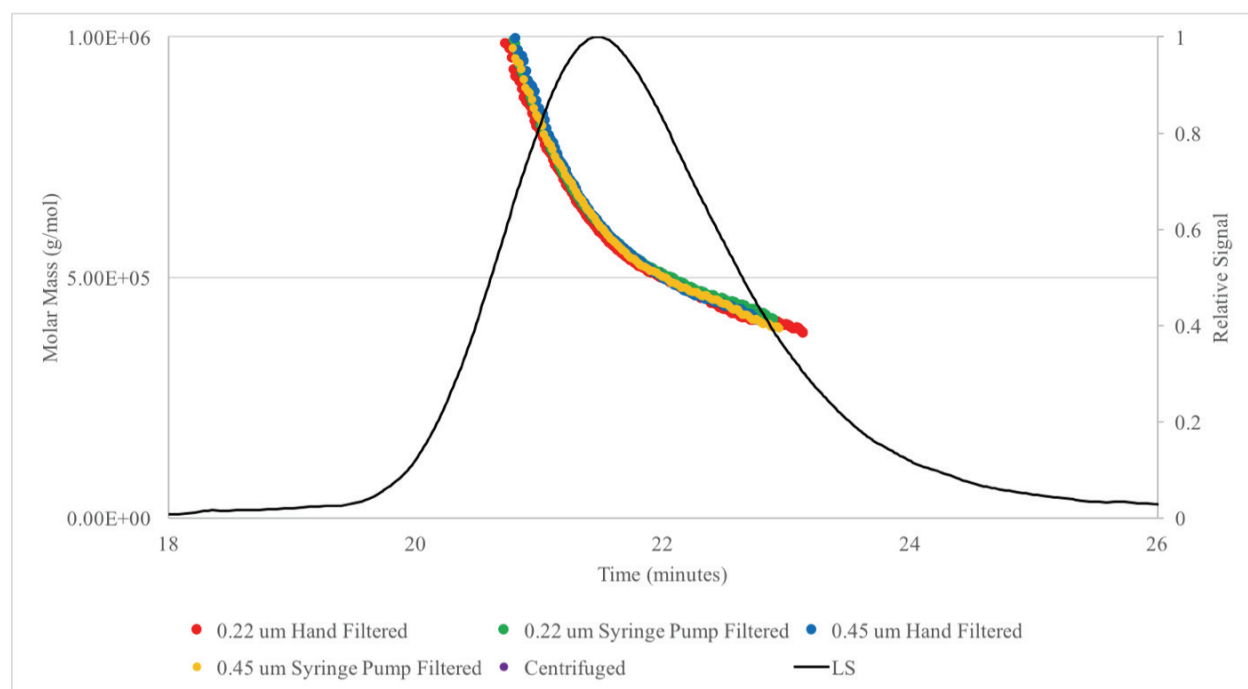


Figure 6.1

Plot of molar mass versus elution time for the HyA samples filtered using five different techniques. The thin solid line is the light scattering chromatogram.

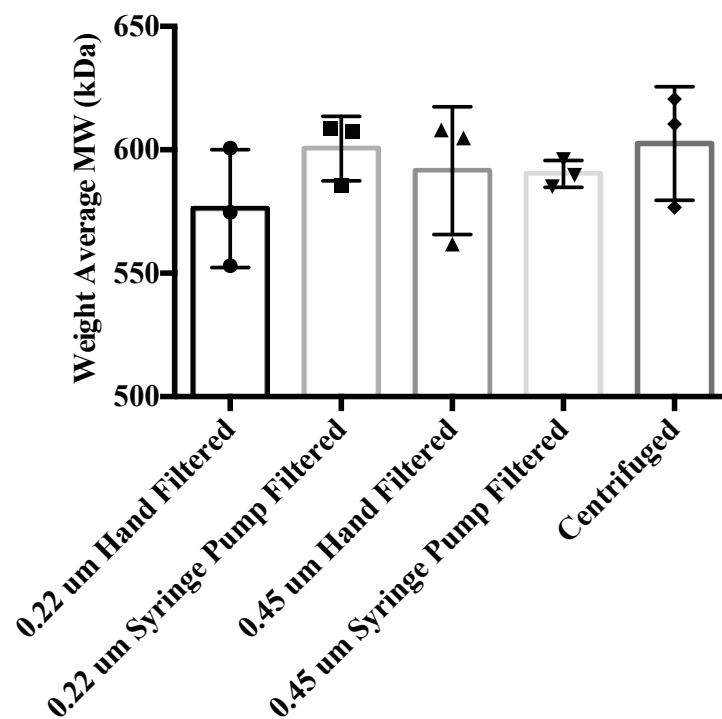


Figure 6.2

Plot of the weight-average molecular weight values measured for each of the HyA samples filtered using a different method.

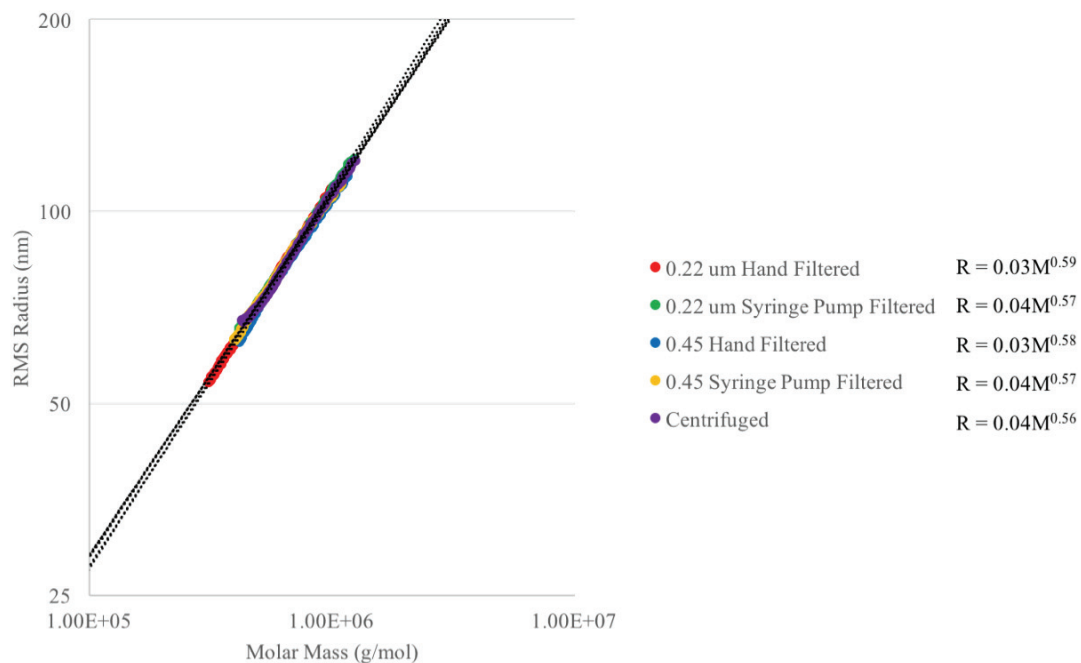


Figure 6.3

Conformation plot for the HyA samples filtered using five different methods. Linear fit lines were applied to obtain equations in the form of $R = kM^b$, where R is the RMS radius, M is the molar mass, and b is the slope.

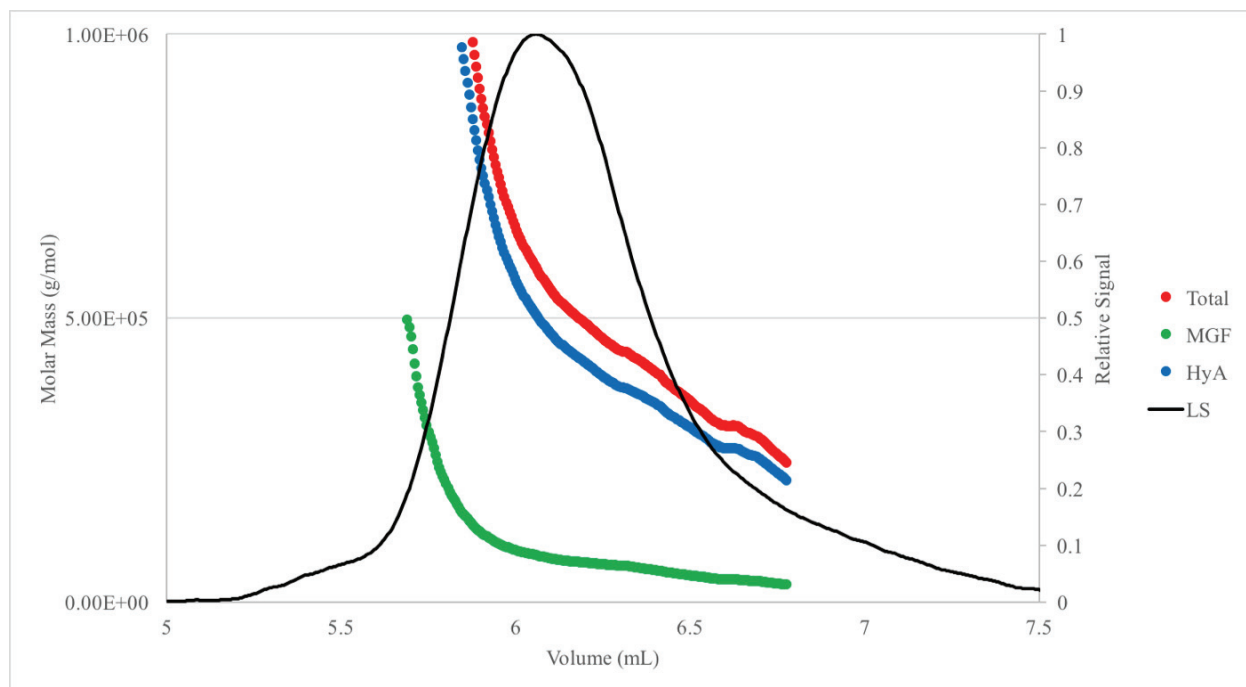


Figure 6.4

Plot of the molar mass versus elution volume overlaid with the LS chromatogram for the mvMGF synthesized using EMCH chemistry. This plot shows the separate contributions from the MGF and HyA components, as well as for the overall conjugate molecule.

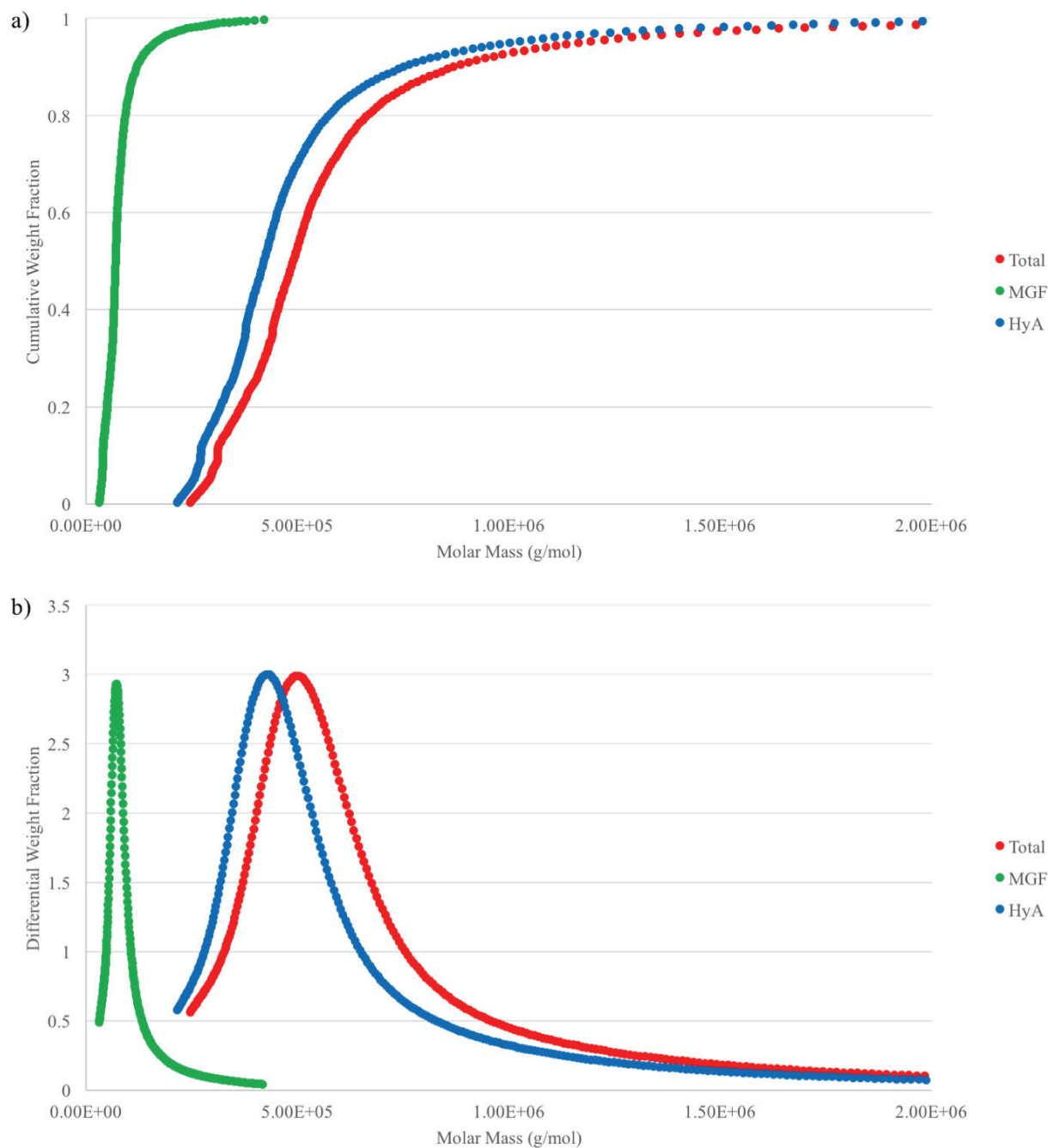


Figure 6.5

(a) Cumulative weight fraction versus molar mass and (b) differential weight fraction versus molar mass for the mvMGF synthesized using EMCH chemistry. These plots show the separate contributions from the MGF and HyA components, as well as for the overall conjugate molecule.

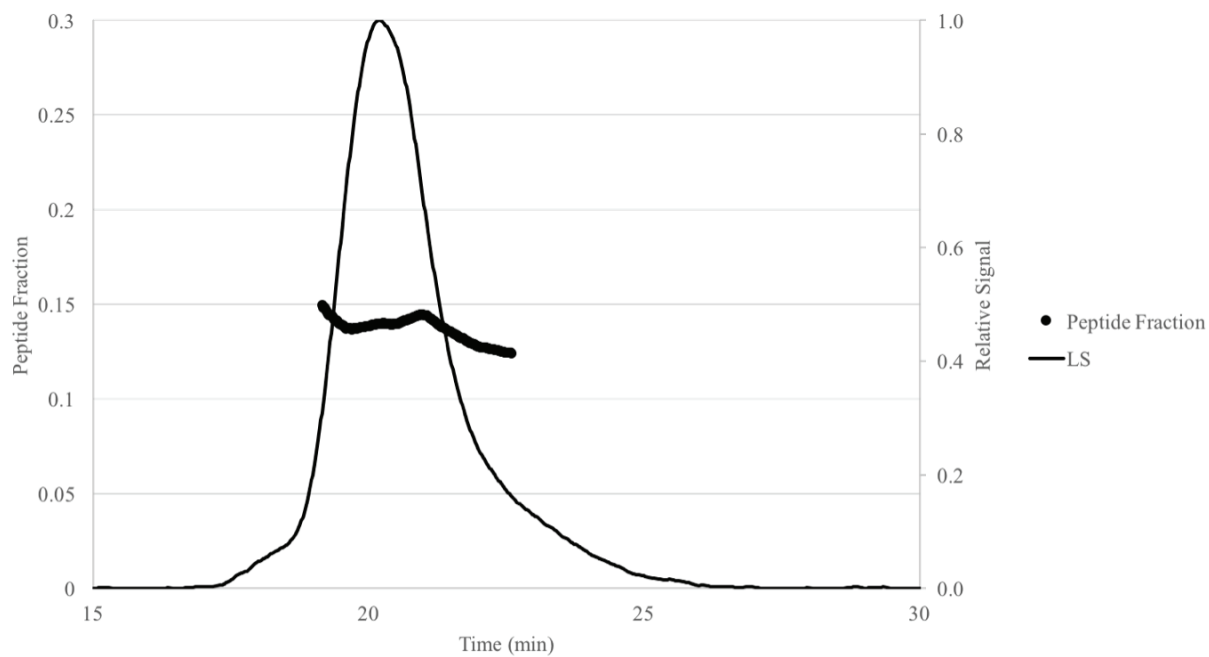


Figure 6.6
Peptide fraction versus elution time overlaid on the LS chromatogram for the mvMGF synthesized using EMCH chemistry.

CHAPTER 7:

DEVELOPMENT OF IN VITRO ASSAYS TO ASSESS THE BIOACTIVITY OF MULTIVALENT MECHANO-GROWTH FACTOR

7.1 Abstract

Mechano-growth factor (MGF) peptide is a promising therapeutic due to its cardioprotective effects. It has been shown to interfere with the caspase cascade to inhibit apoptosis in stressed cardiac cells. Multivalent conjugates (MVCs) of MGF could reduce its proteolytic degradation, improve its pharmacokinetics, and increase its bioactivity. In order to access the bioactivity of the multivalent MGF (mvMGF), it was necessary to develop *in vitro* assays that produce functional impairment of human induced pluripotent stem cell (iPSC)-derived cardiomyocytes (CMs). Two separate assays were developed that resulted in functional impairment of the CMs, which will allow for the *in vitro* assessment of the cardioprotective ability of the mvMGF. The first assay used hypoxia to mimic the ischemic environment of the cardiac tissue during a myocardial infarction (MI), while the second assay stressed CMs through cryopreservation and subsequent thawing. Some preliminary studies show promising data that mvMGF treatment may help recover function in stressed CMs, but further studies are required to confirm these findings.

7.2 Introduction

As was discussed in Chapter 3, it was desirable to synthesize MVCs of MGF peptide since it exhibits promising cardioprotective effects. MGF is a 24-amino acid peptide that is derived from a splice variant of the protein insulin-like growth factor-1 (IGF-1). Recent studies have shown that MGF expression is temporally regulated in cardiac tissue in response to ischemia. MGF is upregulated within an hour post-MI and remains upregulated for up to 8 weeks.^{1,2} This suggests that it may play an important role in mitigating cardiac tissue damage. Studies of stressed cardiac cells treated with MGF peptide demonstrated that MGF inhibits the intrinsic apoptosis pathway through interference with the caspase cascade. Caspase-3 activity was shown to be significantly reduced in MGF-treated cells compared to controls.³ Treatment with MGF immediately post-MI in mice and sheep models improved preservation of cardiac function and reduced apoptosis of cardiac cells at the infarct.^{3,4} Additionally, MGF has been shown to stimulate the migration and proliferation of mesenchymal stem cells^{5,6} and to stimulate pro-angiogenic activity of vascular endothelial cells⁷, which may play a role in repairing damaged cardiac tissue. For these reasons, it is of interest to study MGF as a potential therapeutic to mitigate tissue damage post-MI, which could help in preventing the development of congestive heart failure (CHF).

During an MI, blood flow to the heart is prevented by a blockage of the coronary artery, which creates an ischemic environment in the cardiac muscle tissue. This leads to cardiac cell death, leaving an area of scar tissue at the infarct site. This scarred area is mechanically compromised, so over time, the damaged area will thin and dilate. This reduces the pumping efficiency of the heart, which results in CHF. The weakened heart is no longer able to pump enough blood to provide oxygen and nutrients to the body's organs.⁸⁻¹⁰ Approximately 5.1 million Americans suffer from CHF, and about half of those people will die within 5 years of being diagnosed with the disease. In 2009, CHF contributed to 1 in 9 deaths in the United States.¹¹ According to the

American Heart Association, CHF cases result in approximately \$32 billion worth of healthcare costs each year in the United States.¹²

In order to investigate the cardioprotective benefits of mvMGF, it was necessary to develop an *in vitro* assay to mimic the ischemic environment of the cardiac tissue during an MI. It was decided that subjecting *in vitro* cultures of CMs to hypoxia would be an effective mimic of ischemia for these studies. Different hypoxia assays have been successfully used to study cardiac cell behavior in numerous previous publications.¹³⁻¹⁷ For the development of this hypoxia assay, it was desirable to use adult, human CMs, since these cells make up the cardiac muscle tissue that is affected by ischemia during an MI. However, it is difficult to obtain human CMs, and they are a non-proliferative cell type that cannot be maintained in long term culture. For this assay, CMs were differentiated from human iPSCs using a directed differentiation protocol that has been well established.¹⁸⁻²² This directed differentiation protocol is shown in Figure 7.1.

Based on the previous studies of MGF peptide, the peptide seems to protect cells that are undergoing stressful conditions. Therefore, in addition to developing a hypoxia assay, it was of interest to investigate the effects of mvMGF on cells undergoing another type of stress. Cryopreservation is a crucial step in developing tissue engineering strategies using iPSC-derived CMs, because these cells must be able to be reliably stored and transported before use. Cryopreservation is a harsh process that induces stress in the cells, which could lead to cell death or impairment of cell function after thawing. Therefore, a significant amount of research focuses on effective methods for cryopreserving CMs.²³⁻²⁶ To develop a secondary assay for assessment of the cardioprotective effects of mvMGF, iPSC-derived CMs were cryogenically frozen in the presence of mvMGF and then assessed post-thaw to determine the effect on cell viability and function.

7.3 Materials and Methods

7.3.1 Assessment of iPSC-Derived CM Attachment on Different Culture Substrates

Before developing the *in vitro* assays, it was first desirable to determine the best culture substrate for the iPSC-derived CMs. hESC-qualified Matrigel (Corning), Synthemax II-SC substrate (Corning), and collagen I (Devro) were each used to coat tissue culture polystyrene cell culture plates. iPSC-derived CMs were plated on the coated dishes at 312,500 cells/cm² in RPMI 1640 medium (Gibco) plus B27 supplement (Gibco) and 1 μ L/mL Y-27632 (ROCK inhibitor) (Stem Cell Technologies). Cells were given 3.5 hours to attach to the plates, and then the plates were rinsed gently with PBS and frozen at -80 °C overnight. The number of cells attached to the different culture surfaces was assessed using CyQuant Assay (Thermo Fisher Scientific).

7.3.2 Hypoxia Exposure of iPSC-Derived CMs

iPSC-derived CMs were plated onto Matrigel coated cell culture plates at a density of 31,250 cells/cm². The cells were given 3 to 4 days to recover spontaneous beating before the assay, and were fed once per day with RPMI 1640 medium (Gibco) plus B27 supplement (Gibco). The day before the assay, RPMI 1640 medium minus glucose (Gibco) plus B27 supplement was placed in the Biospherix C-Chamber hypoxia chamber set to 0.1% oxygen and 5% carbon dioxide to

remove the oxygen from the media. To begin the assay, the media in the cell culture plates was replaced with the de-oxygenated media, and then the plates were placed in the Biospherix C-Chamber hypoxia chamber. The hypoxia chamber was maintained at 0.1% oxygen, 5% carbon dioxide, and 37 °C for 22 hours. During this time, control cells in RPMI 1640 medium (containing glucose) plus B27 supplement were maintained at 37 °C in the incubator under normoxia. After 22 hours, the cells were removed from the hypoxia chamber and assessed for functional impairment and viability.

7.3.3 Motion Tracking Analysis of Beating CMs to Measure Functional Impairment Following Hypoxia Exposure

Prior to the hypoxia assay, 10 second videos were recorded of the robust beating of the CMs in both the control and experimental plates. Immediately following the 22-hour hypoxia exposure, 10 second videos were recorded again. The next day, the cells were fed with RPMI 1640 medium plus B27 supplement and then allowed to recover for several hours before recording another set of 10 second videos. All of the videos were analyzed for beat rate and contraction velocity using motion tracking software.²⁷

7.3.4 Alamar Blue Assay to Assess CM Viability Following Hypoxia Exposure

Immediately following the hypoxia assay, the cell culture medium was replaced with RPMI 1640 medium plus B27 supplement and 10% Alamar Blue (Thermo Fisher Scientific). The cells were incubated at 37 °C in the Alamar Blue media for 4.5 hours, and then the fluorescence was measured on a Molecular Devices SpectraMax i3x plate reader at a wavelength of 570 nm for excitation and 610 nm for emission.

7.3.5 Assessment of mvMGF-Treated CM Function and Viability Following Cryopreservation

iPSC-derived CMs were cryogenically frozen in freezing medium containing either unconjugated MGF, 8:1 mvMGF, 15:1 mvMGF, or 30:1 mvMGF at a concentration of 1 μ M. Vials of control cells were frozen without any MGF treatment. The cell vials were thawed after approximately one month in cryogenic storage. Some of the cells were resuspended in fresh culture medium and stained with LIVE/DEAD cell viability assay (Thermo Fisher Scientific). The stained cells were run on a Logos Biosystems LUNA automated cell counter to count the number of live and dead cells. This cell count was used to assess viability of the thawed CMs. The rest of the unstained CMs were plated at a density of 15,800 cells/cm² on Matrigel in RPMI 1640 medium plus B27 supplement and Y-27632. After 24 hours, 30 second videos were recorded of the beating CMs and were analyzed using motion tracking software.²⁷ Then the plates were rinsed with PBS, and the cells were fixed with paraformaldehyde for 10 minutes. The cells were stained with DAPI (nuclear stain), and then a Molecular Devices ImageXpress Micro automated cell imager was used to take fluorescent images of 25 different sites per well. The images were analyzed using MetaExpress software to obtain a total cell count per well, which was used to assess cell attachment.

7.4 Results and Discussion

7.4.1 Assessment of iPSC-Derived CM Attachment on Different Culture Substrates

As part of the assay development, it was desirable to determine the best culture substrate for the iPSC-derived CMs used in these studies. Three different common culture substrates were tested for CM attachment: Matrigel, Synthamax II, and collagen I. Figure 7.2 shows the fraction of CMs attached for each of the substrates. The data was normalized to the Matrigel substrate since it is the current gold standard for iPSC culture. As can be seen from the data, the Matrigel substrate had a greater amount of CM attachment than the other two substrates. The Synthamax II substrate had about 50% of the attachment of Matrigel, while the collagen I substrate had about 30% of the attachment of Matrigel. Based on these results, Matrigel was used as the culture substrate for all of the following experiments.

7.4.2 Assessment of CM Function and Viability Following Hypoxia Exposure

The videos that were recorded prior to, immediately after, and 24 hours after the hypoxia assay were analyzed using a motion tracking software. This analysis yielded the beating traces of contraction velocity versus time that are shown in Figure 7.3. It can be seen from these traces that prior to hypoxia treatment, the CMs were beating regularly. However, after hypoxia exposure, the CMs ceased beating, which implies that they were functionally impaired by the oxygen deprivation. The CMs were given 24 hours to observe if they would recover any of their function, but as can be seen from Figure 7.3c, there was no recovery of beating. The normoxia control CMs maintained regular beating throughout the duration of the assay. The videos were further analyzed to yield the beat rate in beats per minute (bpm) and the maximum contraction velocity of the CMs. A summary of this data is shown in Table 7.1. Prior to the assay, the CMs were beating at around 30 bpm with a maximum contraction velocity of around 18 $\mu\text{m}/\text{second}$. After oxygen deprivation, the CMs ceased beating, so that there was no measurable beat rate or contraction velocity. These results indicate that a measurable functional impairment of the CMs occurs as a result of the hypoxia assay. This will allow for the assessment of mvMGF as a treatment for functional recovery.

Additionally, the oxygen deprived CMs were assessed for metabolic function and viability using Alamar Blue immediately following the hypoxia assay. Alamar Blue is processed by metabolically active cells to produce a fluorescent byproduct. Therefore, cells that are more metabolically active, or viable, will produce a higher fluorescence reading than cells that are less viable. As can be seen from Figure 7.4, the CMs that were exposed to hypoxia were significantly less viable than the normoxia control CMs. The oxygen deprived CMs had only about 57% of the metabolic activity of the normoxia control CMs. This significant decrease in CM viability post-hypoxia will allow for the use of Alamar Blue as an additional assay to assess the cardioprotective effects of mvMGF.

7.4.3 Assessment of mvMGF-Treated CM Function and Viability Following Cryopreservation

iPSC-derived CMs that were cryogenically frozen with either unconjugated MGF, 8:1 mvMGF, 15:1 mvMGF, or 30:1 mvMGF were assessed for their viability and attachment post-thawing, as

compared to control CMs that were frozen with no MGF treatment. Figure 7.5 shows the data for CM viability and CM attachment to a Matrigel-coated dish after thawing from cryopreservation. The data shows that there is no significant difference between the cell viability, or fraction of live cells, between the control and the different treatment conditions. However, there is improvement of cell attachment for the unconjugated MGF and higher valency mvMGF treatments. This data implies that MGF may not inhibit cell death from cryopreservation, but it may be a useful additive to the cryopreservation medium to preserve cell function post-thaw. To further investigate this hypothesis, videos of the thawed CMs were recorded to compare their functional recovery of spontaneous beating. Figure 7.6 shows that the mvMGF treatment resulted in higher contraction velocity of beating compared to the untreated CMs. This data is only preliminary, so further studies are required to fully assess mvMGF as a treatment to improve functional recovery of CMs following cryopreservation.

7.5 Conclusions

This chapter has discussed the development of two different assays for assessing the bioactivity of mvMGF. The first assay used hypoxia to mimic the ischemic environment of the cardiac tissue during an MI, while the second assay stressed CMs through cryopreservation and subsequent thawing. Both assays resulted in functional impairment of the CMs, which will allow for the *in vitro* assessment of the cardioprotective ability of the mvMGF. Some preliminary studies show promising data that mvMGF treatment may help recover function in stressed CMs, but further studies are required to confirm these findings. This will be discussed more in the future directions section of the following chapter.

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7.7 Tables

Table 7.1

Beat rate and maximum contraction velocity data obtained from motion tracking analysis of videos of the normoxia control and hypoxia-exposed CMs.

		Prior to Assay	Immediately Post-Assay	24 Hours Post-Assay
Normoxia Control	Beat Rate (bpm)	31.4 (± 2.7)	38.6 (± 3.3)	30.4 (± 3.3)
	Max Contraction Velocity ($\mu\text{m/s}$)	18.8 (± 7.2)	32.8 (± 4.0)	35.5 (± 2.1)
Hypoxia Exposure	Beat Rate (bpm)	32.9 (± 1.8)	0 (± 0)	0 (± 0)
	Max Contraction Velocity ($\mu\text{m/s}$)	18.3 (± 2.1)	0 (± 0)	0 (± 0)

7.8 Figures

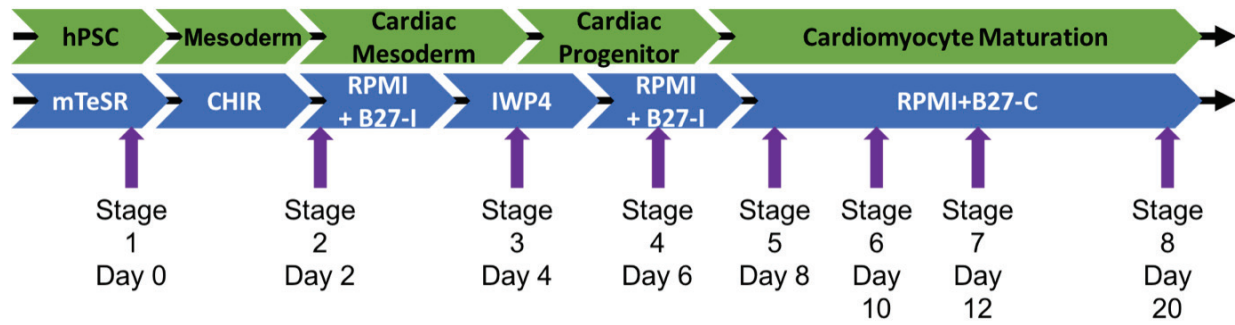


Figure 7.1

Schematic of the directed differentiation protocol for the iPSC-derived CMs. CHIR stands for CHIR99021, which is a WNT pathway activator and a GSK3 inhibitor. Inhibitor of WNT production-4 (IWP-4) inhibits the WNT pathway by inhibiting Porcupine.

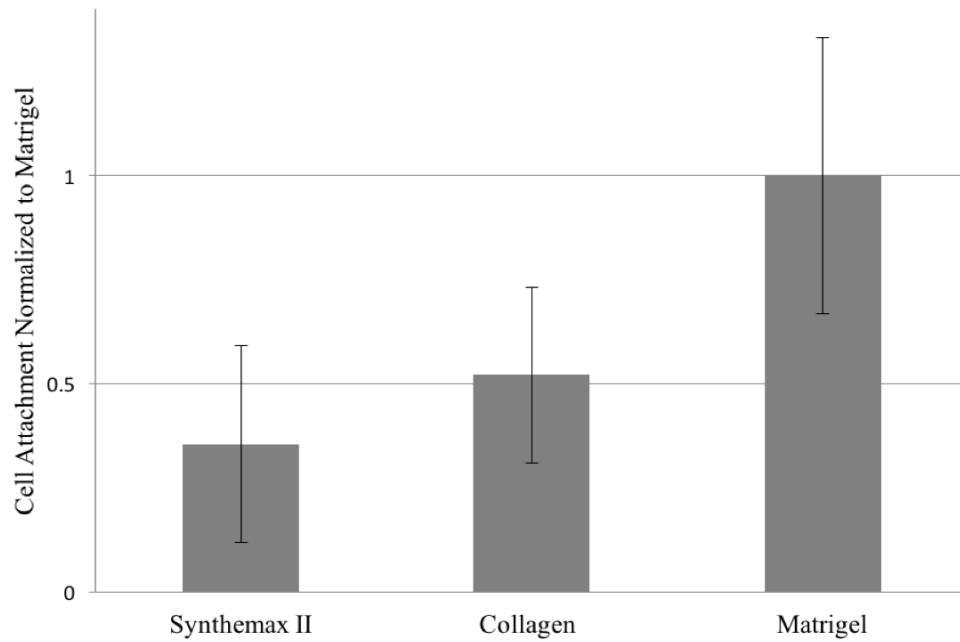


Figure 7.2

Comparison of iPSC-derived CM attachment on different culture substrates. Cell attachment was normalized to attachment on Matrigel.

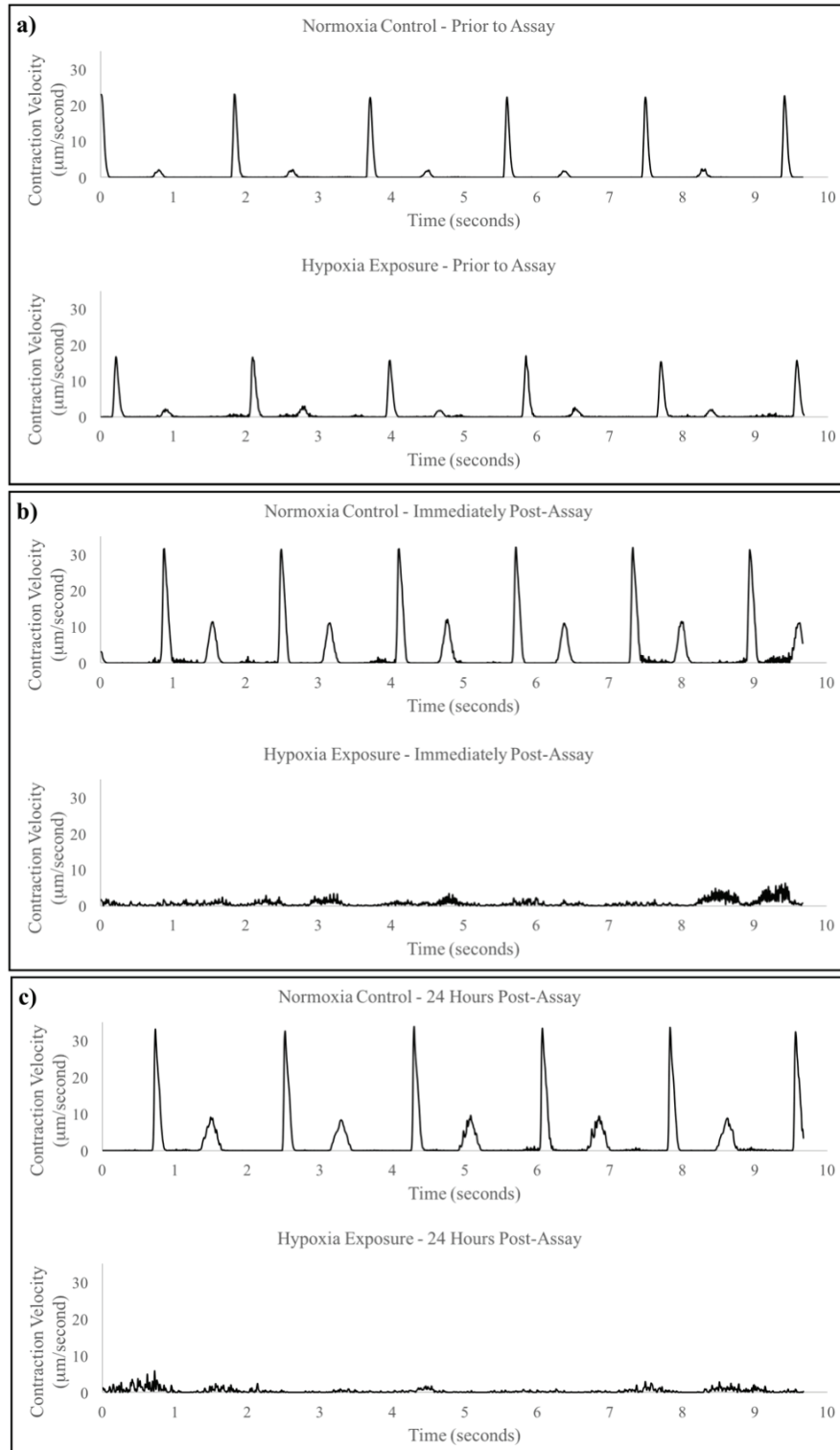


Figure 7.3

Beating traces for the normoxia control and hypoxia-exposed CMs (a) before, (b) immediately after, and (c) 24 hours after the hypoxia assay. Traces were obtained using motion tracking analysis of the cell videos.

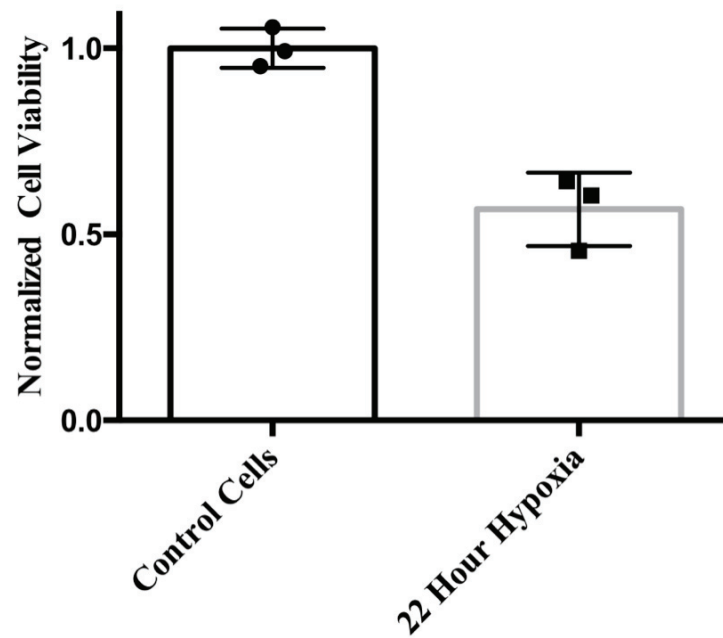


Figure 7.4

Assessment of CM viability post-hypoxia using an Alamar Blue assay. The cell viability was normalized to the normoxia control CMs.

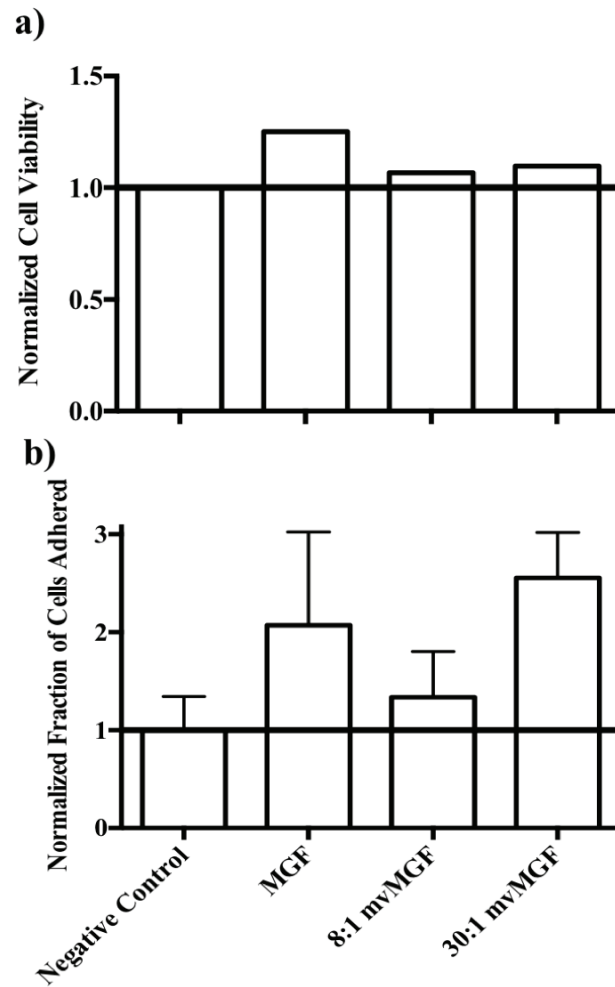


Figure 7.5

(a) Viability and (b) attachment of CMs that were cryogenically frozen with different MGF treatments compared to control cells.

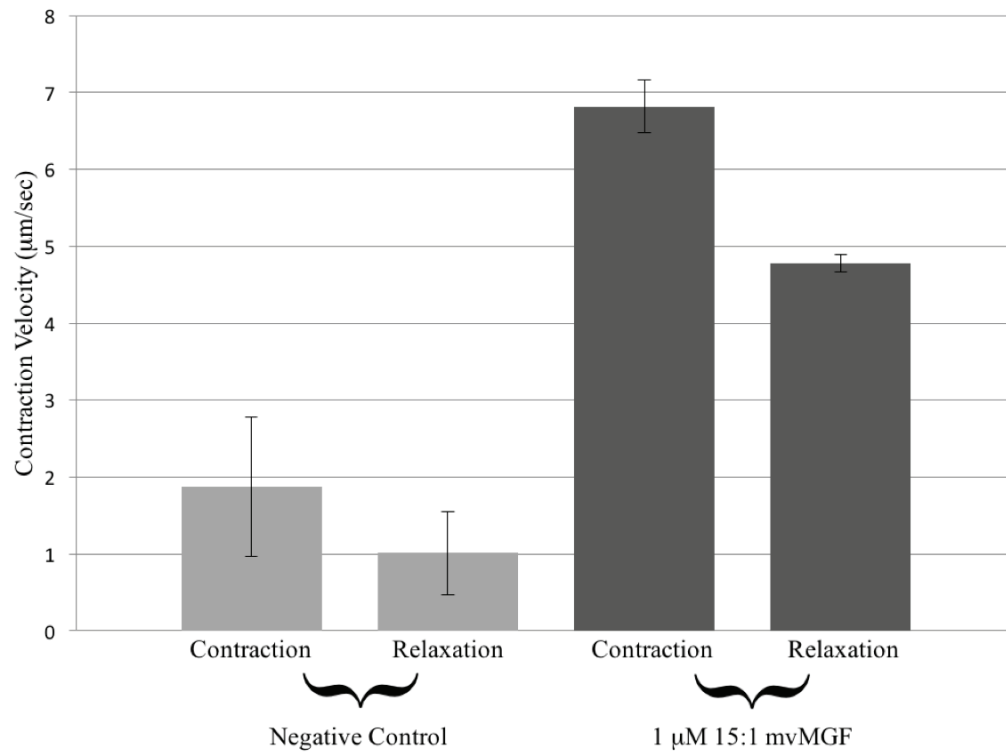


Figure 7.6

Contraction velocity data obtained from motion tracking analysis of videos of CMs that were cryogenically frozen with mvMGF compared to untreated control cells.

CHAPTER 8: CONCLUSIONS AND FUTURE DIRECTIONS

8.1 Conclusions

This dissertation presented the synthesis of a novel multivalent conjugate of mechano-growth factor, applied several different multi-angle light scattering-based characterization methods, and developed *in vitro* assays for the assessment of the conjugate bioactivity.

Chapter 3 presented the development of a reaction method to allow for the multivalent conjugation of MGF peptide to a HyA backbone chain. The conjugation reaction required first the synthesis of a HyA intermediate by the addition of acrylate groups, which were characterized through gelation and NMR spectroscopy. Then the conjugation was achieved by a Michael addition reaction between the acrylate groups on the HyA and the c-terminal cysteine on the MGF peptide. The success of this conjugation reaction was verified through a BCA assay, which also provided an estimate of the final conjugation ratios. The characterization using NMR spectroscopy, acrylated HyA gelation, and BCA was able to confirm the success of conjugation and provide estimates of the peptide concentration and final conjugation ratio.

Chapter 4 provided more thorough characterization of the conjugate molecules through the application of multi-angle light scattering. This analysis utilized an SEC-MALS-UV-RI method, where the inline use of two concentration detectors allowed for the determination of the relative compositions of the MVCs. Using the measured specific refractive index increment and UV extinction coefficient values measured for the two MVC components, it was possible to determine the weight fractions of the MGF and HyA in the total MVC molecule. This analysis confirmed that the bioconjugate chemistry technique used in this work was successful, and that it was possible to determine the total molecular weight, polydispersity, conjugation efficiency, and valency of the MVCs. The SEC-MALS system can be further utilized to assess branching behavior of MVCs, but more in depth branching analysis was not performed for multivalent mvMGF due to the results being so heavily influenced by anchoring in the SEC column.

Chapter 5 presented an alternative SEC-MALS methodology for characterizing MVC molecules. It was first demonstrated that the MVC molecules behave as branched molecules, and then it was shown that branching analysis methods could be successfully applied to the MVCs. Although a linear hyaluronic acid was used as the linear counterpart for the calculations instead of a chemically identical linear counterpart, it was still possible to achieve good agreement between the values calculated by branching analysis and multivalent conjugate analysis.

Chapter 6 further demonstrated the power of SEC-MALS as a characterization technique for macromolecules and MVCs in dilute solutions. SEC-MALS was applied to a variety of different projects in order to provide detailed information about the molecular weight, radius of gyration, polydispersity, and valency of these different macromolecular systems.

Chapter 7 developed two different assays for assessing the bioactivity of mvMGF. The first assay used hypoxia to mimic the ischemic environment of the cardiac tissue during an MI, while the second assay stressed CMs through cryopreservation and subsequent thawing. Both assays

resulted in functional impairment of the CMs, which will allow for the *in vitro* assessment of the cardioprotective ability of the mvMGF. Some preliminary studies showed promising data that mvMGF treatment might help recover function in stressed CMs, but further studies are required to confirm these findings.

8.2 Future Directions

These conclusions still leave some questions unanswered. The future directions of this research should be aimed at answering these questions:

1. What are the limitations of multi-angle light scattering branching analysis as it applies to multivalent conjugate characterization?

As was presented in Chapter 4, branching analysis was unable to be applied to the multivalent conjugates of MGF. However, branching analysis could be successfully applied to characterize multivalent sFlt in Chapter 5. Therefore, the limitations of branching analysis need to be studied and defined, so that it is possible to determine when branching analysis is able to be applied to multivalent conjugates. The abnormal SEC elution behavior of the multivalent MGF was too profound to allow for analysis, so other methods of separation need to be investigated. Field flow fractionation is another separation technique that can be coupled with MALS. Since it does not use a column for separation, it eliminates the issue of anchoring. Additionally, the minimum size cut-off for branching analysis of the conjugated growth factor “branches” should be investigated. Is a peptide like MGF too small to be recognized as a branch in branching analysis?

2. Can multivalent conjugates of mechano-growth factor facilitate functional recovery of stressed cardiomyocytes?

The *in vitro* assays that were developed in Chapter 7 need to be used to study the effects of mvMGF used to treat stressed cardiomyocytes. Hypoxia assays need to be performed comparing functional recovery of the cardiomyocytes between negative control groups, unconjugated MGF treatment, and mvMGF treatment. In addition to the Alamar Blue assay and motion tracking to determine functional recovery, a caspase assay should also be used to assess caspase expression levels, since MGF peptide has been shown to inhibit apoptosis through interference with the caspase cascade. The cryopreservation studies should be repeated with the use of motion tracking analysis, the Alamar Blue assay, and a caspase expression assay to more fully assess the functional recovery of cardiomyocytes treated with mvMGF.

3. Can multivalent conjugates of mechano-growth factor improve cardiac function post-myocardial infarction *in vivo*?

In order for the mvMGF to be assessed as a possible therapeutic for improving functional recovery in stressed cardiac cells, *in vivo* animal studies will need to be performed. A starting point for these studies should be the use of a mouse infarct model with injection of the mvMGF into the cardiac muscle near the site of the infarct. Following recovery

from the infarct, the cardiac muscle should be assessed for cell apoptosis and cardiac function.