

5-2018

Development of a Rapid Small-Scale Purification Method for the Quantitation of Heparin-Like Glycosaminoglycans from Cell Culture Media

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**Development of a Rapid Small-Scale Purification Method for the Quantitation of
Heparin-Like Glycosaminoglycans from Cell Culture Media**

An honors thesis presented to the
Department of Biological Sciences
University at Albany
State University of New York
In partial fulfillment of the
Honors Program Requirements

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May 2018

Abstract

Heparin is an anticoagulant medicinally used to inhibit blood clotting. It is commonly administered to patients requiring surgery or kidney dialysis. Presently, it is produced from animal tissues, but a recent contamination crisis pointed to the need for a safer source of the drug. Our project seeks to develop a rapid, inexpensive, high-throughput assay to quantitate the production of heparin and other glycosaminoglycans (GAGs) from cultured mammalian cells. In order to quantitate heparin from cell culture media, a purification method is needed to separate GAGs from interfering constituents in the media. We developed a purification protocol that absorbs Pluronic and most proteins to Sep-Pak C-18 cartridges, followed by ultrafiltration through 5 kDa cutoff Vivaspin centrifugal concentrators. The purified GAGs are then quantitated by a microcarbazole assay.

Acknowledgments

First and foremost, I would like to thank Dr. Susan Sharfstein and Dr. Trent Gemmill for welcoming me to the Sharfstein Lab and the wonderful world of science research. My time at CNSE has truly been one of the most incredible experiences of my entire life. It has been a tremendous honor to work as a member of this lab for these past two years, and I will cherish these memories forever. I hope and pray for a bright and successful future for the Sharfstein Lab.

I would also like to thank past and present members of the Sharfstein Lab who provided a helping hand during my experiments: Pujhitha Ramesh, Vandhana Muralidharan-Chari, and Vijay Tejwani, as well as the National Science Foundation (NSF) for funding this project.

Last, but not least, I would like to thank my parents, Mario and Analynn, and my sister, Hannah, for their never-ending love and support. To God alone be the glory!

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Introduction

Heparin is a highly sulfated glycosaminoglycan (GAG) composed of repeating disaccharides of L-iduronic acid or D-glucuronic acid and N-acetyl-D-glucosamine¹. It is a hydrophilic molecule with the highest negative charge density among all known biological molecules² and contains chains with molecular weights ranging from 6,000 to 30,000 Daltons³.

Heparin is one of the most widely used anticoagulants in the pharmaceutical industry¹. Currently, it is extracted from animal tissues, including porcine intestines and bovine lungs⁴. A health crisis in 2008 from contamination caused by an oversulfated chondroitin sulfate⁵ led to approximately 100 deaths in the United States², pointing to the need for a safer source of heparin.

One goal of the Sharfstein lab is to metabolically engineer Chinese hamster ovary (CHO) cells to produce heparin⁴. CHO cells are capable of producing heparan sulfate (HS), a less sulfated form of heparin, naturally⁴. Since heparin and HS form a family of GAGs that share the same biosynthetic pathway⁶, CHO cells present a possible new source of heparin. As part of this endeavor, a GAG purification method was previously established to isolate and concentrate GAGs⁴, as well as prevent interference from other components of culture media during the subsequent quantitation step using the microcarbazole assay, which detects the concentration of the purified GAGs⁷. Carbazole-based quantification utilizes sodium tetraborate in sulfuric acid to hydrolyze the GAGs into uronic acid and glycosamine⁷. Carbazole subsequently reacts with the uronic acid, yielding a colored product that can be quantitated spectrophotometrically⁷. The current purification protocol is costly both in time and materials⁴. Thus, our project

seeks to develop a rapid, inexpensive, high-throughput assay to purify and quantitate the production of heparin and other glycosaminoglycans (GAGs) from cultured mammalian cells.

There are two key components of cell culture media that interfere with the microcarbazole assay whose characteristics we focus on to build our new protocol, Pluronic and glucose.

First, Pluronic F68 is used in cell culture as an anti-foaming agent and to protect cell membranes from shearing⁸. Pluronic F68 is a poloxamer and contains a hydrophilic poly(ethylene oxide) and hydrophobic poly(propylene oxide)⁹. Thus, to remove Pluronic and other hydrophobic contaminants, we employ liquid chromatography using Waters Sep-Pak C-18 classic cartridges, which utilize a strongly hydrophobic silica-based bonded phase¹⁰.

Second, glucose is added to cell culture systems to act as a primary source of fuel for animal cells, which are heterotrophs who obtain energy from coupled oxidation-reduction reactions¹¹. Glucose is a strongly hydrophilic hexose sugar¹² with a molecular weight of 180 Da. Thus, to remove glucose and other small interfering molecules, we employ ultrafiltration using Sartorius Vivaspin centrifugal concentrators, which utilize a semi-permeable membrane to separate solutions based on size¹³.

Our hypothesis is that the new GAG purification protocol utilizing liquid chromatography and ultrafiltration can achieve its purpose of heparin and GAG isolation while greatly reducing the time and cost required to run the experiments.

Materials & Methods

Sodium heparin (lot #SLBL6351V) was from Sigma-Aldrich. Sep-Pak C-18 classic cartridges (WAT051910) were from Waters. Vivaspin 6 (3 kDa cutoff) (lot #1703005VS) and Vivaspin 15R (5 kDa cutoff) (lot #1701014VS) centrifugal concentrators were from Sartorius. A Thermo Scientific 8 x 50 mL fixed angle rotor was used for all centrifugations.

A. Microcarbazole Assay¹⁴

To quantitate the concentration of heparin, the colorimetric microcarbazole assay was employed. Two separate revisions of the microcarbazole assay were used.

Revised Microcarbazole Assay #1

A revised assay was developed to reduce the time required by combining the two heating steps and separate addition of reagents into one. Additionally, sulfamide was used to react with some of the byproducts that give false color. Twenty five- μ L samples were added to a Thermo Scientific PCR plate. 2.5- μ L of 4M sulfamide was added, as well as 175 μ L of reagent A (2-mL of 1.25 M sodium tetraborate decahydrate in hot water and 98-mL of 99% sulfuric acid) to hydrolyze and dehydrate the uronic acid of heparin into furans, and 5 μ L of reagent B (1.25 g/L carbazole in absolute ethanol) to react the furans with carbazole to form a colored compound. The plate was covered with a plastic film and heated in the thermal cycler at 100°C for 15 minutes to enable the hydrolysis and carbazole reactions, followed by chilling to 4°C. 150 μ L of each sample was transferred to a Costar 96-well plate and the absorbance was measured at 525 nm using a Fisher

Scientific microplate reader (instrument: Infinite 200, equipment number: 10294608) and Tecan i-control application.

Heparin Standard Curve Using Revised Microcarbazole Assay #1

To test the effectiveness of the revised microcarbazole assay #1, a 1 mg/mL heparin solution was used to create a standard curve. The sample concentrations used were 0, 0.5, 5, 10, and 20 $\mu\text{g}/25 \mu\text{L}$ sample.

Revised Microcarbazole Assay #2

A second revised assay using a lower reaction temperature was developed to reduce the effects of a high temperature, such as charring that produces false color. Additionally, to reduce the propagation of pipetting errors, an Eppendorf Repeater M4 Manual Handheld Pipette Dispenser was used to add all reagents (using 0.1 mL pipette tips for sulfamide and reagent B and 5 mL tips for reagent A), and the 25- μL samples were added directly to the 96-well plate. The repeater pipette was used to add 2- μL of 4M sulfamic acid, 5 μL of reagent B, and 150 μL of reagent A. The plate was then incubated on a hot plate at 60°C for 1 hour (a study of different heating times was conducted and 1 hour was chosen as optimal). The absorbance was measured at 525 nm using a Fisher Scientific microplate reader and Tecan i-control application.

Heparin Standard Curve Using Revised Microcarbazole Assay #2

To test the effectiveness of revised microcarbazole assay #2, a 1 mg/mL heparin solution was used to create a standard curve. The sample concentrations used were 0, 2, 2.5, 5, 10, 15, 20, and 25 µg/25 µL sample.

Heparin Standard Curve with Repeater Pipette Using Revised Microcarbazole Assay #2

To increase the accuracy of the standard curve, a repeater pipette was used to create the standard curve using a 1 mg/mL heparin solution. The sample concentrations used were 0, 5, 10, 15, 20, 22, and 24 µg/25 µL sample.

B. Liquid Chromatography Using Waters Sep-Pak C-18 Classic Cartridges

To remove Pluronic F68 in solution, liquid chromatography experiments were performed using Waters Sep-Pak C-18 classic cartridges.

Using Sep-Pak C-18 Classic Cartridges

The Sep-Pak C-18 cartridge was washed using a syringe with 5 mL of ethanol and 2 x 5 mL of water. The sample was then passed through the cartridge, followed by one 1-mL water wash.

Removing Pluronic in Solution

Two-mL of a 10 mg/mL solution of Sigma-Aldrich Pluronic F68 was passed through one Sep-Pak C-18 cartridge prepared as described above (but followed by two water washes to maximize recovery) with the objective of retaining the Pluronic in the column. The

flow-through and washes were placed in a pre-weighed 15 mL conical centrifuge tube, frozen, lyophilized, and reweighed to determine how much Pluronic did not adsorb to the C-18 resin.

C. Ultrafiltration Using Sartorius Vivaspin 6 Centrifugal Concentrators

To test the ability of the Vivaspin centrifugal concentrators to retain heparin and remove glucose from the solution, ultrafiltration experiments were conducted using Sartorius Vivaspin 6 centrifugal concentrators.

Retaining Heparin in Solution

Six-mL of a 2 mg/mL heparin solution was added to a Vivaspin 6 centrifugal concentrator and two 25- μ L samples were reserved. The filter was centrifuged at 10,000 x g at 18°C for 45 minutes and two 25- μ L samples of the permeate were reserved. Six mL of water was added to the retentate and two 25- μ L samples were reserved. The centrifugal concentrator was then centrifuged once more and the samples reserved as before. All samples were transferred into a PCR plate and analyzed by the revised microcarbazole assay #1.

Removing Glucose in Solution

Six-mL of a 0.1% glucose solution was added to a Vivaspin 6 centrifugal concentrator and two 25- μ L samples were reserved. The filter was centrifuged at 10,000 x g at 18°C for 45 minutes and two 25- μ L samples of the permeate were reserved. Six mL of water was added to the retentate before two 25- μ L samples were reserved. The centrifugal

concentrator was then centrifuged once more and the samples reserved as before. All samples were transferred into a PCR plate and analyzed by the revised microcarbazole assay #1.

D. GAG Purification Experiments Using Ultrafiltration with Sartorius Vivaspin 15R Centrifugal Concentrators & Liquid Chromatography with Waters Sep-Pak C-18

Classic Cartridges

To determine if heparin could be isolated in various larger solutions, GAG purification experiments were performed using liquid chromatography with Waters Sep-Pak C-18 classic cartridges and ultrafiltration with Sartorius Vivaspin 15R centrifugal concentrators.

Isolating Heparin from Pluronic and Glucose in Solution

In one conical tube, 4 mL of nanopure water was added to 1-mL of a 2 mg/mL heparin solution. 100 mg of glucose (a higher concentration was used to better see any residual glucose after spins) and 100 mg of Pluronic F68 was added to 10 mL of water, and 4-mL of the Pluronic-glucose solution was added to a second conical tube containing 1-mL of a 2 mg/mL heparin solution. Three 25- μ L samples were reserved from both conical tubes. Both solutions were passed through three Sep-Pak C-18 cartridges prepared as described above, and three 25- μ L samples were reserved from the total eluent. The solutions were then each placed in a Vivaspin 15R centrifugal concentrator and centrifuged at 10,000 x g at 18°C for 45 minutes, and three 25- μ L samples were reserved from the permeate. Ten mL of water was added to the retentate before three 25- μ L samples were taken. The

centrifugal concentrator was then centrifuged twice more and the samples reserved as before. All samples were transferred into a 96-well plate and analyzed by the revised microcarbazole assay #2.

Isolating Heparin from Interfering Components in Fresh Medium

In one conical tube, 4 mL of nanopure water was added to 1-mL of a 2 mg/mL heparin solution. In a second conical tube, 4 mL of medium was added to 1-mL of a 2 mg/mL heparin solution. In a third conical tube, 4 mL of medium was added to 1 mL of nanopure water. Three 25- μ L samples were reserved from each tube. All three solutions were passed through three Sep-Pak C-18 cartridges prepared as described above, and three 25- μ L samples were reserved from the total eluent. The solutions were then each placed in a Vivaspin 15R centrifugal concentrator and centrifuged at 10,000 x g at 18°C for 45 minutes, and three 25- μ L samples were reserved from the permeate. Ten mL of water was added to the retentate before three 25- μ L samples were taken. The centrifugal concentrator was then centrifuged twice more and the samples reserved as before. All samples were transferred into a 96-well plate and analyzed by the revised microcarbazole assay #2.

Recovery of Heparin-Like Glycosaminoglycans from Conditioned Medium Samples

Eight mL of conditioned medium sample (names are specified in Table 1) was added to a conical tube and three 25- μ L samples were reserved. The samples were purified using a Sep-Pak C-18 cartridge, and three 25- μ L samples were reserved. This larger-volume sample was added to a Vivaspin 15R centrifugal concentrator and centrifuged at 10,000 x

g at 18°C (centrifugation times specified in Table 1). The centrifugations were repeated twice more. After each centrifugation, three 25- μ L samples were reserved from the permeate and 10 mL of water added to the retentate (except for the final retentate, which was filled to the volume indicated in Table 1) before three 25- μ L samples were reserved. Upon exploring various centrifugation times, 45 minutes was determined to be optimal when the tip of the membrane was properly oriented towards the center of the centrifuge. All samples were transferred into a 96-well plate and analyzed by the revised microcarbazole assay #2.

Table 1: Specifications of the medium sample name, time for each centrifugation, and amount of water added to the final retentate before samples were taken from experiment of the recovery of heparin-like glycosaminoglycans from conditioned medium samples.

Name of Medium Sample Used	Centrifugation Time (Minutes)	Water Added to Final Retentate (mL)
C5 Epi 11/2/17 3rd fed Batch = 50 mL	75	0.5
C5 Epi 2 mM B 7/13/17	45	10
C5 Epi 4 mM C 7/18/17	110	10

E. Comparing Old GAG Purification Method & New GAG Purification Method

To compare the recovery of heparin-like glycosaminoglycans from conditioned media samples, the old GAG purification method⁴ and new GAG purification method (liquid chromatography using Sep-Pak C-18 classic cartridges and ultrafiltration using Vivaspin 15R centrifugal concentrators) were performed three times each using the same conditioned media sample (combined ChS Excell A 8/20/17 and ChS Hycell B 8/19). Six 8-mL samples of conditioned media were added to separate conical tubes. GAGs from three of the samples were purified using the original GAG purification method⁴,

and the resulting purified GAGs were reserved in separate Eppendorf tubes. The other three samples were purified using the new GAG purification method: solutions were passed through three Sep-Pak C-18 cartridges prepared as described above, and three 25- μ L samples were reserved from the total eluent. The solutions were then each placed in a Vivaspin 15R centrifugal concentrator and centrifuged at 10,000 x g at 18°C for 45 minutes, and three 25- μ L samples were reserved from the permeate. Ten mL of water was added to the retentate before three 25- μ L samples were taken. The centrifugal concentrator was then centrifuged twice more and the samples reserved as before with one exception: the final retentate alone was transferred to an Eppendorf tube, and 100 μ L of water was added to the centrifugal concentrator to recover all product, which was also transferred to the Eppendorf tube. Two 25- μ L samples of all six experiments were transferred into a 96-well plate and analyzed by the revised microcarbazole assay #2.

Results

A. Microcarbazole Assay

Heparin Standard Curve Using Revised Microcarbazole Assay #1

To reduce the amount of time required for the microcarbazole assay and the production of false color, the two heating steps were combined into one and sulfamide was added to prevent false color. The revised microcarbazole assay #1 was performed to detect the concentration of heparin by measuring the absorbance at 525 nm. In Figure 1, the absorbance values of a standard curve that utilizes the revised microcarbazole assay #1 are reported. The revised microcarbazole assay #1 was able to accurately quantitate heparin in solution.

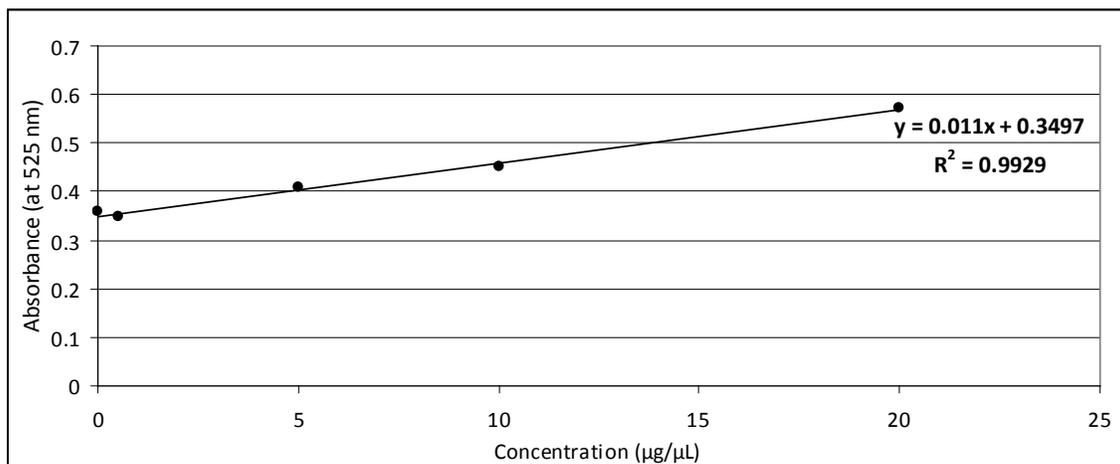


Figure 1: Heparin standard curve using the revised microcarbazole assay #1.

Heparin Standard Curve Using Revised Microcarbazole Assay #2

To reduce the propagation of pipetting errors and the effects of a high temperature, the revised microcarbazole assay #1 was further revised to utilize a repeater pipette to add the

reagents, add the samples directly to a 96-well plate, and perform the assay at a lower temperature (60°) by using a hot plate instead of a thermal cycler to heat the samples. The revised microcarbazole assay #2 was performed in triplicate to detect the concentration of heparin by measuring the absorbance at 525 nm. In Figure 2, the absorbance values of a standard curve that utilizes the revised microcarbazole assay #2 are reported. The revised microcarbazole assay #2 was able to accurately quantitate heparin in solution.

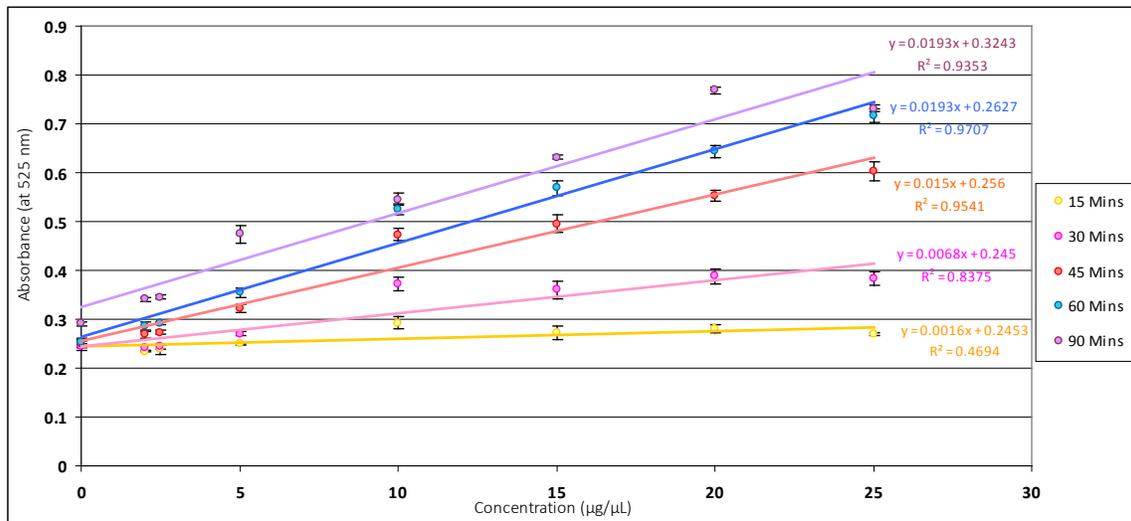


Figure 2: Heparin standard curve using a revised microcarbazole assay #2 at various heating times. The revised microcarbazole assay #2 was performed in triplicate. Error bars represent the standard deviation of triplicate measurements of revised microcarbazole assay #2.

Heparin Standard Curve with Repeater Pipette Using Revised Microcarbazole Assay #2

To increase the accuracy of the standard curve using the revised microcarbazole assay #2, a repeater pipette was used to add the heparin samples. The revised microcarbazole assay #2 was performed in triplicate to detect the concentration of heparin by measuring the

absorbance at 525 nm. In Figure 3, the absorbance values of the standard curve that utilizes a repeater pipette and the revised microcarbazole assay #2 are reported. The repeater pipette was able to improve the quantitation of heparin using the revised microcarbazole assay #2.

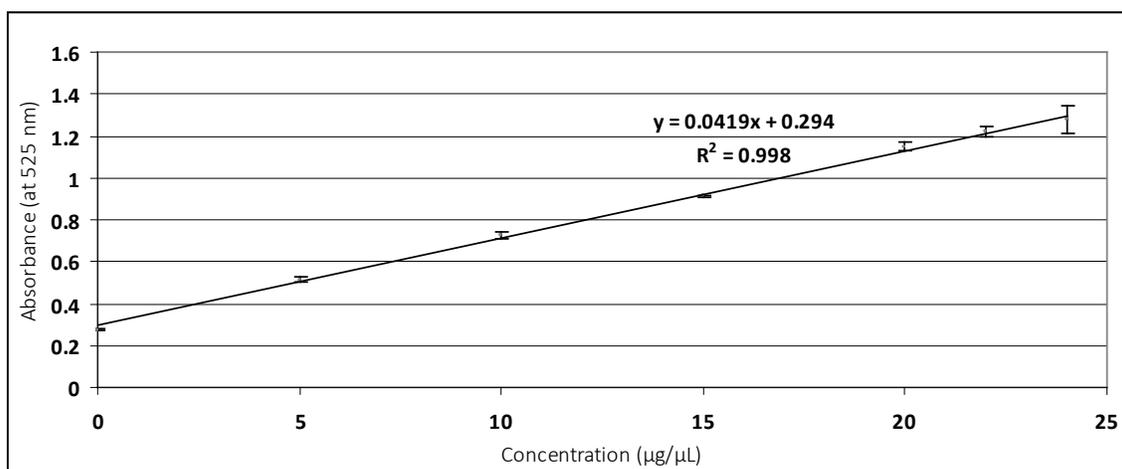


Figure 3: Heparin standard curve with a repeater pipette using the revised microcarbazole assay #2 for 1 hour. The revised microcarbazole assay #2 was performed in triplicate. Error bars represent the standard deviation of triplicate measurements of the revised microcarbazole assay #2.

B. Liquid Chromatography Using Waters Sep-Pak C-18 Classic Cartridges

Removing Pluronic in Solution

To remove Pluronic from water, a liquid chromatography experiment using Sep-Pak C-18 classic cartridges, followed by a lyophilization experiment, were conducted. In Table 2, the mass of the conical tube is reported both before (prior to the addition of Pluronic) and

after the experiment, as well as the mass of Pluronic added to the conical tube. The cartridges removed all of the starting 2-mL of 10 mg/mL Pluronic.

Table 2: Removal of Pluronic in solution during a liquid chromatography experiment using Sep-Pak C-18 classic cartridges and a lyophilization experiment.

Mass Before Liquid Chromatography & Lyophilization (g)	Mass of Pluronic in 2 mL water (mg)	Mass After Liquid Chromatography & Lyophilization (g)	Difference (mg)
6.8653	20	6.8653	0

C. Ultrafiltration Using Sartorius Vivaspin 6 Centrifugal Concentrators

Retaining Heparin in Solution

To determine if heparin could be retained using centrifugal concentrators, heparin in solution was ultrafiltered using Vivaspin 6 centrifugal concentrators. The revised microcarbazole assay #1 was performed in duplicate to detect the concentration of heparin by measuring the absorbance at 525 nm. In Figure 4, concentrations are reported as a percentage of the starting absorbance value of heparin. The filters retained approximately all of the starting 6-mL of 2 mg/mL heparin.

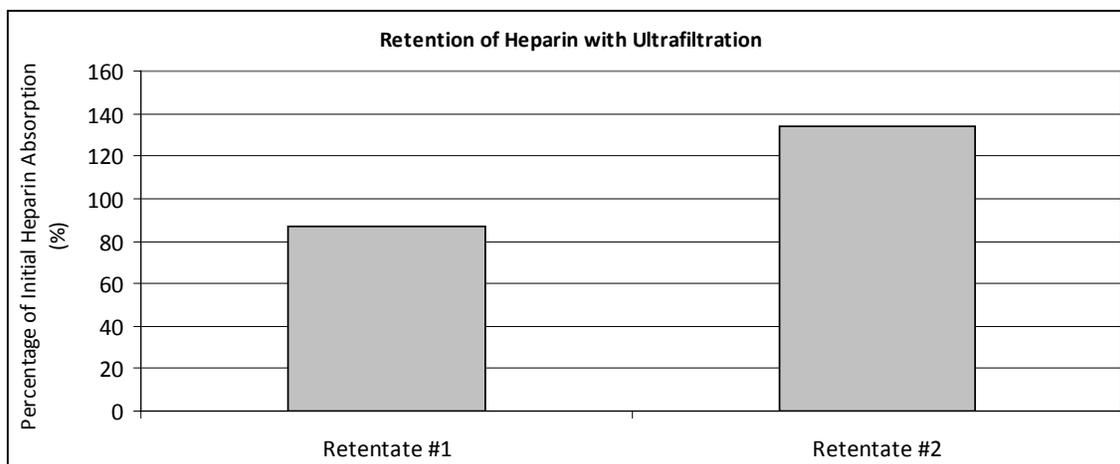


Figure 4: Recovery of heparin from water during each stage of an ultrafiltration experiment using Vivaspin 6 centrifugal concentrators. The revised microcarbazole assay #1 was performed in duplicate.

Removing Glucose in Solution

To determine if glucose could be removed using centrifugal concentrators, glucose in solution was ultrafiltered using Vivaspin 6 centrifugal concentrators. The revised microcarbazole assay #1 was performed in duplicate to detect the concentration of glucose by measuring the absorbance at 525 nm. In Figure 5, concentrations are reported as a percentage of the starting absorbance value of glucose. Although the microcarbazole assay is not quantitative for glucose, the filters appeared to remove approximately 96% of the starting 6-mL of 0.1% glucose.

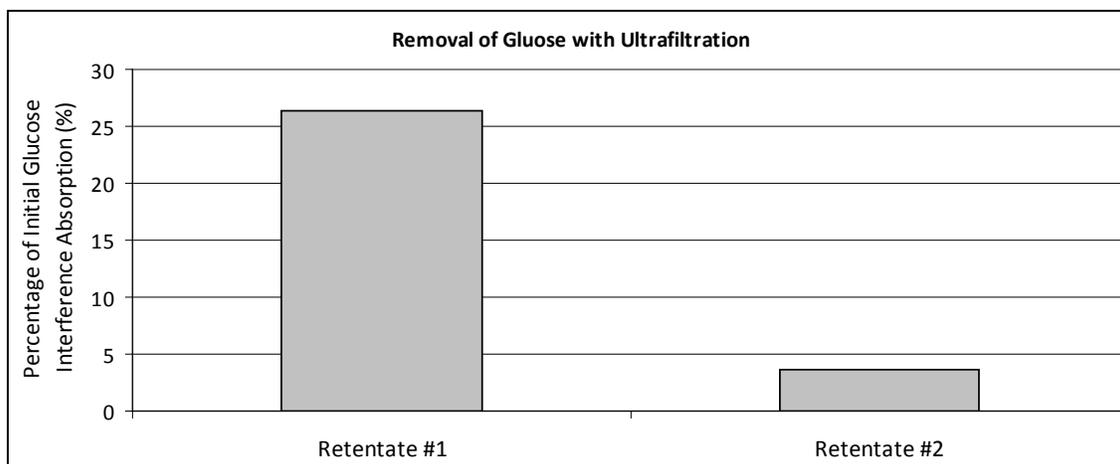


Figure 5: Removal of glucose from water during each stage of an ultrafiltration experiment using Vivaspin 6 centrifugal concentrators. The revised microcarbazole assay #1 was performed in duplicate.

D. GAG Purification Experiments Using Ultrafiltration with Sartorius Vivaspin 15R Centrifugal Concentrators & Liquid Chromatography with Waters Sep-Pak C-18 Classic Cartridges

Isolating Heparin from Pluronic and Glucose in Solution

To isolate heparin from Pluronic and glucose in solution, a GAG purification experiment utilizing liquid chromatography and ultrafiltration was conducted. Two samples (a 5-mL solution of 2 mg/mL of heparin in water and a 5-mL solution of 2 mg/mL of heparin, 10 mg/mL of glucose, and 10 mg/mL of Pluronic) were purified by liquid chromatography using Sep-Pak C-18 cartridges and ultrafiltration using Vivaspin 15R centrifugal concentrators. The revised microcarbazole assay #2 was performed to detect the

concentration of heparin by measuring the absorbance at 525 nm. In Figure 6, concentrations are reported as the absorbance units at 525 nm.

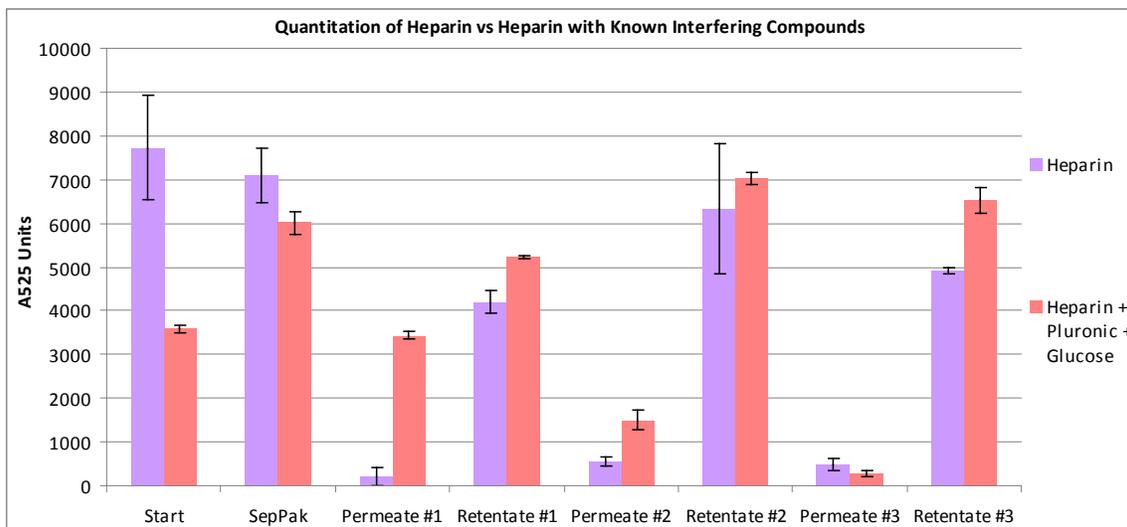


Figure 6: Recovery of heparin from water or known interfering compounds (Pluronic and glucose) during each stage of a GAG purification experiment using liquid chromatography with Sep-Pak C-18 cartridges and ultrafiltration with Vivaspin 15R centrifugal concentrators. Error bars represent the standard deviation of triplicate runs of the GAG purification experiment.

Isolating Heparin from Interfering Components in Fresh Medium

To isolate heparin from interfering components in fresh medium, a GAG purification experiment utilizing liquid chromatography and ultrafiltration was conducted. Three samples (a 5-mL solution of 2 mg/mL of heparin in water, a 5-mL solution of 2 mg/mL of heparin in medium, and a 5-mL solution of 1 mL of medium in water) were purified by liquid chromatography using Sep-Pak C-18 cartridges and ultrafiltration using Vivaspin 15R centrifugal concentrators. The revised microcarbazole assay #2 was performed to

detect the concentration of heparin by measuring the absorbance at 525 nm. In Figure 7, concentrations are reported as the absorbance units at 525 nm.

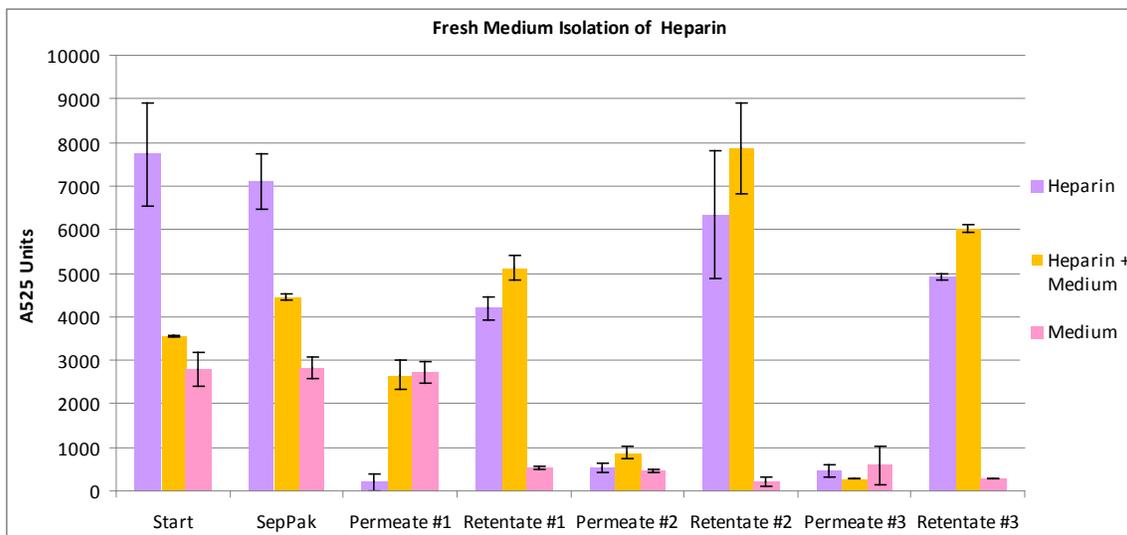


Figure 7: Recovery of heparin from water, heparin from fresh medium, or medium in water during each stage of a GAG purification experiment using liquid chromatography with Sep-Pak C-18 cartridges and ultrafiltration with Vivaspin 15R centrifugal concentrators. Error bars represent the standard deviation of triplicate runs of the GAG purification experiment.

Recovery of Heparin-Like Glycosaminoglycans from Conditioned Medium Sample #1

To determine how much heparin-like glycosaminoglycans could be recovered from conditioned medium sample #1, a GAG purification experiment was conducted using liquid chromatography with Sep-Pak C-18 cartridges and ultrafiltration with Vivaspin 15R centrifugal concentrators. A sample was not taken after the first centrifugation. The revised microcarbazole assay #2 was performed in triplicate to detect the concentration of

heparin by measuring the absorbance at 525 nm. In Figure 8, concentrations are reported as the absorbance units at 525 nm. The amount recovered was 15.9 $\mu\text{g/mL}$.

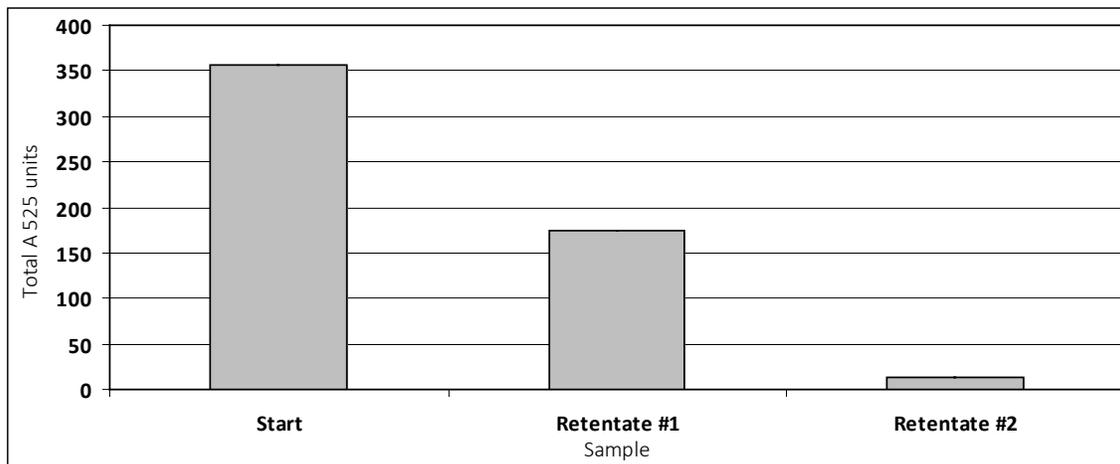


Figure 8: Recovery of heparin-like glycosaminoglycans from conditioned medium sample #1 during each stage of GAG purification experiment using liquid chromatography with Sep-Pak C-18 cartridges and ultrafiltration with Vivaspin 15R centrifugal concentrators. The revised microcarbazole assay #2 was performed in triplicate.

Recovery of Heparin-Like Glycosaminoglycans from Conditioned Medium Sample #2

To determine how much heparin-like glycosaminoglycans could be recovered from conditioned medium sample #2, a GAG purification experiment was conducted using liquid chromatography with Sep-Pak C-18 cartridges and ultrafiltration with Vivaspin 15R centrifugal concentrators. The revised microcarbazole assay #2 was performed in triplicate to detect the concentration of heparin by measuring the absorbance at 525 nm. In Figure 9, concentrations are reported as the absorbance units at 525 nm. The amount recovered was 20.6 $\mu\text{g/mL}$.

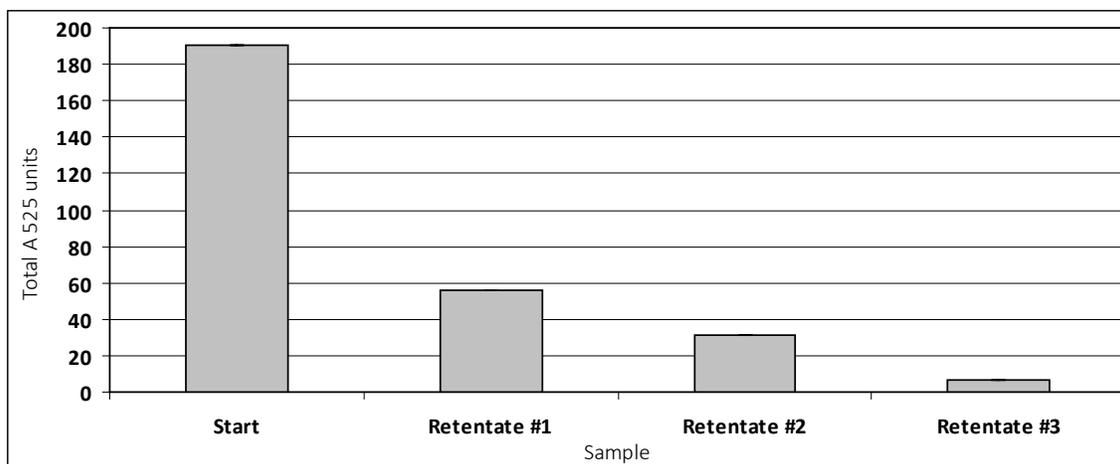


Figure 9: Recovery of heparin-like glycosaminoglycans from conditioned medium sample #2 during each stage of a GAG purification experiment using liquid chromatography with Sep-Pak C-18 cartridges and ultrafiltration with Vivaspin 15R centrifugal concentrators. The revised microcarbazole assay #2 was performed in triplicate.

Recovery of Heparin-Like Glycosaminoglycans from Conditioned Medium Sample #3

To determine how much heparin-like glycosaminoglycans could be recovered from conditioned medium sample #3, a GAG purification experiment was conducted using liquid chromatography with Sep-Pak C-18 cartridges and ultrafiltration with Vivaspin 15R centrifugal concentrators. The revised microcarbazole assay #2 was performed in triplicate to detect the concentration of heparin by measuring the absorbance at 525 nm. In Figure 10, concentrations are reported as the absorbance units at 525 nm. The amount recovered was 10.0 $\mu\text{g/mL}$.

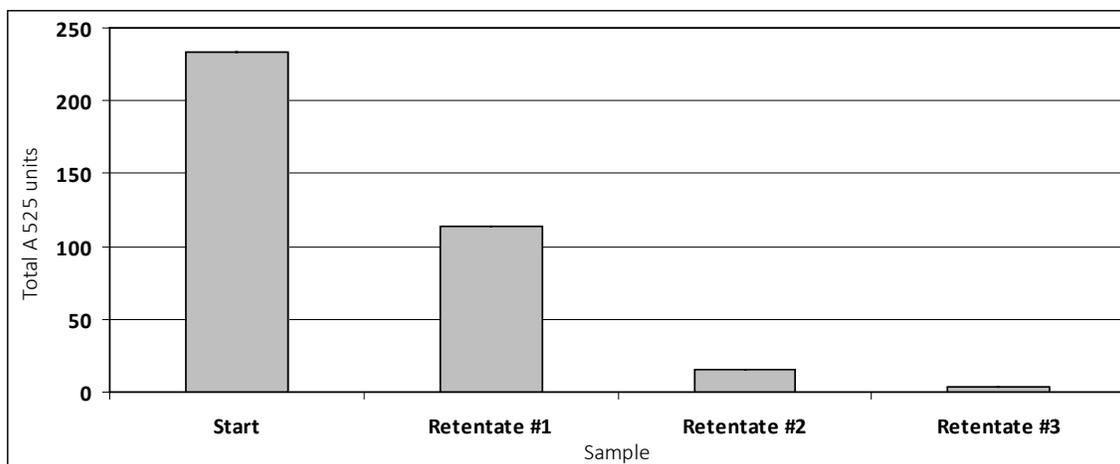


Figure 10: Recovery of heparin-like glycosaminoglycans from conditioned medium sample #3 during each stage of a GAG purification experiment using liquid chromatography with Sep-Pak C-18 cartridges and ultrafiltration with Vivaspin 15R centrifugal concentrators. The revised microcarbazole assay #2 was performed in triplicate.

E. Comparing Old GAG Purification Method & New GAG Purification Method

To compare the old and new GAG purification methods, three trials of each protocol were performed using the same conditioned media sample. The revised microcarbazole assay #2 was performed in duplicate to determine the concentration of heparin by measuring the absorbance at 525 nm. In Figure 11, the concentrations of the heparin-like glycosaminoglycans recovered are reported. Both protocols were repeatable, and the new GAG protocol yielded a higher concentration.

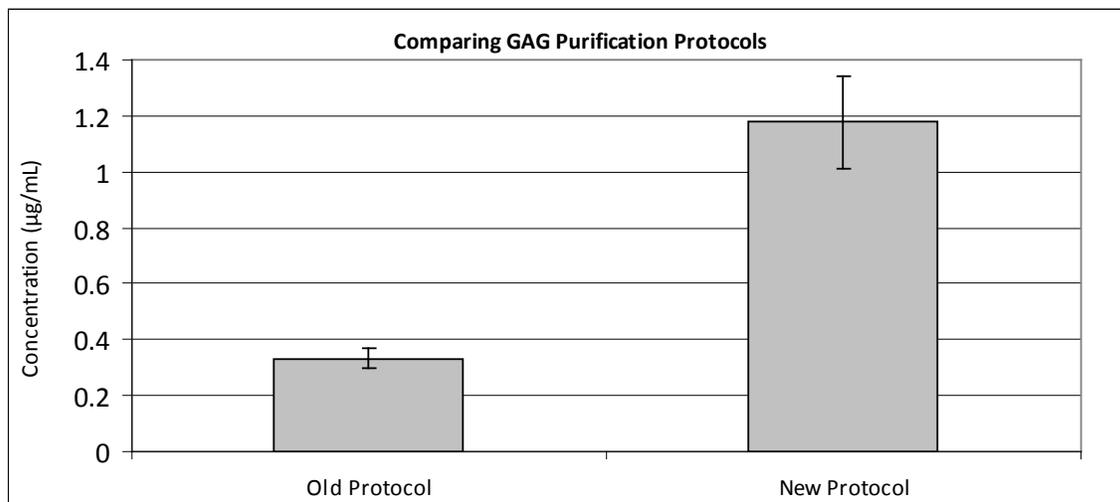


Figure 11: Recovery of heparin-like glycosaminoglycans from conditioned media using the old and new GAG purification methods. The revised microcarbazole assay #2 was performed in duplicate. Error bars represent the standard deviation of triplicate runs of the GAG purification experiments.

Discussion

We were able to develop a new GAG purification method that utilized liquid chromatography using Waters Sep-Pak C-18 classic cartridges and ultrafiltration using Sartorius Vivaspin 15R centrifugal concentrators. Compared to the old GAG purification method, the new GAG purification method reduces the required time from 3 days to less than 4 hours and the cost from \$38.18 to \$22.61 per sample (Table 3).

Table 3: The costs per sample to run the old GAG purification method and new GAG purification method. The manufacturer website links are in the appendix.

<i>New GAG Purification Protocol</i>					
Item	Price Whole Set (\$)	Items Per Set	Price Per Item (\$)	Items Needed	Total Price (\$)
Sep-Pak C18 Classic Cartridge	218	50	4.36	3	13.08
Sartorius Vivaspin 15R Centrifugal Concentrators	457.31	48	9.53	1	9.53
Total Price Per Sample					22.61

<i>Old GAG Purification Protocol</i>					
Item	Price Whole Set (\$)	Items Per Set	Price Per Item (\$)	Items Needed	Total Price (\$)
Amicon Ultra-15 Centrifugal Filter Units	281	24	11.71	1	11.71
Syringe Filter, PVDF, 0.22µm, 30mm Diameter, Sterile	58	30	1.93	1	1.93
Amicon Ultra-0.5 mL Centrifugal Filters	386	96	4.02	2	8.04
Sartorius Vivapure Q Mini H IEX Spin Columns	198	24	8.25	2	16.50
Total Price Per Sample					38.18

Our study first confirmed that Sep-Pak C-18 cartridges could remove Pluronic from solution. One Sep-Pak was sufficient for the removal of Pluronic alone, but since the conditioned medium samples needed 3 Sep-Pak cartridges for the maximum removal of color, we used that standard for all our experiments except for the three experiments using conditioned medium samples #1, #2, and #3 since these experiments were conducted before we determined that 3 Sep-Pak cartridges was optimal.

We also showed that ultrafiltration with Vivaspin centrifugal concentrators could remove almost all the glucose while retaining almost all the heparin in water. However, we observed a large deviation from 100% in the final retentate of the heparin experiment. We suggest that this may have resulted from the fact that glucose interferes with the microcarbazole assay and can give either diminished or increased absorbance depending on the amount of charring that results from the high temperature required by the assay. In addition, the microcarbazole assay was found to be prone to contamination from the covering film and is more sensitive to errors when pipetting from one plate to another. As a result, the microcarbazole assay was revised to a lower temperature using a hot plate instead of a thermal cycler to heat the samples. It was evident from the heparin standard curve that this method worked to quantitate the heparin and that one hour, which had the most linear curve, was the optimal time. Further, using a repeater pipette to create the standard curve and add the reagents increased the accuracy. Revising the microcarbazole assay also reduced the amount of time required for the microcarbazole assay to one hour.

We moved forward with the Vivaspin 15R centrifugal concentrators to work with larger sample volumes. Using the new GAG purification method, we were able to isolate almost all the heparin from glucose and Pluronic in solution, observing that most

interference was removed in the permeate resulting from the Vivaspin centrifugal concentrators. We were also able to isolate heparin from other potentially interfering contaminants in fresh medium, again observing the removal of interference following the chromatography and ultrafiltration steps. Medium lacking heparin showed no reaction when the retentate was assayed by the microcarbazole assay, showing that the interfering compounds were removed in the permeate.

We then showed that the new GAG purification method recovered varying amounts of heparin-like glycosaminoglycans from three different conditioned medium samples. Conditioned medium sample #3 required a longer centrifugation time, suggesting that different conditioned medium samples may require varying centrifugation times. We determined that, when the edge of the membrane was oriented towards the center of the centrifuge, a 45-minute spin achieved our desired retentate volume below 0.5 mL, so we decided that 45 minutes was the optimal time for each centrifugation.

We were then able to determine and compare the amount of heparin-like glycosaminoglycans recovered from the old and new GAG purification methods. We found that the new purification method recovered a higher concentration, which we presumed was because the old purification method had an anion-exchange step that was selective for the most highly charged glycosaminoglycans. Future work will focus on analyzing and comparing the constituents of these samples.

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Appendix I

Links to the manufacturer websites for each item used in the old GAG purification method and new GAG purification method.

Old GAG Purification Method

Amicon Ultra-15 Centrifugal Filter Units:

http://www.emdmillipore.com/US/en/product/Amicon-Ultra-15-Centrifugal-Filter-Units,MM_NF-C7715#ordering-information

Syringe Filter, PVDF, 0.22µm, 30mm Diameter, Sterile:

<https://www.celltreat.com/syringe-filter-pvdf-022µm-30mm-diameter-sterile>

Amicon Ultra-0.5 mL Centrifugal Filters:

http://www.emdmillipore.com/US/en/product/Amicon-Ultra-0.5mL-Centrifugal-Filters-for-DNA-and-Protein-Purification-and-Concentration,MM_NF-C82301#ordering-information

Sartorius Vivapure Q Mini H IEX Spin Columns:

<https://www.fishersci.de/shop/products/sartorius-vivapure-q-mini-h-iex-spin-columns/11760855>

New GAG Purification Method

Sep-Pak C18 Classic Cartridge:

http://www.waters.com/waters/partDetail.htm?partNumber=WAT051910&xcid=ext5005&gclid=Cj0KCQjw2pXXBRD5ARIsAIYoEbfQmx4mNygOawQhD7kj1zZXFITm8T9IBcFrZvgEvanbESZwuSsU3ZoaAtbXEALw_wcB

Sartorius Vivaspin 15R Centrifugal Concentrators:

<https://www.fishersci.com/shop/products/sartorius-vivaspin-15r-centrifugal-concentrators-8/p-4187753>