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An ELISA-based Assay for Measuring Heparanase Activity

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Bernice Hwang

Committee in charge:

Professor Jeffrey D. Esko, Chair
Professor Randy Hampton, Co-Chair
Professor Gulcin Pekkurnaz

2024

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The Thesis of Bernice Hwang is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2024

DEDICATION

I would like to dedicate this thesis to my boyfriend, Alex, my dog, Molly, my parents, and my family for supporting me.

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Hwang_Gel_Electorphoresis.pdf

LIST OF ABBREVIATIONS

HSPG	heparan sulfate proteoglycans
HS	heparan sulfate
HPSE	heparanase
HSV	herpes simplex virus
ELISA	enzyme-linked immunosorbent assay
HRP	horseradish peroxidase
TMB	3,3',5,5' tetramethylbenzidine
HS-BSA	heparan sulfate-bovine serum albumin
BSA	bovine serum albumin
FGF2	fibroblast growth factor 2
PBS	phosphate-buffer saline
GAG	glycosaminoglycan
IEX	ion exchange
DEAE	diethylaminoethyl
BCN	bicylcononyne
DTT	dithiothreitol
LB	Luria-broth
HEK	human embryonic kidney
DMEM	Dulbecco’s Modified Eagle’s Medium
FBS	fetal bovine serum
PS	Pen-strep
PEI	polyethylenimine

OD	optical density

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ABSTRACT OF THE THESIS

An ELISA-based Assay for Measuring Heparanase Activity

by

Bernice Hwang

Master of Science in Biology

University of California San Diego, 2024

Professor Jeffrey D. Esko, Chair
Professor Randy Hampton, Co-Chair

Heparanase, an endo-β-glucuronidase, is expressed by mammalian cells and plays essential roles in viral infection. For example, in herpes simplex virus, the virus induces the host to produce heparanase and which facilitates release of viral progeny from the infected cell. SARS-CoV-2 may use a similar mechanism to escape from cells. Heparanase acts upon cell surface heparan sulfate proteoglycans, cleaving the heparan sulfate chains into fragments, and

releasing any bound proteins or viral particles. Assays for measuring heparanase activity exist, however, the existing assays are not generalizable to tissues or cells, which limits their use for measuring heparanase activity in a variety of biological samples. We developed a sensitive assay using an ELISA-based format in which heparan sulfate-BSA conjugates are used to coat the wells of a 96-well plate. The immobilized heparan sulfate-BSA will bind heparin binding proteins, such as biotinylated FGF2, which can be quantitated by ELISA. Incubation with heparanase cleaves the heparan sulfate chains and reduces FGF2 binding. The assay was optimized with respect to substrate and buffer conditions. Future studies will focus on assaying heparanase in cell and tissue extracts, before and after SARS-CoV-2 infection. The high through-put potential of the assay also would allow screening for heparanase inhibitors.

INTRODUCTION

Coronavirus infection and heparan sulfate. COVID-19 is a disease caused by SARS-CoV-2 and is characterized by respiratory illness with a wide range of symptoms. The World Health Organization (WHO) deemed COVID-19 a "pandemic" in March 2020 and extensive research has been done to target prevention and treatment (WHO Director General, 2020). To be able to treat viruses like SARS-CoV-2, it is crucial to understand how the virus propagates and spreads.

SARS- CoV-2 infect cells through binding to receptors such as ACE2 on the cell surface. Heparan sulfate proteoglycans (HSPGs) act as co-receptors working in tandem with ACE2 (Clausen et al., 2020). HSPGs are proteins that contain covalently bind heparan sulfate (HS) chains, a type of glycosaminoglycan made by virtually all animal cells.

Each proteoglycan has a core protein with one or more linear, negatively charged HS chains. HSPGs reside on the surface, in the extracellular matrix, and in storage granules. On the cell surface, they are part of the glycocalyx (Agelidis & Shukla, 2020). The glycocalyx is a cell coat that consists of glycans and glycoconjugates that acts as a barrier for viruses and a filter for soluble protein entry into cells (Clausen et al., 2020). Research has found that the SARS-CoV-2 spike protein interacts with HSPGs in the glycocalyx to enter the cell and cause infection (Clausen et al., 2020). Once viral progeny are ready to leave and cause further infection, the same HS that helped the virus enter the cell potentially prevents its spread (Thakkar et al., 2017).

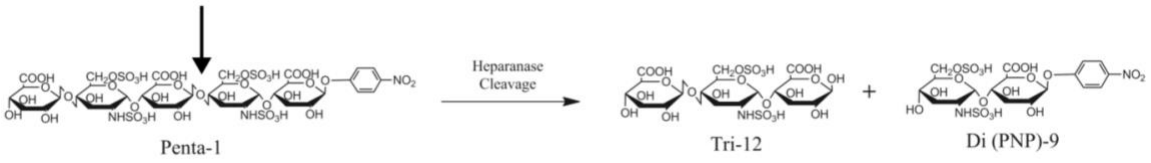


Figure 1. Structure of HS and HPSE cleavage site. HS are negatively charged chains that are cleaved between glucuronic acid and glucosamine residues. Modified from Peterson, S., et al. (2012). The Journal of Biological Chemistry, 287(41), 34836–34843.

Heparanase. Heparanase can modify the HSPGs on the cell surface and could play a role in the exit strategy of viral progeny. Heparanase (HPSE) is a mammalian endo-β-glucuronidase that cleaves HS into oligosaccharides and plays a major role in physiology and pathological conditions (Vlodavsky et al., 2007). HS is cleaved between glucuronic acid and glucosamine residues by HPSE (Peterson & Liu, 2012). Refer to Figure 1. The enzyme is produced as an inactive 65 kDa proenzyme that needs to be proteolytically cleaved to have activity (Figure 2). Proteolytic cleavage yields 50 kDa and 8 kDa subunits (Vlodavsky et al., 2016). A recombinant, constitutively active form of HPSE, called a HPSE GS3, has been made, in which the 50 kDa and 8 kDa subunits are covalently linked with three glycine-serine repeats (Peterson & Liu, 2012).

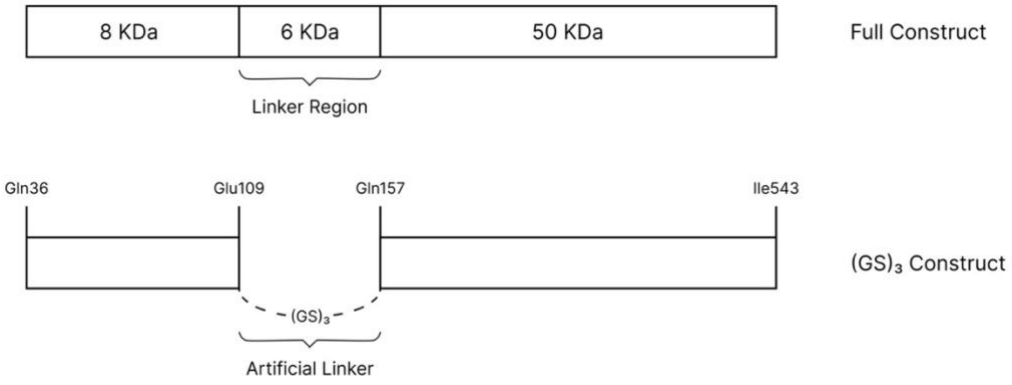


Figure 2. Schematic of full length HPSE and the $(GS)_3$ truncated construct. The $(GS)_3$ construct has an artificial linker, making it constitutively active.

Studies of herpes simplex virus (HSV) showed that HPSE plays a role in viral release. HSV does not encode an enzyme that can degrade HS, but viral infection induces HPSE production in the host, resulting in cleavage of the HSPG chains and release of viral progeny (Hadigal et al., 2015). The induction of HPSE results in loss of HS and active 50 kDa HPSE expression was greater compared to uninfected cells. Flow cytometry and immunofluorescence microscopy also showed enhanced HPSE on the cell surface (Hadigal et al., 2015). The presence of activated HPSE cleaved HS on the cell surface, as visualized by flow cytometry and immunofluorescence microscopy data.

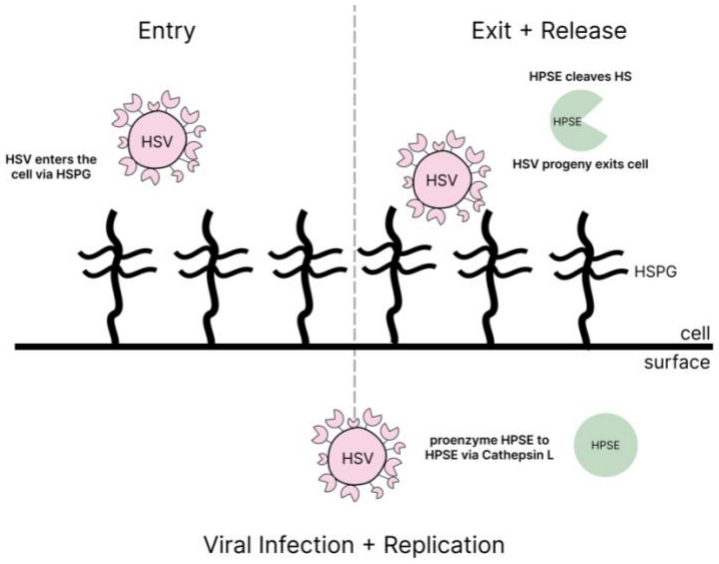


Figure 3. HPSE's effect in herpes simplex infection. The host cell is induced by the virus post infection. This allows viral progeny to leave the cell by digestion of HS on the cell surface.

Studies by our collaborators in Israel (Israel Vlodavsky, Amos Panet, Dana Wolf) showed that HPSE is induced in nasal turbinate and lung tissue after SARS-CoV-2 infection (Figure 4). A related gene HPA2 shows an inverse relationship. HPA2 lacks enzyme activity but can inhibit HPSE by binding to the HS substrates. Thus, infection by SARS-CoV-2 may mimic the behavior of HSV in inducing HPSE and cleaving host tissue HS. These studies were based on

immunohistochemistry using antibodies that recognize HPSE and HPA2. Whether the increase in protein levels correlates with enzyme activity is unknown. This question underlies the need to develop a facile method to assay HPSE activity.

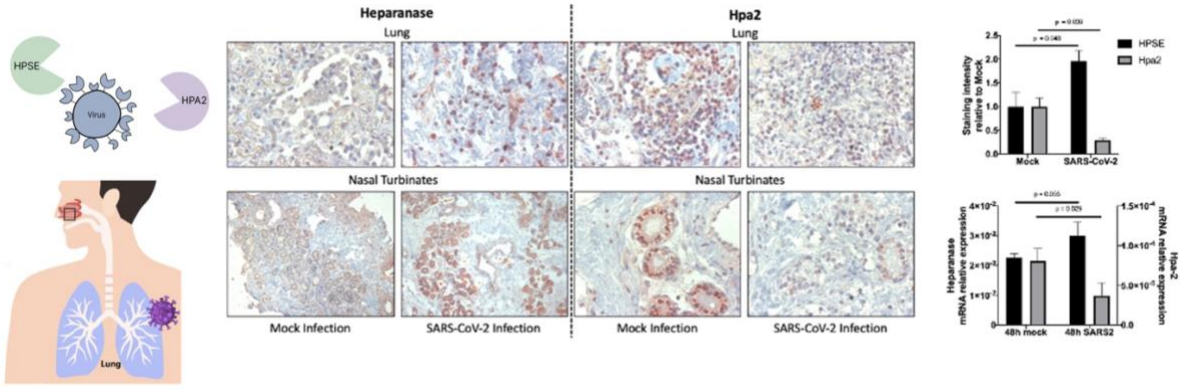


Figure 4. Increased Heparanase Levels Detected in SARS-CoV-2 Infection of Lung and Nasal Tissues. Immunohistochemical staining of HPSE and HPA2 in explanted lung and nasal turbinate tissue before and after infection by SARS-CoV-2 for 2 days. SARS-CoV-2 infection increased staining for HPSE in both nasal turbinate and lung. HPA2 expression was reduced by SARS-CoV-2 infection compared to expression in mock-infected tissue specimens.

Measuring Heparanase. Measuring HPSE activity is difficult and there is a need for a more rapid high-throughput method to measure its activity that might allow for screening potential inhibitors. Existing assays for measuring HPSE activity (Table 1) generally require labeling of HS with radioisotopes (^{35}S, ^{14}C or ^{3}H), fluorophores, or biotin (Chhabra & Ferro, 2018). A commonly used assay using radiolabeled HS chains is based on gel-filtration. Metabolically ^{35}S-labeled HS chains are incubated with enzyme and the products are analyzed through a gel filtration column (Li et al., 2005). Active HPSE cleaves the chains to smaller fragments. Enzyme activity of HPSE is often compared to bacterial heparin lyases, which is commercially available. The bacterial enzymes cleave the chains to disaccharides.

Another approach is based on release of ^{14}C-labeled HS fragments from [^{14}C] HS that is chemically conjugated onto agarose beads (Chhabra & Ferro, 2018). After treating with HPSE,

the radiolabeled HS fragments are released and quantitated. There are numerous variations of using radiolabeled HS and size exclusion chromatography, but they are generally time consuming and low in sensitivity (Chhabra & Ferro, 2018). The gel filtration assays do not enable kinetic studies because of the tedious way the products must be analyzed.

Table 1. Existing Assays for Measuring Heparanase. Modified from Chhabra, M., & Ferro, V. (2018). Molecules, 23(11).

Type of Heparin/HS Assay	Type of HS	Method
Size exclusion chromatography	[^{14}C]-labeled HS	HS is immobilized onto agarose beads and treated with HPSE
Size exclusion chromatography	[^{3}H]-acetylated HS	HS pieces from HPSE digestion are more soluble than undigested HS in the presence of ionic detergents
ELISA	[^{3}H]-labeled biotinylated HS	HS is immobilized on streptavidin-coated plates and treated with HPSE
ELISA	Biotinylated HS (non-specifically with photobiotin)	HS-photobiotin immobilized on a hydrazide-coated plate and treated with HPSE
ELISA	HS-biotin	HS-biotin bound to streptavidin-coated plates and measured by release of fluorescently labeled fragments
ELISA	HS with non-reducing end GlcNAz-label	GlcNAz-labeled HS is captured on an antibody-coated plate, biotinylated, and then treated with HPSE
FRET-based assay	HS labelled with Eu-cryptate	HS-Eu-cryptate is biotinylated and HPSE treated, which is measured by fluorescence

Another way to assay the enzyme is to use enzyme linked immunosorbent (ELISA) assays. Enzyme linked immunosorbent assays are based on the reaction between an antigen and antibody (Sakamoto et al., 2018). In one form of the assay, the antigen is conjugated with enzymes like horseradish peroxidase (HRP), which is then detected with hydrogen peroxide and a chromogenic substrate such as 3,3',5,5' tetramethylbenzidine (TMB), which produces a blue color. The reaction is stopped with a solution like sulfuric acid and the product of the coupled reaction is quantitated with a plate reader (Sakamoto et al., 2018). ELISA assays are simple and highly specific, sensitive, and efficient. This assay also does not require the use of radioisotopes making it safe and eco-friendly (Sakamoto et al., 2018).

Using an antigen or substrate that is like the natural proteoglycan substrate in an ELISA format has been tried. Syndecan-4 is a type of HSPG, consisting of a protein core and several covalently linked HS chains (Elfenbein & Simons, 2013). Recombinant syndecan-4 was made and the HS chains were modified enzymatically to contain a non-reducing end GlcNAz-residue (Wu et al., 2017). The substrate was captured on a plate coated with an antibody to syndecan-4. GlcNAz-labeled HS was then biotinylated using click chemistry through the GlcNAz residue. Treatment with HPSE digests the biotinylated HS-GlcNAz; if the HS is digested, the biotin label is lost, which results in reduced binding of streptavidin. Quantitatively, this is seen as a reduction in signal through a reading at 450 nm on a microplate reader (Wu et al., 2017). This recombinant syndecan-4 HS chain and GlcNAz coupling allows for a highly specific approach in assaying for HPSE. However, the assay involves expression of recombinant syndecan-4, recombinant Ext1/Ext2 to end label the chain, and an expensive nucleotide sugar derivative (UDP-GalNAz). The other assays listed in Table 1 are variations on this theme.

The production of a recombinant proteoglycans as a substrate has many advantages, but it is difficult to produce due to extraction, proteolysis and incorrect folding of the overexpressed recombinant protein. Here, we used heparin/HS-BSA conjugates as a surrogate “neo” proteoglycan. The heparin/HS-BSA conjugates used in the ELISA-based HPSE assay resemble naturally occurring proteoglycans and can be easily produced in large quantities.

In the studies reported here, we developed a sensitive ELISA-based assay with substrates similar to what is found in the human body (Figure 5). We used HS-bovine serum albumin (HS-BSA) conjugates as the substrate, which was produced by reductive amination. The HS was extracted from human lung tissue and bovine serum albumin is commercially available and inexpensive. The HS chains were covalently bound to the BSA to produce HS-BSA conjugates (Trieger et al., 2019). The constitutively active GS3 clone HPSE was produced and used to treat the HS chains. Biotinylated fibroblast growth factor 2 (FGF2) protein was then produced and when added to HS-BSA, it binds to the HS chains of the conjugates. HRP avidin was then added to the wells to measure how much biotinylated FGF2 is present. In a sense, the HS-BSA conjugates coated on a 96-well plate mimic the HSPGs on the cell surface, and the biotinylated FGF2 protein mimics the spike protein of SARS-CoV-2 that binds to HSPG on a cell.

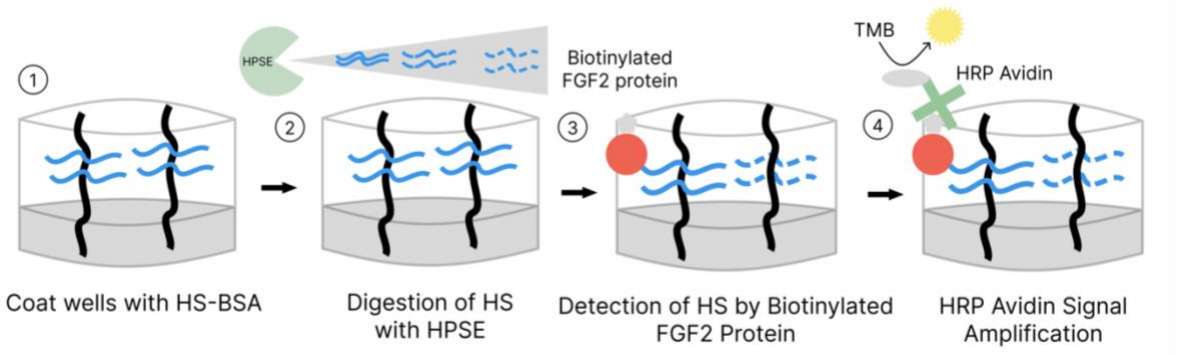


Figure 5. ELISA-based assay for measuring HPSE. The wells are coated with HS-BSA conjugate and a dilution series of HPSE is added to the wells. Biotinylated FGF2 protein is added to the wells to bind to intact HS. HRP avidin binds to FGF2-biotin, which is quantitated by reaction with TMB.

This thesis is divided into 4 chapters. Chapter 1 introduces the Introduction, Chapter 2 describes the Materials and Methods used, Chapter 3 summarizes Results and Discussion of the findings, and Chapter 4 presents Conclusions and future goals.

MATERIALS AND METHODS

Biotinylation of FGF2 on Heparin Column

To biotinylate FGF2, a 500 μL Heparin Sepharose 6 Fast Flow affinity resin bed (GE Healthcare) was put onto a 5 mL column. The column was equilibrated with 2 mL of Dulbecco's phosphate-buffered saline, PBS (Gibco). 250 μg of Recombinant Human FGF-basic (PeproTech) was suspended in 500 μL of Ultrapure water. This was directly loaded onto the column where flow through was collected and reapplied to the column 6 times. The column was then washed with 2 mL of PBS. The protein concentration of the flowthrough was measured to ensure that all the FGF2 protein had bound to the heparin column. The sulfo-NHS-LC-biotin (Thermo Scientific) was prepared at 0.3 mg/mL in PBS and 1 mL was loaded onto the heparin Sepharose column. Before it completely flowed through, the column was stopped, and the biotin solution was incubated on the column for 30 minutes. The column was then washed with 2 mL PBS and biotinylated FGF2 was following eluted with 2 x 250 μL of PBS + 1 M NaCl. Biotinylated FGF2 was then buffer exchanged into PBS to remove excess salt and stored at -80°C.

Purification of Heparan Sulfate from Human Lung Tissue

Human lung tissue (Lifesharing) was prepped by measuring 11.78 g of tissue into eight 50 mL conical tubes. The tissue was digested using a Pronase (Roche) digestion buffer (500 mM Tris pH 7.6, 200 mM $CaCl_2$, 1% Triton X-100, and 10 mg Pronase). The tubes were left overnight at 37°C on a shaker. The temperature of the shaker was increased to 60°C the next day and left overnight. The samples were spun down at 2000 g for 10 minutes on a centrifuge and filtered.

The total volume was mixed 1:4 with diethylaminoethyl (DEAE) equilibration buffer (50mM sodium acetate, 200 mM NaCl, 0.1% v/v 10% Triton X-100, pH 6.0). The first ion exchange (IEX) was performed by preparing a column with 15 mL of the DEAE Equilibration buffer and 5 mL of DEAE beads (Cytiva). The beads settled and the column was equilibrated with 2 x 10 mL DEAE equilibration buffer. The sample was loaded onto the column and allowed to completely enter the resin. Once all the sample was run through, the column was washed with 3 x 10 mL of the DEAE wash buffer (20 mM sodium acetate, 200 mM NaCl, pH 6.0). The desired sample was eluted with 20 mL of DEAE elution buffer (20 mM sodium acetate, 2 M NaCl, pH 6.0). The IEX eluted sample was desalted using PD-10 desalting columns (Cytiva) that were equilibrated 5 x 5 mL with 10% ethanol in Ultrapure water. Each PD-10 column had 2.5 mL of the IEX eluted sample by gravity flow and allowed to completely enter the resin. To elute, 3.5 mL 10% ethanol was used. The desalted samples were collected into 15 mL conical tubes, frozen at -80°C, and lyophilized until dry. The dried samples were resuspended in 5 mL DNase I buffer (500 mM Tris, 500 mM NaCl, 25 mM MgCl2, 5 mM $CaCl_2$, pH to 8 with HCl) and vortexed to mix. DNase I (Sigma-Aldrich) was added to a final concentration of 20 kUnit/mL and digested for 2 hours at 37°C. Next, 20 mUnits of chABC (Sigma-Aldrich) was added to the sample and incubated at 37°C for 2 hours with shaking at 150 rpm. The sample was then brought to 0.4 M NaOH and incubated overnight at 4°C to allow for beta-elimination. The following day the sample was neutralized with acetic acid to reach pH 6. Lastly, the sample was diluted 1:10 with DEAE equilibration buffer. GAG purification was repeated over DEAE beads, desalted with PD-10 columns, and lyophilized. The HS was then used to make HS-BSA conjugates.

Heparan Sulfate-BSA Conjugates by Click Chemistry

Azide-Peg4-NHS (BroadPharm) was added in a 4x molar excess to azido modify the HS. The samples were incubated under rotation in 100 mM phosphate buffer pH 8 at room temperature overnight. The next day, the remaining HS was inactivated by adding 100 mM glycine. The azido modified HS was purified through ion exchange and PD-10 column, freeze dried, and stored at -80°C. To Bicyclononyne (BCN) modify BSA, 8x molar excess of BCN-peg4-NHS (BroadPharm) was added to 20 mg Bovine Serum Albumin (BSA), Fraction V, Fatty Acid-Free (Sigma-Aldrich). This reaction was carried out and rotated for 2 hours at room temperature. To stop the reaction, 100 mM glycine was added and left rotating for 30 minutes at room temperature. The BCN modified BSA was then buffer exchanged into PBS using Zeba™ Spin Desalting Column (Thermo Scientific). To conjugate the azido modified HS to BCN-BSA, 4x molar excess of azido modified HS was added to BCN-BSA and incubated overnight at 4°C. The following day, SDS-PAGE was done to verify the conjugates. The SDS-PAGE was done by using a NuPAGE™ Bis-Tris Mini Protein Gels, 4–12% (Invitrogen) to run protein electrophoresis. The samples made for the gel consisted of 10 μL of the sample, 4 μL of NuPAGE™ LDS Sample Buffer 4X (Invitrogen), and 3 μL of 1M Dithiothreitol (DTT). Protein electrophoresis was run for about an hour and stained overnight with Coomassie blue (Abcam). The conjugates were aliquoted and stored at -80°C.

Plasmid Production for Transfection

200 mL of the LB Broth (Fisher Bioreagents) was prepared and autoclaved. A bacterial glycerol stock of GS3 HPSE plasmid was added to tubes of LB broth with ampicillin (Sigma-Aldrich) using a sterile pipette tip. The tubes were place in a 37°C shaker overnight. The

ZymoPURE II Plasmid Maxiprep Kit (Zymo Research) was used to obtain an endotoxin-free GS3 HPSE DNA plasmid.

Transfection and Production of HPSE

Human embryonic kidney 293T (HEK293T) cells (adherent) were grown in Dulbecco's Modified Eagle's Medium (Gibco) with 10% Fetal Bovine Serum (Sigma-Aldrich) and 1% Pen-strep (Gibco) at 37°C and 5% CO_2 and passaged every 2-3 days. The day prior to transfecting the cells was the day of splitting. 3 mL Trypsin was used to detach the cells for 5 minutes and 7 mL of DMEM 10% Fetal Bovine Serum (FBS) + Pen-strep (PS) media was added to neutralize. 2.5 mL of the volume was added to a T25 flask with 2.5 mL of the DMEM 10% FBS + PS media. Transfection was performed after confirming cell conditions. The media was carefully replaced with DMEM with 2% FBS and incubated at 37°C and 5% CO_2 for 1 hour. The GS3 HPSE plasmid was diluted to a concentration of 10 ng/μL at a volume of 500 μL with OptiMEM (Gibco). The polyethylenimine (PEI) was mixed with OptiMEM to a ratio of 1:1.5 (plasmid to PEI) at a volume of 500 μL. The plasmid and PEI were mixed and incubated at room temperature for 20-30 minutes. The plasmid/PEI mixture was added dropwise to the flask and returned to incubate at 37°C and 5% CO_2. The media was harvested on day 3.

HEK293F cells were grown in Expi293™ Expression Medium (Gibco) at 37°C and 8% CO_2 on an orbital shaker and passaged every 2-3 days. The day prior to transfecting the cells, the HEK293F cells were seeded at 3 x 10^6 cells/mL. On the day of transfection, the cells were checked with a cell counter for viability and cell density to be around 95% and 5.5 x 10^6 cells/mL, respectively, to continue with transfection. The cells were diluted to a density of 3 x 10^6 cells/mL with Expi293 Expression Medium and returned to the incubator while setting up

transfection mixes. ExpiFectamine™ 293 Transfection Kit (Gibco) was used to set up appropriate transfection mixes to add to the flask and incubated in an orbital shaker at 37°C and 8% CO_2. Around 18- 22 hours later, ExpiFectamine™ 293 Transfection Enhancer 1 and ExpiFectamine™ 293 Transfection Enhancer 2 were added to the flask. The flask was then returned to an orbital shaker at 33°C and 8% CO_2.

Harvesting HPSE and Purification via Heparin Affinity Chromatography

To harvest the HPSE, the media was collected in 50mL conical tubes and spun in a centrifuge at 500 g x 10 minutes at 4°C. The supernatant/media was collected to be purified. One cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche) was added per 50 mL to the media and diluted 1:3 with Buffer A (100 mM NaCl, 25 mM Tris-Base, pH 7.5). Heparin affinity chromatography was done using HiTrap Heparin HP 1 mL columns (Cytiva). The column was equilibrated with 10 mL of Buffer A and the sample was loaded on the column at 1 mL/min. Then, the column connected to an ÄKTA Pure 25 chromatography system and was washed with Buffer A. Elution was done using a gradient of Buffer B (1 M NaCl, 25 mM Tris-Base, pH 7.5). The relevant fractions were confirmed to have HPSE by performing an SDS-PAGE and western blot. The western blot was done by making an SDS-PAGE gel and using the Invitrogen™ Power Blotter XL System. The blot was blocked with Fish Serum Blocking Buffer (Thermo Scientific) for an hour at room temperature and Polyclonal Rabbit Anti-Human HSPE antibody (ProSpec, Catalong #ANT-155) was used as the primary at a dilution of 1:1000 overnight at 4°C. The next day, the blot was washed three times with a wash buffer (1:3 fish buffer to TBS-T). The secondary antibody, IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody (LI-COR), was applied to the blot for 1 hour at room temperature. The blot was washed three times with wash

buffer and imaged on LI-COR Odyssey imager. Once doing so, the concentration of the fractions with HPSE was increased by using another heparin column to elute the protein with 100% Buffer B. The eluted protein was buffer exchanged from Buffer B to a 400 mM NaCl, 20 mM NaH_2PO_4, pH 7.5 buffer with a 10 mL Zeba™ Spin Desalting Column. The final concentration of HPSE was measured with a nanodrop at 0.104 mg/mL.

Measuring HPSE Activity in an ELISA-based Assay

HPSE activity was measured using an ELISA. HS-BSA conjugates (100 ng) were coated in duplicates on a high-binding 96-well plate at 4°C overnight (Costar). All the following incubations were done on a shaker at 37°C and after each incubation step, the wells were washed with TSM buffer (20 mM Tris Base, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% Tween-20, pH 7.4) three times. The wells were blocked with TSM blocking buffer (1% (w/v) BSA) for 1 ½ hours and followed with an hour digestion of HS with HPSE. The HPSE buffer used for digestion was 40 mM ammonium acetate, 3.3 mM calcium acetate, pH 6, 0.25% BSA, and 0.05% Tween-20. Then, the wells were incubated with 20 nM FGF2-biotin for 30 minutes, following with 1:2000 HRP avidin (BioLegend) for 30 minutes. Lastly, the wells were incubated with TMB (Thermo Scientific) until there was a gradient color change and before the control BSA-PEG started developing a blue hue and reaction as stopped with 1 M H2SO4. The absorbance was measured at 450 nm using Molecular Devices' SpectraMax M3.

Verifying Biotinylation of FGF2 by Detecting HS

The analysis of binding FGF2-biotin to HS involved setting up an ELISA assay with 1, 2, 4, 8 μg/mL HS-BSA conjugate and a two-fold dilution of FGF2-biotin starting from 100 nM to

1.5625 nM. HS-BSA was incubated with PBS overnight at 4°C. The plate was blocked with TSM blocking buffer for two hours and FGF2-biotin was incubated using TSM blocking buffer. Then, HRP avidin was incubated using TSM blocking buffer. The absorbance was measured at 450 nm using Molecular Devices' SpectraMax M3.

Varying Conditions of the Assay

The ELISA assay was optimized by testing different concentrations of FGF2-biotin by performing a saturation curve, testing different dilutions of HRP avidin, and different volumes of TMB. To find the optimal FGF2-biotin concentration, a saturation curve was done with a two-fold dilution starting at 100 nM. To find the optimal HRP avidin dilution, dilutions of 1:1500, 1:2000, and 1:2500 were tested to see if there was a different in absorbance values that can be obtained at 450 nm. Different volumes of TMB were also tested to see which volume reaches an absorbance value at around 0.4 to 0.6 optical density (OD). The time for the TMB reaction was also tested.

RESULTS AND DISCUSSION

Bioconjugation of Neoproteglycans

To produce a native-like HS proteoglycan substrate, we generated HS-bovine serum albumin (BSA) conjugates using click chemistry (Trieger et al., 2019). HS was purified from human lung tissue and modified with an azido group, and BSA was modified by a bicyclononoyne (BCN) group (Figure 6a). These were conjugated by copper-free click chemistry to produce HS-BSA. Once the HS-BSA conjugate was made, an SDS-PAGE gel was performed to evaluate its coupling (Figure 6b). BSA has a molecular weight of 66 kDa, which is where the band for BSA with no modification migrates. The SDS-PAGE gel shows that there was a noticeable size increase in the conjugates compared to BCN-BSA, indicating successful generation of the HS-BSA conjugates.

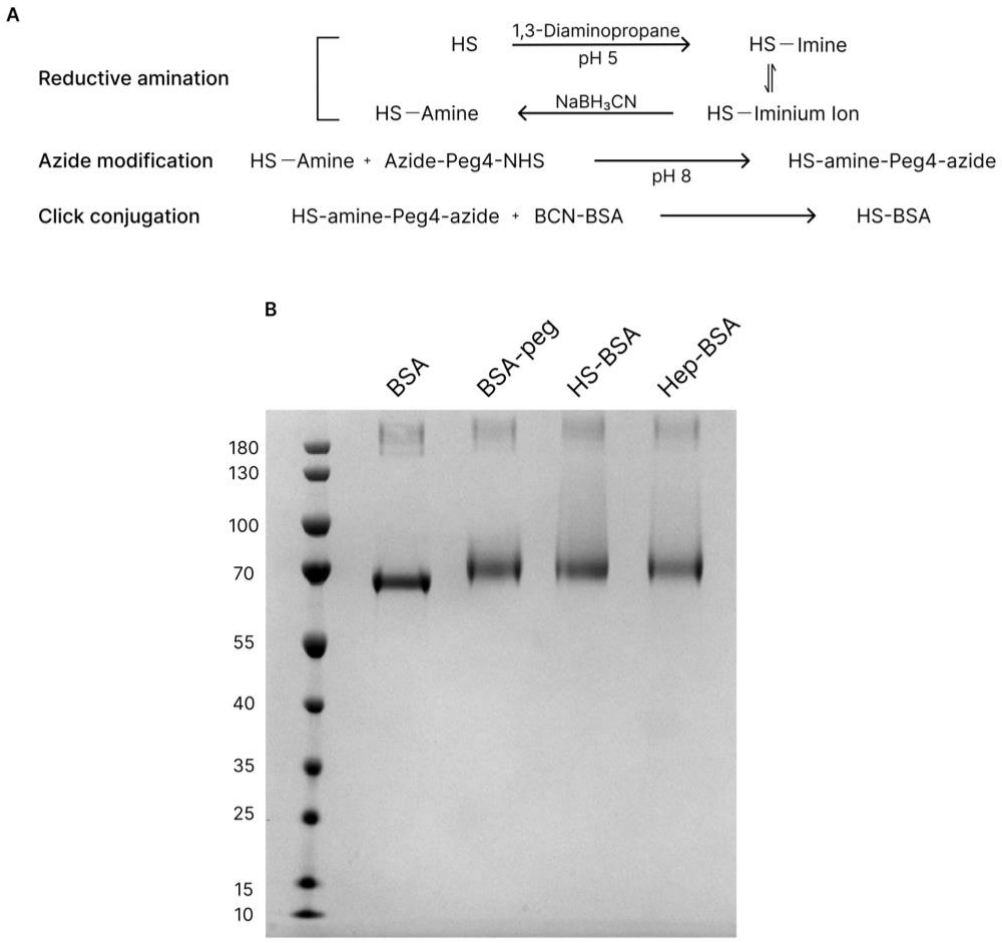


Figure 6. Bioconjugation of neoproteoglycan substrate for ELISA-based HPSE assay. (A) Chemistry of synthesis of HS-BSA conjugate made using HS sourced from human lung tissue. (B) Coomassie stained SDS-PAGE gel showing BSA without any modification compared to BSA conjugates with different modifications. The different conditions are unmodified BSA, BSA modified with a PEG linker, BSA modified with HS, and BSA modified with heparin.

Production of HS-binding Detection Reagent

To produce a HS-binding detection agent, we produced FGF2-biotin. This was done by biotinylating FGF2 on a Heparin Sepharose column to protect the heparin binding sites on FGF2 (Raman et al., 2003). Refer to Figure 7a. To test that FGF2 biotinylation was successful and that HS-BSA conjugates were modified, an ELISA was performed. We observed FGF2-biotin

binding to HS-BSA (1 μg/mL) and no binding of FGF2-biotin to unmodified BSA (Figure 7b). The data showed FGF2-biotin conjugate reaches saturation at 25-50 nM.

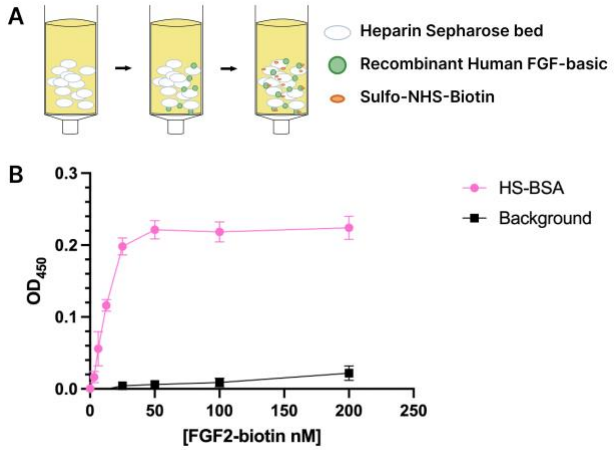


Figure 7. Preparation of biotinylated FGF2 detection agent. (A) FGF2 was biotinylated on a heparin sepaharose column to allow modification of the protein while protecting HS binding sites. (B) Successful biotinylation of FGF2 was confirmed using an ELISA assay. FGF2-biotin binding to HS-BSA (pink) and unmodified BSA (black).

Protein Production of HPSE

To produce HPSE, the GS3 plasmid was first transformed into *E. coli* cells, purified, and a gel electrophoresis was performed (Supplementary data 1). The GS3 plasmid has an artificial linker that allows it to be constitutively active, which is ideal for protein production. Sequencing was performed to confirm the identity of the plasmid (data not shown).

For HPSE production, the HEK293 cell expression system was chosen based on its reported ability to produce high quality recombinant proteins with mammalian-like posttranslational modifications (Abaandou et al., 2021). HEK293 cells are available as both adhesive (HEK293T) and suspension (HEK293F) cell expression systems. To evaluate the two expression systems, HEK293T and HEK293F cells were transfected with 10, 15, 20 ng/μL plasmid at a ratio of 1:1.5 of DNA to transfection reagent polyethyleneimine (PEI). The

supernatant was collected at day 3 and a western blot was performed to evaluate the expression levels of HPSE in the two cell lines under the tested conditions (Figure 8b). For the HEK293F cell line, transfection with 10 ng/μL plasmid resulted in the production of monomeric HPSE with minimal aggregation and degradation (Figure 8b). HEK293T cells also expressed HPSE but analysis of the band intensity was complicated by the presence of other cross reactive protein bands.

The media used for each cell line were also analyzed on the same western blot. HEK293T cells uses FBS conditioned media which contains high amounts of serum proteins. This produced a band at a similar molecular weight to where we would observe a band for HPSE, which is probably BSA as it is 70 kDa in size. Therefore, this raised an issue of determining whether the bands seen in the HEK293T cell transfections were from the cells producing and expressing HPSE or if it could be from the FBS in the media. The Expi293 media used for culturing HEK293F cells is synthetic media with low amounts of protein and did not show any bands on the western blot. This was helpful in determining whether the bands on the western blot were from the harvest that contained HPSE or if it could have been media related. The presence of plasma proteins in the cell media used for HEK293T cells was evaluated for this reason as it has a presence of a band around where HPSE is expected on a western blot. The higher protein expression in HEK293F cells could also be due to the lack of serum in the media (Yamano et al., 2010). Considering the sharp monomeric bands from samples collected from HEK293F cell transfections and the lower protein content of the synthetic media (Expi293 media), we decided to use HEK293F cells as the cell line for producing HPSE.

To see if there is a difference in HPSE expression produced by HEK293F cells using different transfection reagents, PEI and Lipofectamine were compared. We included a no

transfection control to determine endogenous HPSE expression levels (Figure 8c). PEI is a cationic polymer known to have high transfection efficiency, and Lipofectamine differs from PEI as it is a cationic liposome based transfection reagent (Guo et al., 2018). The western blot showed that for the control there were no bands observed at 58 kDa or at 50 and 8 kDa for days 3-5 in the absence of plasmid, indicating that undetectable endogenous HPSE is expressed (Figure 8c). PEI and Lipofectamine transfection conditions both showed bands at around 58 kDa. Lipofectamine transfection showed higher intensity bands compared to PEI. The day 4 Lipofectamine condition shows a smeared lane due to improper storage of the day 4 harvest, which likely caused cell lysis and proteolytic degradation of the sample (Figure 8c). Subsequent transfections were done with HEK293F cells using the ExpiFectamine™ 293 Transfection Kit with enhancers and harvested on Day 3 for efficiency.

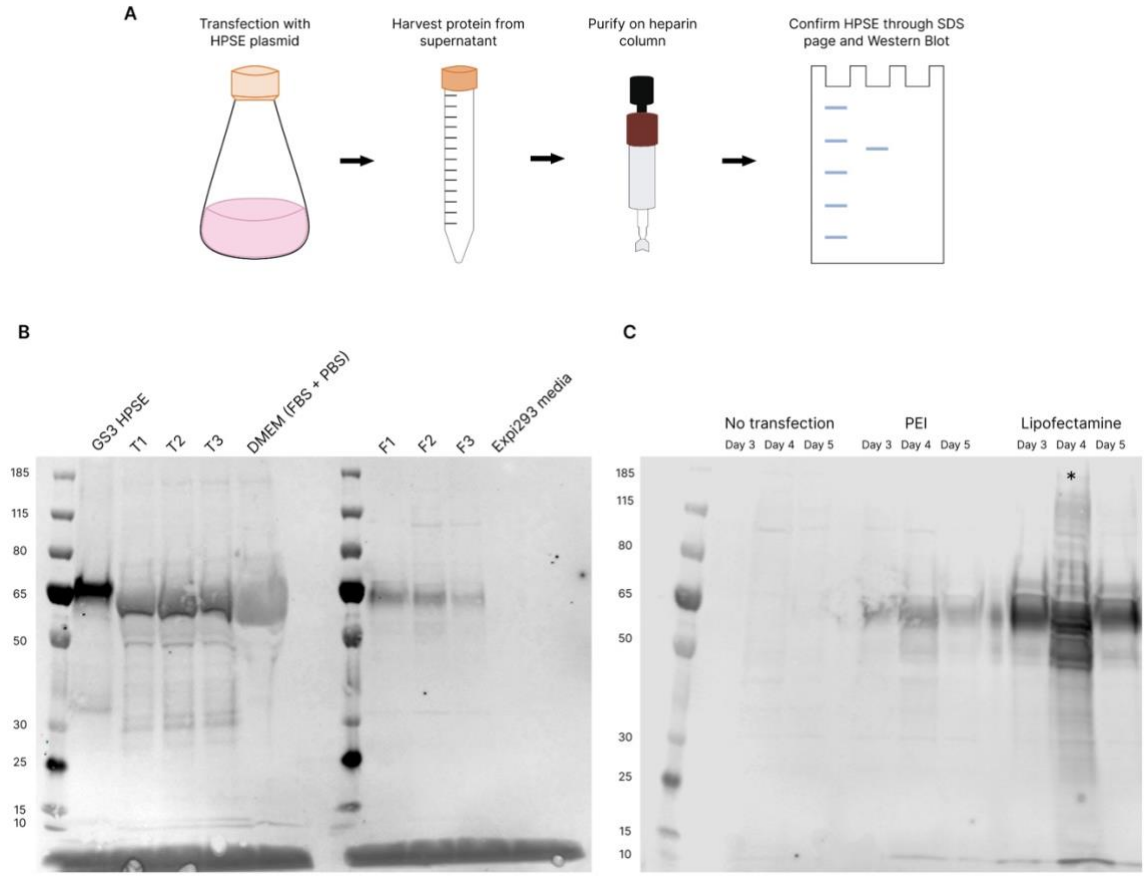


Figure 8. Optimizing HPSE production in HEK293 cell expression systems. (A) Schematic of HPSE expression and purification steps. (B) Comparison of HPSE expression using HEK293T and HEK293F cells. Western blot showing GS3 HPSE expression in cell supernatants of HEK293T and HEK293F cells transfected with different plasmid: transfection ratios. The transfections were done using 10 (T1/F1), 15 (T2/F2), and 20 (T3/F3) ng/μL of DNA plasmid with a ratio of 1:1.5 of PEI transfection reagent. The supernatant was collected on day 3. (C) HEK293F cells were used to compare HPSE expression using PEI or lipofectamine as transfection reagents. Western blot showing no transfection, PEI and Lipofectamine conditions on days 3-5. Lipofectamine had higher expression of HPSE compared to the PEI condition.

*Day 4 lipofectamine condition shows a smeared lane because improper storage after harvest

Purifying HPSE using Heparin Affinity Chromatography

After transfecting HEK293F cells with GS3 HPSE plasmid and ExpiFectamine™ 293 Transfection Kit, the supernatant was harvested on day 3 for purification. The diluted supernatant (1:3 dilution with Buffer A) was loaded on a HiTrap Heparin HP column and

purified using a salt gradient by FPLC. The chromatogram showed that the low affinity binding proteins eluted around fractions 6-12 while HPSE mainly eluted in high salt concentrations around fractions 52-72. Fractions 52-72 were eluted around 650-700 mM NaCl according to the chromatogram (Figure 9a). This correlated with earlier findings showing that HPSE is known to elute at 700-800 mM NaCl (Novick et al., 2014). SDS-PAGE and western blot were used to evaluate the levels of HPSE in the eluted fractions (Figures 9b and 9c). Fractions 56, 60, 64, and 66 showed high intensity bands corresponding to HPSE on western blot (Figure 9c) while only low amounts of contaminating proteins were observed on SDS-PAGE (Figure 9b).

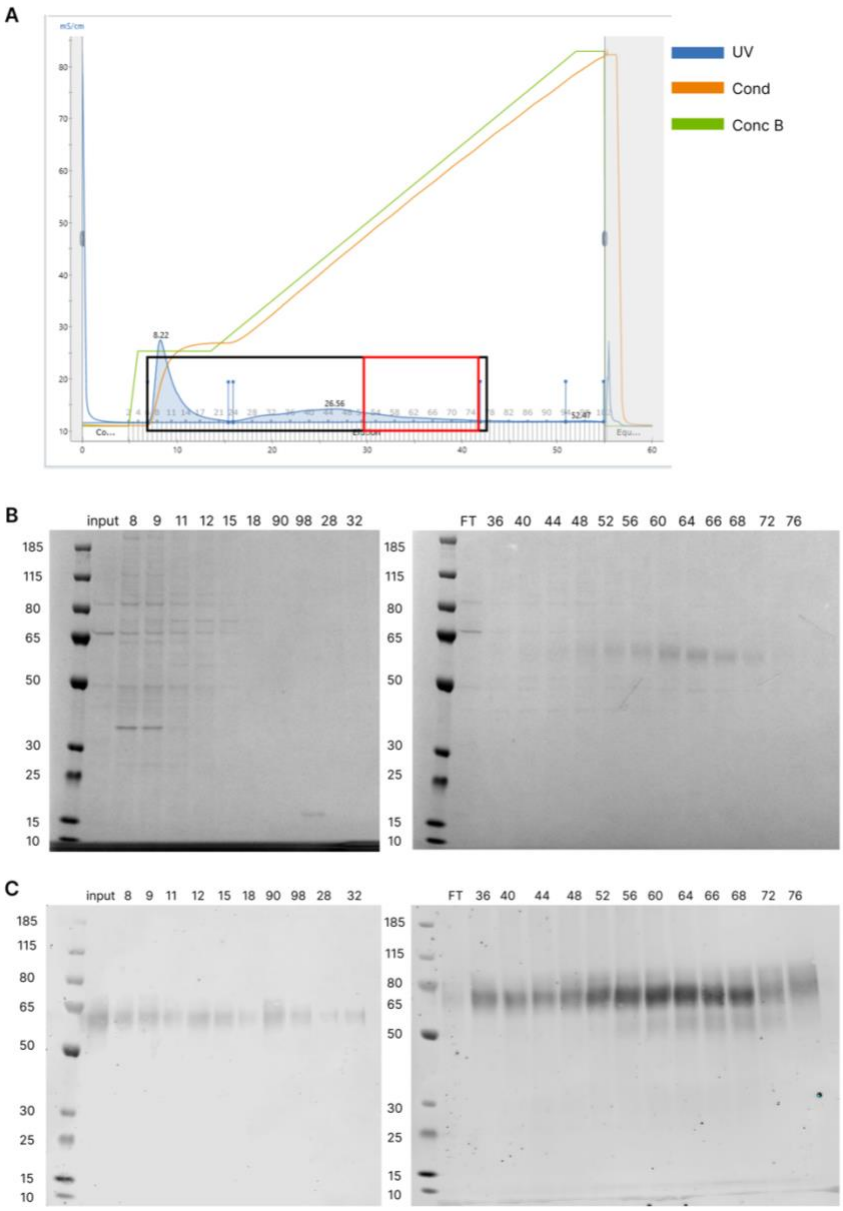


Figure 9. HPSE purification on Heparin Sepharose Column. (A) Cell media from a transfection using HEK293F cells was collected and HPSE was purified using a Heparin Sepharose column. The purification was done using a buffer made with 100 mM NaCl and 25 mM Tris-Base at pH 7.5 and a stepwise and gradient elution using 1 M NaCl and 25 mM Tris-Base at pH 7.5.

Unbound protein came out in a lower salt concentration; HPSE generally came out at a higher salt concentration (red box). Black box indicates the fractions analyzed on SDS-PAGE gel and western blot. (B) Coomassie SDS-PAGE gels of the fractions in the red and black box (Figure 9a). (C) Western blot gel of the fractions in the red and black box (Figure 9a).

These findings showed that the HPSE fractions eluted at high salt contained highly pure HPSE. To concentrate the enzyme, these fractions (52-72) were desalted, re-loaded onto a new HiTrap Heparin HP column, and eluted in 1 M NaCl. The chromatogram showed a clean peak where HPSE eluted in fractions 5 and 6 yielding 2 mL of 0.104 mg/mL HPSE protein. (Figure 10a). The fractions were then pooled, desalted, and evaluated by SDS-PAGE gel (Figure 10c) and western blot (Figure 10b). These data showed a monomeric HPSE band around 58kDa with few contaminants (Figure 10c).

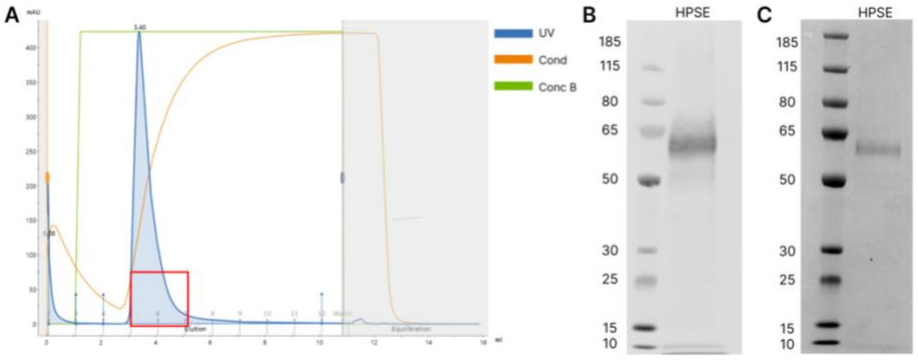


Figure 10. Increasing the concentration of pooled HPSE fractions. (A) Fractions 52-72 from the purification were pooled, loaded onto a heparin column and eluted in 100% NaCl to concentrate the purified HPSE. Western Blot (B) and SDS-PAGE (C) of final concentrated HPSE.

Obtaining an Optimal Signal-to-noise Ratio in the ELISA-based HPSE Assay

To obtain the best signal-to-noise ratio in the ELISA-based HPSE assay, different components of the assay were optimized (ligand coating density, secondary HRP avidin, and TMB substrate). Refer to Figure 11a. The ELISA-based HPSE assay consists of coating the wells with HS-BSA conjugate, using FGF2-biotin as a detection agent for HS, adding HRP avidin to bind to the biotin, and using TMB to quantitate the reaction. First, we tested how much HS-BSA conjugate was needed to saturate the surface and obtain the highest coating density of HS substrate. This was done by coating the plate with 1, 2, 4 and 8 μg/mL HS-BSA and measuring

binding of FGF2-biotin. No significant differences in FGF2-biotin binding were observed between the different coating concentrations, indicating that 1 μg/mL (50 ng/well) of HS-BSA was sufficient to saturate the surface (Figure 11b). FGF2-biotin binding to HS reached saturation at 20-25 nM of FGF2-biotin, meaning all binding sites were occupied. Based on these data, 1 μg/mL HS-BSA and 20-25 nM FGF2-biotin were set as standard conditions for the ELISA assay.

Next, we optimized the amount of HRP avidin to add. Testing dilutions of 1:1500, 1:2000, and 1:2500 HRP avidin showed that the OD values for 1:2000 were higher compared to a 1:2500 dilution (Figure 11c). There wasn't a noticeable difference in OD values comparing 1:2000 and 1:1500 HRP avidin, therefore 1:2000 HRP avidin was used for the assay. TMB is a chromogenic substrate that upon processing by HRP produces a blue color which turns yellow upon addition of 1 M H_2SO_4 (Sakamoto et al., 2018). Testing different volumes (50, 100, 150, and 200 μL) of TMB showed that 100 μL was the optimal amount (Figure 11d). While obtaining a higher signal-to-noise ratio is desired, using too much TMB could have led to less accurate reading of the plate. Higher OD values are less accurate with less light being transmitted through the sample (Dell, 2021). Longer incubation of TMB with HRP avidin allows for more processing of the chromophore substrate and producing a higher OD. To find the optimal TMB incubation time, assays were tested at 1, 2.5, 5, 10, and 20 minutes (Figure 11e). This showed that there was a gain in OD as the reaction proceeded for a longer period as it allows HRP avidin to interact longer with TMB, but the BSA-PEG values also increased in OD with an increase in time, resulting in a lower signal-to-noise ratio (Figure 11e). BSA-PEG acted as a control for ensuring that the reaction was stopped when there started to be visual signs (BSA-PEG wells turning blue) of the reaction running for too long.

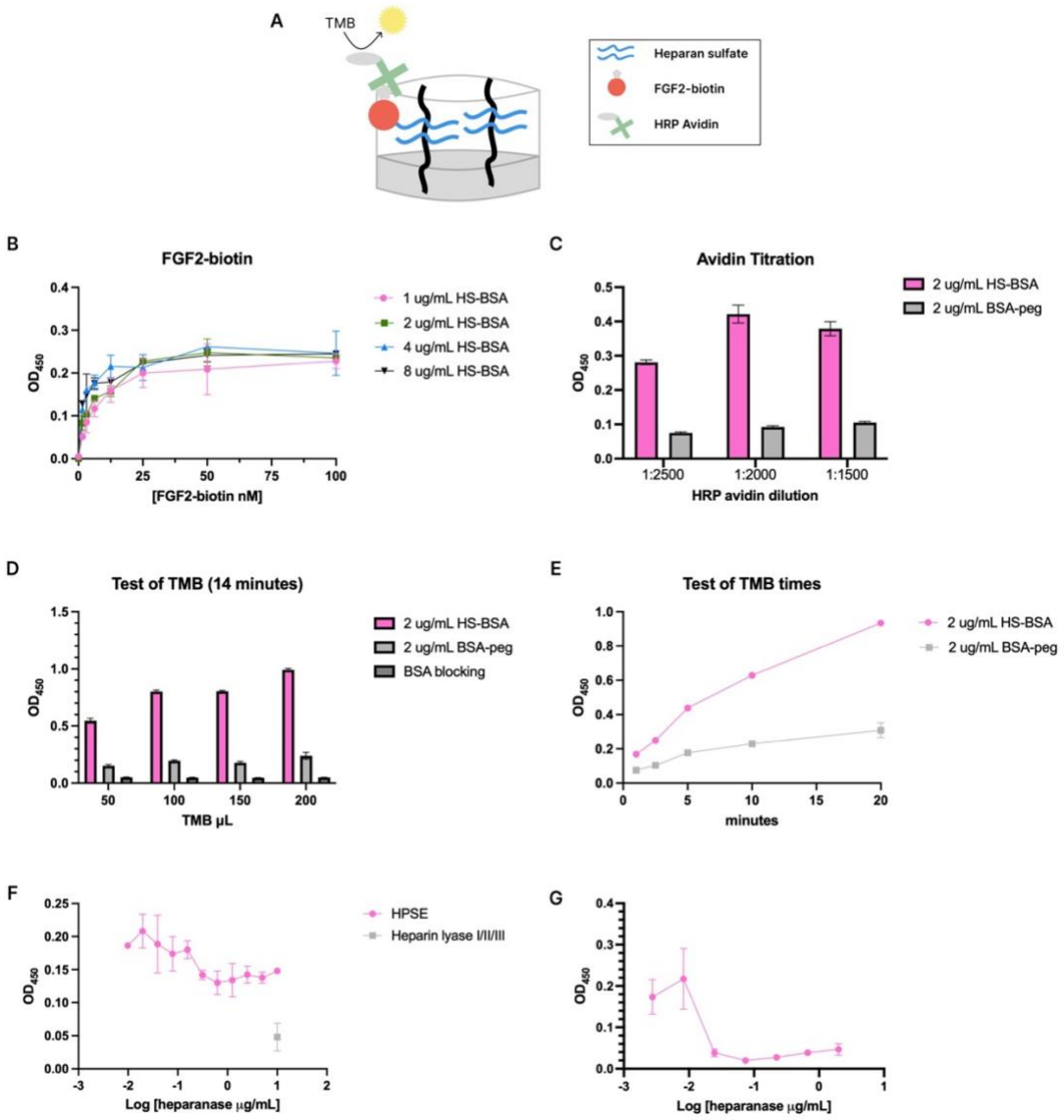


Figure 11. Optimization of ELISA-based HPSE assay. (A) Schematic of assay components (HS-BSA conjugate, FGF2-biotin, HRP avidin, TMB) that were tested to optimize the ELISA assay. (B) FGF2-biotin was tested against HS-BSA to see how much FGF2-biotin is needed to reach full coating of the wells. Concentrations of 1, 2, 4, 8 µg/mL HS-BSA were tested against a titration of 100nM of FGF2-biotin. We observed no significant difference in coating concentrations, (C) ELISA assay with dilutions of 1:2500, 1:2000, and 1:1500 of HPR avidin measure at OD 450nm. (D) ELISA assay comparing 50, 100, 150, and 200 µL of TMB measured at OD 450nm. (E) ELISA assay linearly comparing 1, 2.5, 5, 10, and 20 minutes of TMB color development before stopping the reaction with 1M H_2SO_4. (F) HPSE activity of (2 µg/mL to 0.02 µg/mL what concentration) of HPSE provided by collaborators. (G) In-house produced HPSE activity of (10 µg/mL to 0.01 µg/mL) HPSE. HPSE is shown in pink and bacterial heparin lyases I/II/III control is shown in grey.

Measuring HPSE Activity Using an ELISA-based Assay

With optimized assay conditions and in-house produced HPSE in place, we next tested whether we could use our ELISA assay for measuring HPSE activity. To test this, we treated the HS-BSA with a dilution series of the in-house produced HPSE and detected undigested HS using FGF2-biotin. We observed some activity but not the outcome we had hoped. Digestion with bacterial heparin lyases I/II/III was used as control of this assay and resulted in complete loss of signal (Figure 12b). Therefore, we treated the HS-BSA with a dilution series of HPSE enzyme provided by our collaborator, Israel Vlodavsky. We saw that the Vlodavsky's batch was active and showed a dose-dependent decrease in signal corresponding to the digestion of HS (Figure 12a). This shows that the most likely reason for not being able to replicate the dose-dependent decrease is due to lack of activity of the in-house produced HPSE.

Possible reasons for in-house HPSE inactivity in the assay could be due to incorrect protein folding, long purification process, or buffers conditions used in the purification and assay techniques. Failure to produce HPSE with the correct folding could have led to enzyme inactivity. Without a tag on the protein, it is a longer process to purify. To use the FPLC, we needed to load the 1:3 diluted supernatant onto the 1 mL heparin column. Loading such a large volume (200 mL) on a 1 mL column at 1 mL/min took over three hours to complete for loading onto the machine. While this step was completed at 4°C and protease inhibitors were added, this could have made it difficult to minimize protease activity (Ryan & Henehan, 2017). Western blots performed after each purification to identify the fractions containing HPSE and desalting steps also took several days to complete, which could have caused the enzyme to lose activity in the process. Buffer A and Buffer B used for purification could have affected the activity of the protein. The sensitivity of enzymes to pH and buffer conditions could also be problematic. The

HPSE buffer used in the ELISA-based assay was a 40 mM ammonium acetate, 3.3 mM calcium acetate, pH 6, 0.25% BSA, and 0.05% Tween-20 buffer. This was optimized compared to a phosphate/citrate buffer (pH 6.0) to better detect enzyme activity in the assay. Enzymes tend to have the most activity in a certain pH range, so incompatibility in the buffers used for digestion could be a factor in the inactivity observed (Schomburg et al., 2012). However, the buffers used for our assays are recognized as standard HPSE digestion buffer indicating that buffer conditions alone does not explain the observed enzyme inactivity (Kayal et al., 2023). More work is needed to optimized buffer conditions.

While the in-house HPSE failed to show activity, we were able to use our ELISA-based assay to determine the activity for HPSE provided by our collaborators. Compared to earlier available gel filtration assays using an ELISA based assay for measuring HPSE activity provide a fast and reliable platform that can easily be adapted for high-throughput screening. Other ELISA based assays are available but use recombinant proteoglycans like syndecan-4 (Elfenbein & Simons, 2013). The production of recombinant proteoglycans presents additional challenges due to their large size, complexity, and replicating sulfation levels, which often leads to non-native GAG modification of the protein core (Lord & Whitelock, 2013). In contrast, our ELISA-based assay uses HS isolated from human lung tissue and following coupling to BSA, securing a high-quality HS substrate. Additionally, some proteins bind to the core of syndecan-4 while BSA is an inert molecule used wildly as blocking reagent as it rarely interferes with biochemical interactions (Zhang et al., 2010).

CONCLUSION

In conclusion, we here developed an ELISA-based assay for measuring HPSE activity. For this, we coated 96-well plates with HS-BSA substrate, then added a dilution series of HPSE and finally detected undigested HS using biotinylated FGF2. To achieve this, we produced the HS-BSA substrate, a FGF2-biotin detection agent, recombinant HPSE enzyme, and partially optimized the ELISA conditions. Using this assay, we were able to measure HPSE digestion of HS and determine activity of in-house and collaborator-provided HPSE. We detected low activity using our in-house HPSE, while dose-dependent digestion of HS was observed using collaborator-provided HPSE. Further work is needed to optimize HPSE production conditions to produce active enzyme. Once this is done, the next step will be to use this assay to measure HPSE activity in biological samples such as lung and nasal tissue extracts to determine how viral infection affects HPSE activity.

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