

Design of Phantoms and Targeted Agents for Ultrasound Contrast Imaging of
Microvessels and Fibrin Clots

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

The author contributions for each Chapter are described below.

Chapters 1, 2, and 6: Hanyue Shangguan wrote these chapters.

A version of **Chapter 3**, titled “A Forked Microvascular Phantom for Ultrasound Localization Microscopy Investigations” has been published in IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control. This article was co-authored by Hanyue Shangguan, Billy Y.S. Yiu, Adrian J.Y. Chee and Alfred C.H. Yu. Hanyue Shangguan carried out the design, creation and testing of the phantoms, analyzed the results, and wrote the manuscript draft. Billy Yiu provided the super-resolution algorithm, provided feedback for super-resolution results and phantom design revisions, and provided input on manuscript editing. Adrian Chee provided input on phantom design and maintenance, and feedback for manuscript editing. Alfred Yu reviewed the results and edited the manuscript for final publication.

Chapter 4, titled “Fibrin Targeting Microbubble Contrast Leveraging Biorthogonal Conjugation” is being prepared for submission, co-authored by Hanyue Shangguan, Billy Y. S. Yiu, and Alfred C.H. Yu. Hanyue Shangguan carried out the reaction design, experimental set-up design, performed all experiments, analyzed the collected data, and wrote the manuscript draft. Billy Yiu provided feedback on the main results figures as well as providing writing recommendations in editing. Alfred Yu reviewed the results and edited the manuscript for final publication.

A version of **Chapter 5**, titled “Sawing the Branch You Sit On: The Efficacy of Inhibited tPA for Fibrin Targeting” is being prepared for submission, authored by Hanyue Shangguan and Alfred C.H. Yu. Hanyue Shangguan carried out the reaction design, experimental design, performed all experiments, analyzed the collected data, and wrote the manuscript draft. Alfred Yu reviewed the results and edited the manuscript for final publication.

Abstract

Venous thromboembolisms (VTE) are common cardiovascular events including deep vein thrombosis and pulmonary embolism. Early diagnosis and management of VTEs can significantly improve patient quality of life and mortality. Such clinical insights made possible through medical imaging are critical for clinicians to manage patient risk and treatment efficacy. As the linchpin in point of care medical imaging, Ultrasound (US) is the workhorse modality in patient diagnosis and treatment monitoring within the clinic. However, specific detection of the molecule fibrin which is the current clinical treatment target for VTEs remains elusive for US in the clinic.

In this dissertation, I designed an ultrasound contrast agent with fibrin targeting properties. The baseline contrast agent was used to test a novel *in vitro* flow phantom with tissue mimicking characteristics to validate a contrast specific super-resolution ultrasound algorithm. The contrast agent was further modified to target fibrin leveraging the existing fibrin targeting properties of a therapeutic agent and demonstrated to be adherent to a fibrin surface as well as enhancing the detection of fibrin surfaces when imaging with a clinical scanner. Finally, a comparison study was performed examining the fibrin targeting ligand options in ultrasound literature and to further investigate the robustness of using anti-fibrin therapeutics for pure imaging applications in more physiologically realistic flow scenarios.

This work aims to raise current developments in ultrasound molecular imaging to address the existing clinical need to detect fibrin as a treatment target. Baseline MBs were shown to be versatile enablers for specialized contrast imaging applications. Moreover, the significant improvement to ultrasound signal using fibrin targeting contrast confirms that ultrasound molecular imaging can be a robust solution to improve fibrin detection. Finally, the insights gained through comparing the existing MB targeting strategies provides new directions to consider for future development of fibrin targeting contrast.

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List of Abbreviations

Abs	Antibody
B-mode	Brightness Mode
CAD	Computer Aided Design
CEUS	Contrast Enhanced Ultrasound Imaging
CREKA	Cys-Arg-Glu-Lys-Ala peptide
D-dimer	Fibrin degradation protein
DBCO	Dibenzocyclooctyne
DI	Dionized [water]
DiD'	1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, perchlorate
DLS	Dynamic Light Scattering
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
DSPE	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine
DVT	Deep Vein Thrombosis
ELIP	Echogenic Liposome
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
fXIII	Human blood factor XIII
FWHM	Full width half maximum
GPU	Graphics processing unit
GSH	Glutathione
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
LU	Lipase unit
MB	Microbubble Contrast Agent
MI	Mechanical Index
MRI	Magnetic Resonance Imaging
MW	Molecular Weight
NHS ester	N-Hydroxysuccinimide ester
NIH U	Unit of activity used with blood clotting enzymes

PBS	Phosphate buffered saline
PEG	Polyethylene Glycol
PEG(4,2k)	Longer chain PEG structures
PET	Positron Emission Tomography
PET	Polyethylene terephthalate
PFB	Perfluorobutane, decafluorobutane (C ₄ F ₁₀)
pH	Unit for acidity/basicity (potential of Hydrogen)
PLA	Polylactic acid
PPACK	D-phenylalanyl-l-prolyl-l-arginine chloromethyl ketone
PVA	Polyvinyl alcohol
QoL	Quality of Life
RCF	Relative Centrifugal Force
RF	Radiofrequency
RGD	Arg-Gly-Asp Peptide
RoI	Region of Interest
RPM	Rotations per Minute
SPAAC	Strain promoted azide-alkyne cycloaddition reaction
SVD	Singular Value Decomposition
tMB	Targeted Microbubble
tPA	Tissue-type plasminogen activator
Tris	2-Amino-2-(hydroxymethyl)propane-1,3-diol
ULM	Ultrasound Localization Microscopy ()
US	Ultrasound
UV	Ultraviolet
VALAP	A mixture of Vaseline-Lanolin-Paraffin
VEGF	Vascular Endothelial Growth Factor
VTE	Venous Thromboembolism

List of Symbols (Order of Appearance)

Z	Acoustic Impedance
ρ	Density
c	Speed of sound
B	Bulk modulus
a	Reflection fraction
λ	Wavelength
f	Frequency
τ	Time of flight
d	Distance
Tx	Transmit
Rx	Receive
X	Location in Cartesian (x,z)
t	Time
s	Signal
P	Pressure
γ	Surface tension
R	Radius
C	Concentration
R_b	Ideal gas coefficient
T	Absolute temperature
V	Volume
ω_0	Resonance frequency
γ	Liquid medium specific heat
v_i	Velocity of particle i
η_2	Fluid viscosity
g	Coefficient of gravity
D_τ	Stokes diffusion coefficient
k_B	Boltzmann coefficient

R _h	Hydrodynamic radius
wt%	Mass percentage
dB	Decibel
Da	Dalton

Chapter 1

1.1 Introduction

1.1.1 Contrast Ultrasound and Targeted Ultrasound Imaging

The objective of my thesis is to explore topics specific to the use and design of microbubble (MB) contrast agents in specialized ultrasound imaging applications such as super-resolution imaging or the targeted improvement of fibrin clot detection. By developing a novel *in vitro* microvessel model, we can improve the evaluation of the accuracy in developing contrast enabled super resolution algorithms. To improve the robustness of fibrin clot detecting contrast agents, we detail a novel process of modifying fibrin targeting ligand to introduce modern click conjugation in fibrin targeting ultrasound study. Further study was also made on ligand selection, in comparing the predominant two ligand types in contrast ultrasound literature to identify their role in fibrin targeting ultrasound study.

Ultrasound (US) is a non-ionization, and non-invasive imaging modality that is used to image features within the body (Cobbold, 2006; S. Wang et al., 2020). Its merits of being a reliable and portable instrument has positioned US as one of the workhorse modalities within the clinic compared to the more involved but heavier modalities of magnetic resonance imaging (MRI), positron emission tomography (PET), and X-ray based methods (Abhisheka et al., 2024). The prevalence of US is especially true in the case of venous thromboembolism (VTE) where the extent of US's integration within existing diagnostic/prognostic strategies is mostly only limited by patient anatomy (Lim et al., 2018). As with all disease management strategies, treatment of VTEs balances the efficacy of treatment and the risks associated with a procedure given the state of information of the patient (Ortel et al., 2020). At its current state, knowledge of the disease condition is collected through the administration of established clinical scoring questionnaires and medical lab testing in conjunction with applying the appropriate medical imaging methods (Lim et al., 2018; Ortel et al., 2020). At present, clinical information available through current diagnostic pathways is sufficient in dealing with the disease at large but the implementation of key

improvements to existing clinical tools undeniably lowers the health/economic burden of treatment on the patient.

Fibrin is a key structural molecule to consider in venous clot treatment: 1) The presence of elevated fibrin degradation derivatives is a key indicator of a clot during a diagnostic D-Dimer test (Kogan et al., 2016; Tripodi, 2011), 2) Anticoagulant treatment acts to directly decrease the accumulation of additional fibrin to venous clots (Heestermans et al., 2022; Kattula et al., 2017), 3) Thrombolytic treatment dissolves the existing fibrin structure of a clot to destroy it (L. Zhang et al., 2021). Yet despite significant attention towards fibrin in the treatment pipeline, a pervasive problem exists where clinical instruments cannot interact with fibrin outside of medical lab testing. That is not to say that current methods cannot monitor the practical effects of treatment such as the restoration of flow or the reduction of symptoms (Stevens et al., 2021). However, the evolution of a clot's structural proteins over time (e.i, low density fibrin > high density fibrin > collagen) has significant implications on treatment efficacy which current instruments struggle to differentiate (Czaplicki et al., 2017). Recent recognition of this gap sees progress in contrast enhanced imaging using fibrin targeting contrast to support the dominant heavyweight modalities of MRI (Lohmeier et al., 2022; Overoye-Chan et al., 2008; Stracke et al., 2007), and PET (Izquierdo-Garcia David et al., 2022). Here, US contrast agents hold a unique position of enabling targeted imaging, drug carrying, and treatment options (Ferrara et al., 2007; S. Wang et al., 2020). Yet the disproportionate emphasis on development of novel treatment has progress in contrast enhanced ultrasound's (CEUS) fibrin imaging capabilities to be wanting. Existing MB carriers in this context use ligand loading methods that can be impacted by local environment (Electrostatic(Li et al., 2022)), or feature carrier types that are incongruent with existing therapy directions (polymer (Gormley et al., 2019), ELIPs (Klegerman et al., 2008)) (Bader et al., 2016). Therefore, a robust agent design that is compatible with current finding in MB contrast aided treatment would form a critical bridge in improving the value added of clinical US in venous clot management.

To assess the clot fibrin concentration, novel ultrasound contrast agents using modern targeted agent design principles are proposed. These agents exploit new bioconjugation strategies to elevate the methods used in current ultrasound fibrin targeting literature. Further, we also examine the theranostic potential unique to tissue plasminogen activator as both a thrombolytic agent and a binding ligand. Consequently, we hypothesize that novel ultrasound contrast agents will provide further insights within fibrin targeting technologies in VTE studies to advance future adoption of these techniques to drive more informed clinical indications in venous clot management.

1.2 Background

1.2.1 Ultrasound Imaging Mechanics

Ultrasound imaging is a medical imaging technique that uses the interactions between physical waves and differences in material acoustic properties to visualize boundaries and flow within the body. At the fundamental level in M-mode (single line) ultrasound, ultrasound imaging functions through a transmit and receive sequence. A transmitted pulse will generate a reflection at an intensity associated with the acoustic impedance mismatch of the materials at an encountered interface. Acoustic impedance is described as follows:

$$Z = \rho * c \quad (1)$$

where ρ is the density of the medium and c is the speed of sound within the medium. Additionally, the speed of sound is related to the material properties through the following:

$$c = \sqrt{\frac{B}{\rho}} \quad (2)$$

thus,

$$Z = \sqrt{B * \rho} \quad (3)$$

where B is the bulk modulus, the resistance of a material to deformation under uniform compression. Reflected signal intensity, a , is related to the material impedance as follows:

$$\mathbf{a} = \frac{(Z_2 - Z_1)^2}{(Z_2 + Z_1)^2} \quad (4)$$

Having i in Z_i associated with its respective medium. One important takeaway is that interfaces containing media of different states (e.g. liquid-gas interface) will generate higher intensity signals compared to similar state interfaces.

On the topic of interfaces, it is also important to consider the resolution limit of ultrasound. Imaging resolution is limited by λ , the transmit wavelength:

$$c = \lambda * f \quad (5)$$

with an inverse relation to the center frequency f . Accordingly, higher resolution images can be achieved through a higher frequency transmission but at the cost of increased signal attenuation thus lowering depth of penetration. Medical ultrasound scanners operate in the center frequency range between 2 and 12 MHz (Miller et al., 2012). Biological membranes (e.g. cell membranes, the shelled MBs monolayers here) are well below the acoustic diffraction limit.

In B-mode (2D-Brightness Mode), signals from multiple array elements are used to create the final image in a Delay-and-Sum process (Gong et al., 2005) (Perrot et al., 2021). Consider a single transmitted wavefront with a scattered signal reflected in all directions from a scatterer located at a pixel location X_s with cartesian co-ordinates (x, z) for the respective lateral and axial directions relative to the array. The distance that the advancing wavefront travels to interrogate the pixel location is consistent for all receiving array element $d_{TX}(X_s)$. However, the distance that the reflected wave travels to reach the i th element depends on the relative location of the element to the pixel $d_{RX}(X_s, x_i)$. For a constant speed of sound, a time of flight can be assigned to the transmit-reflect pair for the location as τ_i . The relation is expressed as follows:

$$\tau_i(X_s) = \frac{d_{TX}(X_s) + d_{RX}(X_s, x_i)}{c} - t_0 \quad (6)$$

with t_0 as the acquisition start time.

$d_{RX}(X_s, x_i)$ is expressed as:

$$d_{RX}(X_s, x_i) = \sqrt{(x_i - x_s)^2 + z_s^2} \quad (7)$$

The analog signals received by the transducer, s_i , are demodulated by downmixing (conversion of the analog signal to its real and imaginary components, or In-phase, I, and Quadrature, Q, components) and low-pass filtering. The final beamformed intensity for a given pixel is obtained through a summation:

$$s_b f(X_s) = \sum_{i \in [1, N_e]} s_i(\tau_i(X_s)) \quad (8)$$

1.2.2 Contrast Enhanced Ultrasound Imaging

To enhance the existing merits of ultrasound, Contrast Enhanced Ultrasound (CEUS) uses contrast MBs to significantly improve the signal to noise ratio between its immediate surroundings and endogenous features (Ferrara et al., 2007). CEUS exploits two mechanisms: 1) Strong linear response from acoustic reflections at the gas-liquid interface as described in Eq. (4), 2) Unique non-linear response in the formation of super-harmonic $n f_c$ ($n \in 2, 3, 4, \dots$ integer multiples of f_c), ultra-harmonic, ($n \in 1.5, 2.5, \dots$ fractional multiples of f_c), or sub-harmonics f_c/n (integer fractions of f_c) through the interaction of the oscillating MB with the transmitted wave (Gong et al., 2005). CEUS is vital for imaging highly vascular tissue in clinical settings. For example, the environment inside liver focal lesion presents the ideal case to use MBs because of the need to differentiate irregular vessel formations within vascularized tissue (Ferrara et al., 2007).

The presence of MBs also significantly decreases the energy barrier required for acoustic cavitation to take place to which the acoustic cavitation of these agents directly impact their stability under ultrasound imaging. To explain this effect in greater detail, acoustic cavitation in a homogenous medium such as pure water occurs when the local pressure dips below that of the fluid vapor resulting in the formation of a cavitation nucleus. Where the formation of a nucleus facilitates the growth and eventual collapse of the vapor bubble resulting in local temperature and pressure changes, as well as fluid jet and shockwave formation (Wu et al., 2022). A heterogenous solution containing pre-existing nuclei such as free gas bubbles or gas stabilizing defects bypasses the need to reach the fluid vapor pressure allowing for the direct growth

into collapse of those existing nuclei. With this context in mind, MBs act as nuclei in heterogenous acoustic cavitation and can be similarly popped to create regions of low-pressure cavitation to induce physical effects on its surroundings (Rich et al., 2022). Localized damage caused by cavitation induced phenomena known as sonoporation has been leveraged in new approaches in therapy to exploit the transient bioeffects that rupture can impart. Briefly, such effects include temporary blood-brain-barrier opening (McMahon & Hynynen, 2022), cell membrane perforation and cell destruction (Chen et al., 2014; Kooiman et al., 2020). It is key to note that permanent destruction of MBs is possible at sufficiently high-pressure pulses. The Mechanical Index (MI) is a dimensionless metric used to monitor the mechanical output of an imaging transducer (Kollmann, 2013):

$$MI = \frac{P_r}{\sqrt{f_c}} \quad (9)$$

where f_c is the transmission frequency and P_r the peak rarefaction pressure. To prevent unwanted popping of MBs during a CEUS scan, images are taken at MI threshold value below ~0.2 to 0.3 (Jang et al., 2013; Porter et al., 2018; Porter Thomas R. & Xie Feng, 2010). The popping effect can be further exploited in imaging to determine the rate of perfusion of contrast agents into the visualized cavity through a destruction and replenishment cycle.

MBs have seen prolific use within the past decades. Bubbles in ultrasound were observed with “bubble studies” involving injections of agitated saline paired performed during echocardiograms of the heart over 30 years ago. More complex MBs have since been approved for commercial use and adoption by clinics around the world. Table 1 denotes commercially available MBs and their variations in formulation:

Microbubble Brand	Composition	Company of Origin	Region of Availability
Definity	Octafluoropropane, Lipid shell	Lantheus Medical Imaging	North America, Europe

SonoVue	Sulfur hexafluoride, Lipid shell	Bracco Imaging	Europe, Asia
Optison	Octafluoropropane, Albumin shell	GE Healthcare	North America
Lumason	Sulfur hexafluoride, Lipid shell	Bracco Imaging	North America, Europe
Sonazoid	Decafluorobutane, Lipid shell	GE Healthcare	Asia Norway

Table 1. Summary of common commercially available MBs with short breakdown of composition and sources of availability.

As shown in Table 1, clinically approved MBs are typically comprised of polypeptide, phospholipid, or polymer stabilizer shell with variations of medically inert gases such as perfluorocarbon or sulfur hexafluoride. Each variety of MB coating has merit (Navarro-Becerra & Borden, 2023; Sirsi & Borden, 2009):

- 1) Phospholipid coatings are thin and soft (on the scale of 3-5 nm) and are the most responsive to acoustic radiation of the three coating materials (Navarro-Becerra & Borden, 2023). Consequently, they are the best suited coating for contrast imaging. As an aside, the thin shells of the lipid stabilized MBs also result in an inherently lower drug loading capacity and lower circulation lifetime. Further the amphiphilic nature of phospholipids results in polarized drug loading onto the membrane. Lipophilic drugs are limited to loading within the lipid tails whereas hydrophilic drugs prefer the solution facing headgroup. These limitations can be somewhat mitigated by tuning coating characteristics with longer chain lipids and modifying the surface architecture of the coating.
- 2) Protein coated microbubbles offer a middle ground in their thicker (15-150 nm) shells (Navarro-Becerra & Borden, 2023). The stiffer shell offers a lower acoustic response than lipid MBs but has a stronger capacity for drug loading. It follows that these coatings can also be tuned for higher drug loading capacity or other functions. MBs inherently suffer from elimination by the immune system

where exogenous proteins such as albumin are a particular cause for concern. The addition of polyethylene glycol has been shown to cloak these MBs from immune response (Upadhyay et al., 2017).

- 3) Polymer coated MBs have the thickest and stiffest coating layer of the common coatings (50-500nm) (Navarro-Becerra & Borden, 2023). This characteristic results in the largest drug carrying capacity of the coating groups with a non-ideal acoustic response for imaging ultrasound because of the rigidity of the shells.

A rough summary of coating choice on MBs functionality is that MBs with thicker coatings have longer circulation times at the cost of their imaging performance. While part of the choice to use phospholipid shelled bubbles with perfluorocarbon core in this work is because of a precedent within the lab. The rapid clearance and superior acoustic response of lipid MBs also make them the most practical choice of a clinical imaging agent.

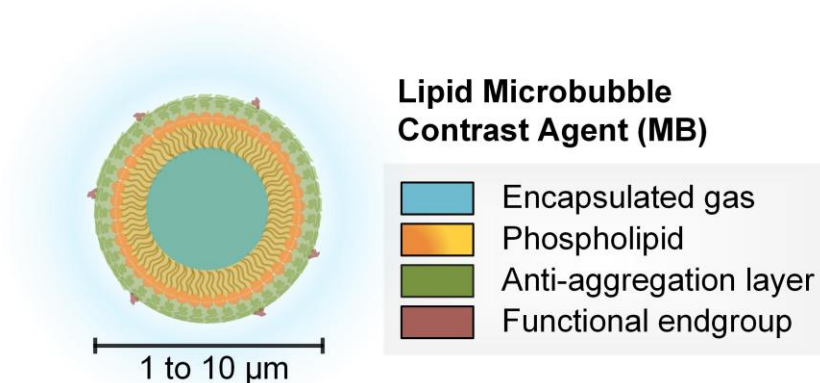


Figure 1.1 A sketch of a phospholipid coated microbubble (MB) noting the encapsulated gas, phospholipid, anti-aggregation brush layer and functional end group. Monolayer elements are not depicted to scale.

The necessity for a coated MB may be simply explained: 1) MBs are inherently thermodynamically unstable given the large Laplace pressure resulting from their small size, 2) The pressure difference causes gas particles to diffuse or solubilize into the surrounding solution (Duncan & Needham, 2004; Epstein & Plesset, 1950), 3) Surface interactions drive further destruction through aggregation or coalescence (Segers et al., 2017). Practically 1) and 2) cause gas molecules to diffuse out from small bubbles and are taken up

by larger bubbles in a process called Ostwald Ripening (Uematsu, 2025) whereas surface modifications such as PEGylation can indirectly improve resistance to 3) through the introduction of forces such as Osmotic repulsion.

The corresponding solution to the loss of gas to diffusion are thus: 1) Adding a stabilizing shell to reduce the surface tension as briefly described below:

$$\Delta P = \frac{2\gamma}{R} \quad (10)$$

eq. (1) the surface tension, γ , is proportional to the rate of diffusion through the driving pressure difference. This is further expanded upon in the Gibb-Thomson relation describing the equilibrium surface concentration in relation to particle size and surface characteristics (Baldan, 2002):

$$C_R = C_e \exp \left[\frac{2\gamma V_{molar}}{R_B T} \frac{1}{R} \right] \approx C_e \left[1 + \frac{2\gamma V_{molar}}{R_B T} \frac{1}{R} \right] \quad (11)$$

with C_R and C_e as the solubility of the species at the surface of a particle with the radius R , and at a plane surface respectively. V_{molar} as the molar volume of the species, R_B and T as the ideal gas constant and absolute temperature. Where the relation describes an increase in species solubility at a surface inversely proportional to the particle's size.

and 2) exchanging the solubility of the gas of the bubble to heavier less soluble gases to mitigate outward diffusion. The improvement in MB stability because of changes to both shell and core gas is well documented to be more stable than those used for bubble studies in the past (Ferrara et al., 2007).

Disease pathologies are often associated with molecular biomarkers that are also exposed to the blood solution. Thus, using CEUS with MBs imparted with the function to target such markers has become a topic of recent interest (Navarro-Becerra & Borden, 2023). Examples of such markers include Vascular Endothelial Growth Factor (VEGF) and integrin where both are associated with abnormal vascular generation to support the large metabolism of tumors. Methods of MBs functionalized with anti-VEGF Abs or cyclic RGD proteins corresponding to the biomolecules have been reported within the literature (Li et

al., 2019; Rojas et al., 2018; J. Zhong et al., 2023). Likewise, targeted commercial MBs have also been marketed by companies such as Bracco's BR55 (Smeenge et al., 2017).

Conjugating ligands to MBs vary between passive methods of binding through charged or intermolecular interactions or through direct covalent bond to the MB (Hermanson, 2013a, p. 3; Navarro-Becerra & Borden, 2023). An example of the former comes with using phospholipids cationic heads to passively bind negatively charged particles such as DNA or charge compatible polypeptides. Although not a covalent bond fitting the latter, avidin-biotin's conjugate structure finds prolific use in target MB research because of its high bond strength (Hermanson, 2013b, p. 11; Navarro-Becerra & Borden, 2023). Regardless of the method of attaching a ligand to the bubble, the choice of binding the MB to the target site is equally important. Like charged ligand binding, passive targeting of charged pathologies such as negatively charged cancer cells or active modes using antibodies or proteins specific to a molecule related to the pathology as mentioned previously. In summary, targeted CEUS forms a next step highlighting abnormalities caused by disease but often require knowledge of the pathological physical or biomolecular conditions associated with the disease to appropriately choose the targeting strategy.

1.2.3 Venous Thrombosis and Clotting

Blood flow is essential for material transport through the human body and the systems involved in maintaining this network are equally important. Of relevance to this work is the clotting cascade. At the scale that we typically observe, this ensures that minor cuts or bruises do not result in life threatening injury. More specifically, blood clotting forms the first portion of the wound healing response before more permanent tissue is settled to close the wound (Troisi et al., 2023). This process can be compared to a temporary scaffold placed around a building in construction. Part of the long-term healing process involves the activation of proteins for the breakdown of the clot structure. Overall, this system is maintained by procoagulant and anti-coagulant processes to ensure that clotting is well managed. However, unwanted blood clotting does occur and can be fatal if untreated.

The cause of abnormal clotting can vary. Often patients can be at higher risk of developing unwanted blood clots due to factors such as pregnancy, old age, immobility, cancer, obesity, medication, or genetic disposition towards clotting (Stone et al., 2017). Regardless of the cause, clotting can occur in both arteries and veins with differences in clot make up between the two. Arterial clots are rich in platelets and venous clots contain more fibrin (Lippi, 2018; May & Moll, 2024). Treatment of the respective clots lean towards anti-platelet medication for arterial clots and anti-coagulant for venous clot management. Conceptually, these strategies reduce the continued build-up of each clot type relying on the body to manage a gradual breakdown. In serious VTE cases thrombolytic agents can be introduced via catheter at the clot to increase clot breakdown to a manageable level or clots can be surgically removed from the vessel (Ortel et al., 2020). However, the more intensive options here pose a sizeable risk to the patient. Drugs used for thrombolytic treatment directly destroys fibrin indiscriminately meaning that patients are at risk to hemorrhaging from the loss of healthy clotting located elsewhere in the body (Ouriel et al., 1995). An additional complication is that thrombi may also recur post-operation because of clotting's role in the body's natural healing process after surgery. In summary, managing abnormal clotting at the early stages allows clinicians to avoid complications associated with aggressive means of clot treatment such as unintended death by hemorrhaging through thrombolytics or post-operative recurrence. At the core the issue stands that early diagnosis and early management allow for more patient friendly treatments. To segue back to medical imaging despite access limitations outside of the extremity's ultrasound stands to be "old reliable" when it comes to diagnosis here. Accordingly, the choice in tackling deep vein thrombosis (DVT) in this work is because of Ultrasound's dominance in thrombosis diagnoses at the limbs.

1.2.4 Current Clot Diagnosis Pathway and Limitations

For suspected thrombosis or pulmonary embolism (PE), possible patients are first scored through the Wells Score questionnaire for DVT risk (Lim et al., 2018). In greater detail, the Wells Criteria covers symptoms associated with the physical effects of vessel occlusion that can be immediately identified without imaging with examples such as 1) swelling/redness caused by decreased venous return and local vessel damage 2) formation of non-varicose collateral veins to restore venous return. Negatives to the Wells

Score are further screened by the D-dimer test which looks for elevated levels of dissolved clot biomarkers in the event of a false negative. Positive patients are then diagnosed by ultrasound through the physical characteristics of a clot. Specifically, leveraging B-mode ultrasound to image the compression response of a suspect vein by pressing at the location with the ultrasound probe as well as visualizing flow within the vessel for signs of occlusion (Needleman et al., 2018). Overall, ultrasound is a well incorporated tool within a typical DVT treatment pipeline and is especially valuable because of its ease of accessibility compared to its peers in x-ray venography or magnetic resonance imaging.

However, ultrasound's more heavyweight contemporaries have seen recent developments to address a core need of clot characterization within treatment. Intuitively, since the current non-invasive clinical treatment pipelines typically attempt to control fibrin accumulation or degradation, it is the key target to visualize in venous clot treatment. Strides in MRI and PET research have shown that fibrin targeted contrast for those specific modalities can offer more information than that achieved in the clinic. In ultrasound's case the proteins responsible for fibrin binding can be readily adapted to use with MB contrast with the bioconjugation techniques available for protein loading.

1.2.5 Focus in Ultrasound with Clots is Treatment Centric

Although therapy is not the focus of this work, it is necessary to acknowledge that much of current research interest for thrombosis in general ultrasound (and fibrin targeting ultrasound by extension) is shaped through a therapeutic lens. Some examples of these strategies leverage ultrasound to physically disrupt the fibrin of acute clots or even collagen of fully retracted clots presents a non-pharmaceutical alternative to traditional thrombolytic treatment. Cavitation histotripsy provides a viable method for breaking up stubborn clots even in a retracted state (Bansal et al., 2025; Xu et al., 2024). Initial human trials for using invasive catheter-based methods to ameliorate uptake of traditional drug based thrombolytic therapy in retracted clots show much promise in improving patient QoL although the bleeding risks associated with tPA still presents itself (Garcia et al., 2020). Current methods of aspirating the site are notably more invasive through the use of direct mechanical manipulation or fluid aspiration, It follows that

current developments in ultrasound-based clot treatment follow the line of logic in significantly reducing the risk associated with the worst-case scenario of contemporary tPA treatment (Vedantham Suresh et al., 2017). Naturally, the ability to identify and spot treat clots can bring a new image guided therapy approach to clot treatment. There is an irony in using state-of-the-art treatment with ultrasound but relying on the old horse of duplex ultrasound and questionnaires to indicate the treatment and to survey the outcome. While it must be reiterated that the therapeutic dimension was not explored in this work, the above both emphasizes the reliability of ultrasound and highlights the gap in advancements between ultrasound's role in treatment and prognosis.

1.3 Central Hypothesis

I hypothesize that MB ultrasound contrast can improve the detection of fibrin surfaces imaged on a clinical ultrasound scanner in addition to enabling the assessment of next generation imaging solutions.

This will be accomplished through the following:

1. **Modular ligand-based fibrin targeting.** The tunable coating of MBs allows flexible changes to the fibrin targeting strategy. This allows for quick pivots to ligand choices that are optimal for the situation at hand according to new findings in research.
2. **Using a physiological relevant testing platform.** By controlling the flow and chemical inputs of testing model, changes in MBs can be systematically evaluated for individual changes in MB or imaging algorithm design.

1.4 Thesis Outline

Chapter two outlines general test protocols and theories that were common to all the subsequent chapters and modules. The chapter includes the ground-work MB fabrication and characterization set up to enable **Module #1** but also used in all following chapters, MB size selection used in **Modules #4** and **#5** to remove excess liposomes prior to decorating the MBs with ligands.

Chapter three describes **Module #1**, where undecorated ultrasound contrast was used for super resolved imaging in a novel *in vitro* representation of branched microvessels. The newly developed ultrasound imaging phantom allowed for a ground truth assessment of a super-resolution algorithm.

Chapter four documents **Module #2**, which outlines the creation of a novel fibrin targeting ultrasound contrast to allow the investigation of fibrin's presence using a clinical scanner. By leveraging the existing fibrin targeting capability of an inhibited therapeutic in tissue-type plasminogen activator and a recently developed MB conjugation technique, fibrin targeting capability was imparted onto ultrasound contrast.

Chapter five details **Module #3**, which compares common types of fibrin-targeting ligand strategies used in ultrasound contrast literature. The imaging guided therapy potential of MBs is a recent topic of interest where contrast agents can function to both improve imaging and act as therapy carriers. Given the treatment centric focuses with contrast agents in this field, this study aims to determine if residual enzyme activity of inhibited tPA makes for an unviable ligand when strictly used for imaging applications.

Chapter six summarizes the contributions in each work and lists current limitations within those works and their methodologies as well as future directions that those studies can be taken in.

Chapter 2

2.1 Introduction

This chapter details the common experiments carried out throughout the thesis document to avoid repeats in chapter specific sections and to give some context to the readers. These experiments are well documented within ultrasound contrast literature but falls outside layman knowledge.

2.1.1 Contrast Microbubble Fabrication Overview

Phospholipid microbubbles were prepared in a two-step process involving 1) Solubilization in a liposome precursor solution, 2) Mechanical agitation to form microbubbles. This process is summarized in **Figure 2.1**. To elaborate on this process, isolated phospholipids are commonly purchasable in either powder form or dissolved in an organic solvent and both forms are difficult to safely dissolve in aqueous solution

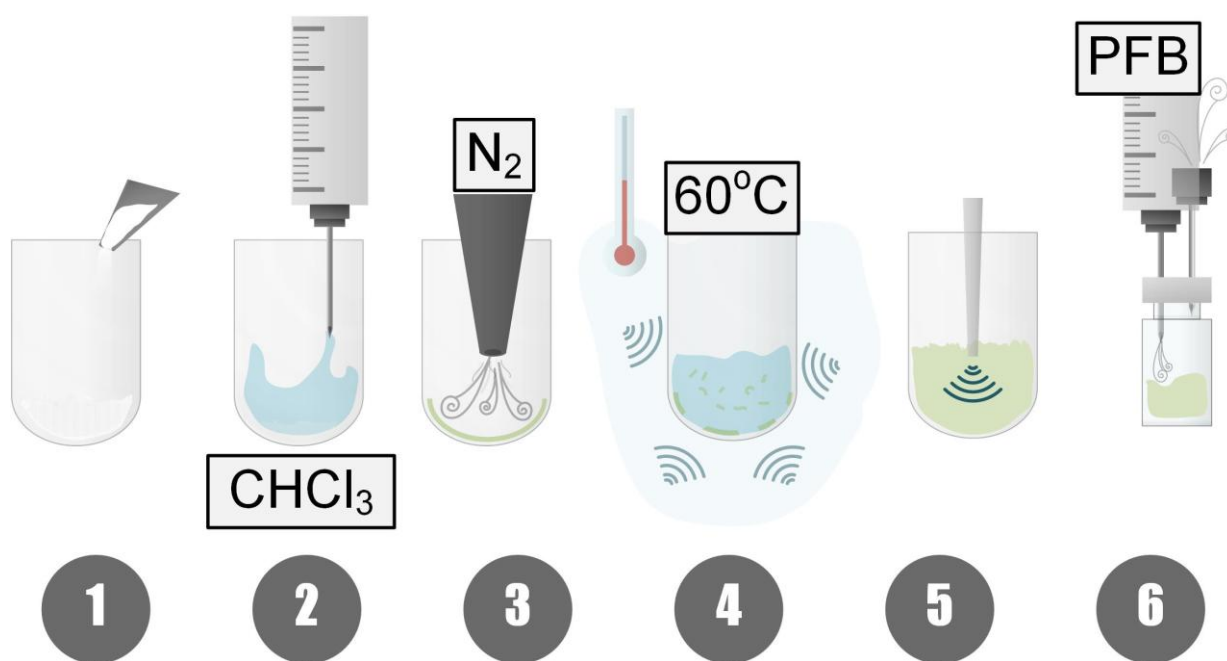


Figure 2.1. Cartoon overview of liposome vial preparation before mechanically shaken into bubbles. 1) Measurement of solid phospholipid powder, 2) Dissolution of powder into organic solvent (chloroform), 3) Removal of organic solvent by aspirating with nitrogen gas and vacuum at 24 Torr, 4) Initial dissolution of phospholipids in storage medium by bath sonication at 60°C (DSPC LC-Gel Transition Temperature), 5) Solution homogenization using horn sonicator, 6) Aliquoting liposome solution and replacement of air headspace with perfluorobutane gas.

without additional steps. For the chosen process in this work, liposome solutions were formed through the

film hydration process where: 1) Powdered phospholipid were measured and mixed before dissolution into chloroform, 2) Solvent evaporated to form a phospholipid film (**Figure 2.6**), 3) Hydrated in an aqueous solution and solubilized with bath and horn sonication to homogenized the solution (**Figure 2.2-3**). Following solubilization, the solutions are then aliquoted into vials for storage and later use. Vials were agitated using a dental amalgamator (**Figure 2.4**) to create microbubbles for imaging or modification.

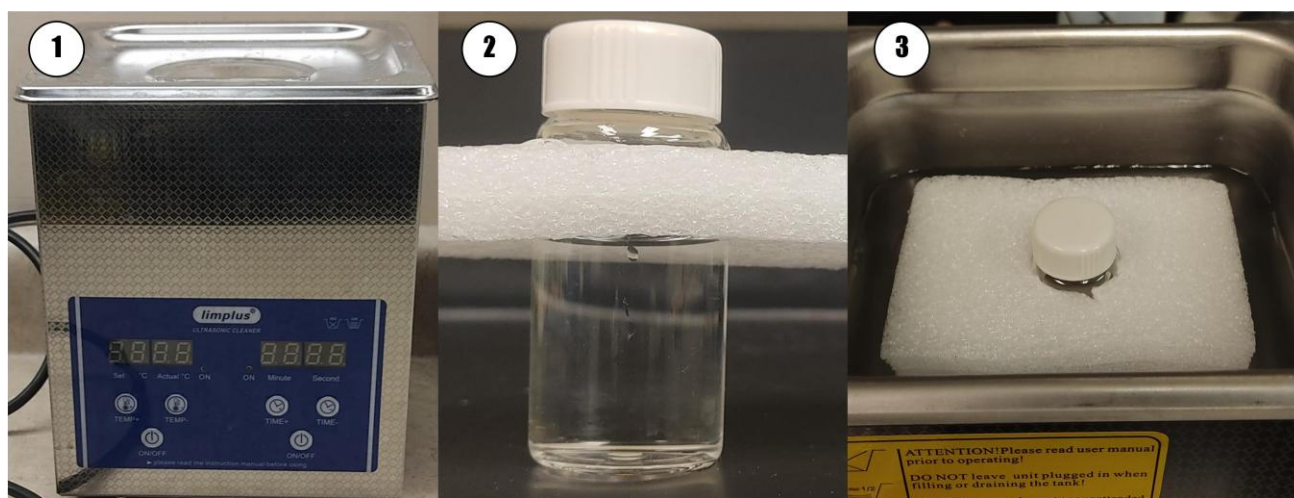


Figure 2.2. How the bath sonicator is used in this thesis: 1) Exterior of bath sonicator for temperature and sonication time controls, 2) Vial donning a buoyant section of packing insulation to ensure the contents are positioned below the waterline, 3) Floating the vial inside the sonicator, water level was made sure to be above the heating element of the device.

2.1.2 Gas and Phospholipid Choice for Design

Microbubbles are inherently unstable because of 1) High surface area to volume ratio resulting in a large Laplace Pressure driving diffusion of gas out of the bubble (Ostwald effect), and 2) Drive to aggregate forming a larger more thermodynamically stable structure (Ferrara et al., 2007; Uematsu, 2025). However, the overall stability outcomes of MB blends are well established within literature. Most common in-house lab blends of MBs are often adjacent to clinically approved commercially available MBs. The in-house MBs used in this work are based on Definity® MBs produced by Lantheus which are lipid perfluorocarbon based systems and are differentiated by using higher MW gas cores and phospholipids.

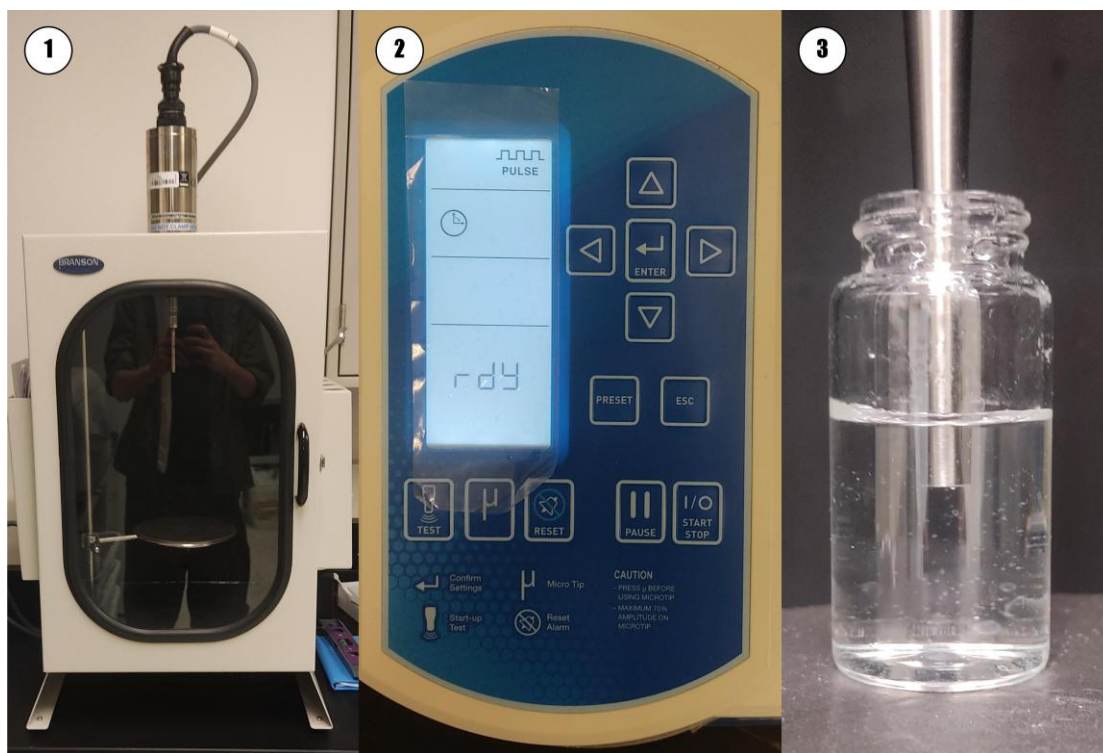


Figure 2.3. Overview of the Branson Horn Sonicator used in this thesis: 1) Tip sonicator enclosure without an attached tip, 2) Tip sonicator was operated on pulse mode and time controlled, 3) Tip sonicator platform was position so that the tip was immersed to 1/3 of the depth of the vial.

2.1.3 Gas Core Choice

Starting with the gas, gas cores with lower blood-gas partition coefficients as with the decafluorobutane used in this work further reduces outward diffusion. Perfluorocarbon gas core choice is somewhat trivial with a trend of more stable microbubbles towards higher molecular weight fluorocarbons. Of the choices available, decafluorobutane is the highest molecular weight perfluorocarbon that is a gas at room temperature.

2.1.4 Phospholipid Choice

Generally, coated microbubbles are more stable than uncoated bubbles through a reduction in surface tension and the overall theory behind phospholipid coating choice is also simple. Phospholipids with longer fatty acid chains have stronger van der Waals interactions and thus have more physically rigid bubble monolayers than shorter chain lipids (Navarro-Becerra & Borden, 2023). More rigid monolayers are noted to have a higher liquid crystal transition temperature which has some impact on the mixing/annealing temperature used for a liposome precursor. It is noted that lipid chain length is consistent

within literature with common lengths at 16 (palmitic) or 18 (stearic) carbons. The 18-carbon chain saturated distearic phospholipid was used to carry on previous precedents within the lab as well as for its commercial availability.

Around 90 mol% of lipids used in the mixture have zwitterionic (neutral charge) phosphatidylcholine heads as the matrix lipid. The matrix makes up the bulk of the monolayer and is important for its general structure. The remaining 10% is composed of phosphatidylethanolamine groups functionalized as received with a polyethylene glycol bridge. The bridge molecule serves two purposes in the shell design: 1) The large chain reduces MB aggregation through steric hindrance (spatially blocking aggregation + coalescence) or hydration repulsion (diffusion gradient of water into the space between two hydrophilic molecules), 2) Functionalized end group on the polyethylene glycol enables future covalent linkages to functional proteins. For pure imaging applications, DSPE with a methyl (-CH₃) end group was used as a generic low interaction end group for the remaining 10 mol %. In functionalized cases, 5% of the PE lipids used bioconjugate compatible end groups with the remaining 5% as methyl capped.

2.1.5 Microbubble Processing and Outcomes

Phospholipid MBs are formed by continuously disrupting the monolayer at the gas-liquid interface causing pinch offs to enclose a portion of the gas headspace. Accordingly, the degree of control over the size characteristics of pinched bubbles are dependent on the consistency of the pinch off method. Flow based methods such as membrane extrusion or flow focusing enable a high output of bubbles of a narrow size range. These “monodisperse” bubble are generally valued within this field because size is a key factor when considering the physical interactions of ultrasound with MBs. For example, the resonance frequency of an unencapsulated bubble is described by the Minnaert formula as (Doinikov et al., 2009):

$$\omega_0 = \frac{1}{R_0} \left[\frac{3\gamma P_0}{\rho_0} + \frac{2(3\gamma-1)\sigma}{\rho_0 R_0} \right]^{1/2}$$

describing an inverse relationship of resonance frequency ω_0 to a gas bubble with radius R_0 and specific heat γ in a liquid medium of density ρ_0 and hydrostatic pressure P_0 . Having MBs of similar sizes

through a monodisperse production method has unique benefit in a more efficient response to specific inputs of ultrasound. On the other hand, mechanical methods such as mechanical agitation or interface horn sonication produce MBs that have a polydisperse distribution. These MBs are created through stochastic pinch offs at the interface as well as simultaneous breakdown of larger bubbles deeper within the solution.

Despite the advantage of monodisperse creation methods, a mechanical process was chosen for this work strictly for infrastructure reasons. To elaborate, large quantity of liposomes sharing the same coating material are often retained alongside processed bubbles. Consider that the echogenicity of a liposome is significantly lower than a MB. In a targeting imaging context such as this, having liposomes in solution consuming ligands that would otherwise be reacted with echogenic MBs would significantly reduce the process yield of targeted ultrasound agents. It was until fairly recently that a method for liposome washing that did not disrupt the monodispersity of input MBs was developed (van den Broek et al., 2024). On the other hand, the predominant method of size selective centrifugal washing improved the monodispersity of polydisperse input MBs. With the context in mind, since the output of monodisperse MBs after a necessary centrifugal washing step was likely similar, the mechanical agitation method was chosen because it is difficult to justify using monodisperse MBs if the process was limited by the wash step.



Figure 2.4. Examples of dental amalgamators used to form MBs through mechanical agitation. Two commercial amalgamators sold for TRUST Biosonic bubbles are pictured and operate at a static 4.4kHz. An amalgamator purchased from Amazon with adjustable shake rates. We chose a shake rate of 4.4kHz to be consistent with the commercial bubbles used at our lab.

2.1.6 Size Selective Washing

Excess coating liposome were removed through the centrifugation method (Feshitan, Chen, Kwan, & Borden, 2009). Briefly, polydisperse microbubbles were drawn into a clean 6ml syringe and diluted to 5ml. Excess “foam” that easily form a dense white layer were removed by allow the foam to gather at the outlet and manually removing by pushing the plunger or wiping with Kim-wipes. Remaining foam or large bubbles in the solution were removed by pulling air into the plunger and vigorously hitting the side of the syringe against a hard surface to force the foam to coalesce into larger bubbles which were then removed. MBs were then swirled by hand within the syringe to suspend MB sediment to finish preparing the sample for selection.

Differential centrifugation works by exploiting the Stokes Rise Principle to accentuate the effects of buoyancy differences between MBs of different sizes (Kvåle et al., 1996):

$$v_i = \frac{2(\rho_2 - \rho_{1i})}{9\eta_2} R_i^2 g$$

describing the relative velocity v_i of a sphere with size bin i with radius R_i in a fluid with density ρ_2 and viscosity η_2 under the influence of gravity g . Wherein g is represented by the relative gravitation

force (RCF) in the context of a centrifuge. Velocity can further be broken down into the distance traveled by the microbubble inside the syringe as well as the centrifugation time. Given these inputs, a RCF can be calculated for the procedure to isolate populations based on their size R. It should be noted that the force associated with the machine's rotations per minute (RPM) varies on the arm of the centrifuge bucket and should be converted to a force value for this to work.

The following protocol was modified from the protocol developed by the Borden Research Lab hosted ultrasound contrast agent summer camp for knowledge sharing. RCF values were selected for cutoffs at rough 0.8 to 3 μm . The upper population was removed by placing a capped syringe outlet faced upwards (**Figure 2.5 (1)**) allowing large MBs to gather at the nozzle for removal under 15 RCF spin for 5 minutes. The non-foam contents were then transferred into a clean syringe using a 14-gauge needle, topped to 5ml and capped. The lower population containing small bubbles and liposomes were separated by placing the capped syringe with the plunger side up at 140 G for 5 minutes (**Figure 2.5 (2)**). The contents of the selected MBs were expelled leaving the foam near the plunger. The foam is then suspended in the diluent solution to a working volume for use or reactions.

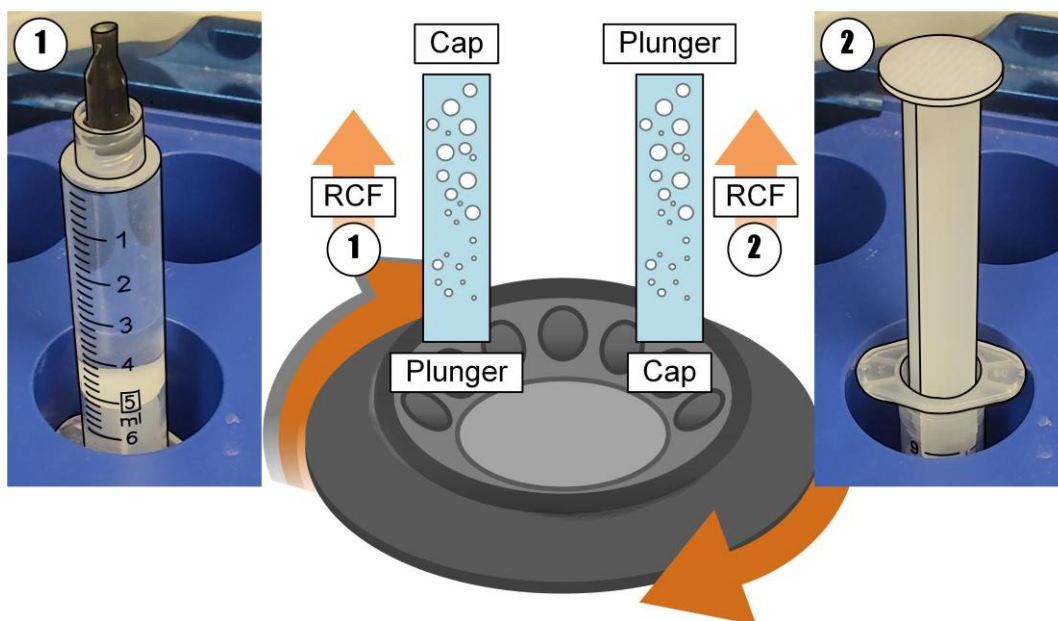


Figure 2.5. Aid to visualize syringe orientation and MB separation under the influence of centrifugal force, 1) MBs rise towards the cap of the syringe under the influence of the centrifuge to expel oversized foams, 2) MBs float towards the plunger to form a foam cake allowing users to expel the non-MB fraction of the solution.

2.1.7 Thin Film Hydration Specific Notes

While MB formation occurs at the gas liquid interface it is also important to consider the rest of the solution because of a need to replenish phospholipids forming the monolayer during the process. Presence of excess coating liposomes is key to ensuring the pinched MBs were coated during the agitation process. At present, thin film hydration is the least complex method solubilizing phospholipids. In most circumstances, hydration can be directly ended after bath sonication at slightly above DSPC's gel-liquid

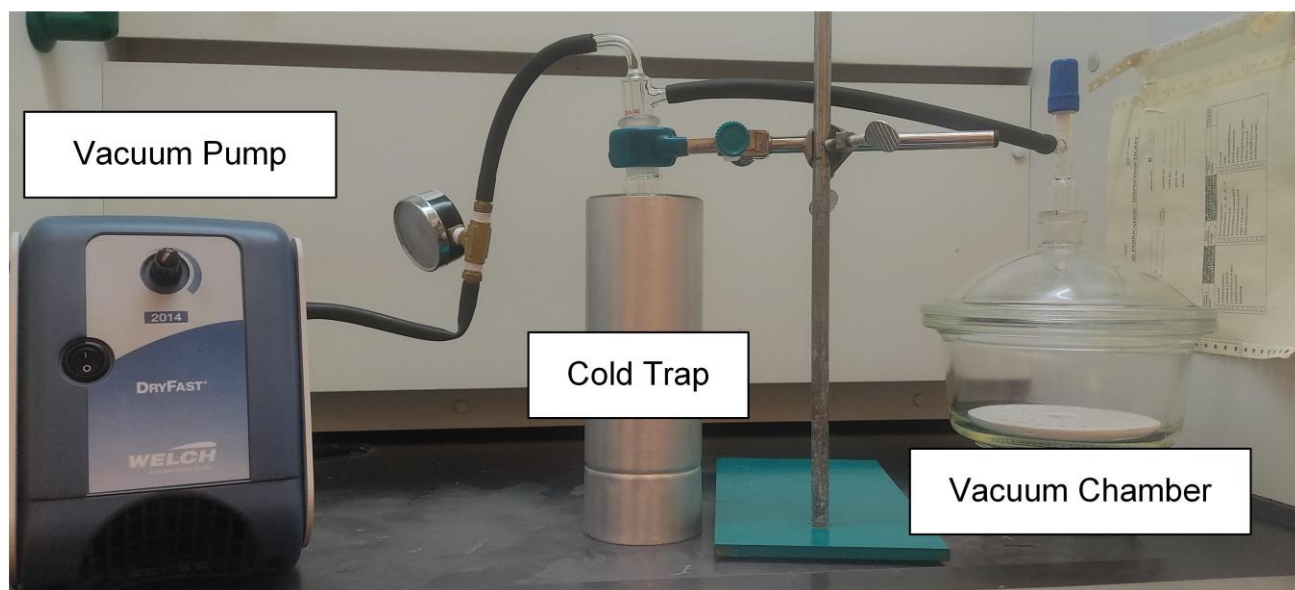


Figure 2.6. Simple vacuum chamber setup used to remove excess organic solvent in this study. A chemically resistant pump and vyton tubing were used in combination with a dry-ice ethanol cold trap to improve the lifetime of the pump.

crystal transition temperature of 55°C for around 60 minutes. The low energy output of bath sonicators does result in the formation of larger lamellar particles which is observed as cloudiness within the solution. Here, a horn sonicator was used to further reduce the size of these lamellar particles to improve the transparency of the solution. However, to my knowledge, reducing the size of the lamellar vesicles in the solution only improves the visual appearance of the mixture. Given that shaking bubbles can be a black box compared to methods like flow focusing it might not be worth the time investment to look at these effects. It is noted that the coating phospholipids can be directly mixed into solution with a horn sonicator to save time by skipping film hydration altogether but is sometimes inconsistent with powder being fully solubilized. In

addition, a notable amount of heat is added to the solution when using a horn sonicator or a bath sonicator which can degrade the lipids over time.

2.1.8 Horn Sonicator Specific Notes

Overall, horn sonicators are known to produce titanium nanoparticles as a potential contaminant. The particles can adsorb molecules in solution such as ligands, or other proteins which may impact reactions or general outcomes of these reactions. Given the generous use of horn sonicators here there may be some accumulation of titanium particles in the stock liposome solution but accounted for by controls. Finally, the titanium particles will disrupt reagents such as the thiols within the maleimide thiol reaction hence the reason it is not used in this work. A time-based pulsed homogenization scheme was used to allow for the solution to cool between each pulse to mitigate temperature increase in the absence of an ice bath.

2.2 Characterization

2.2.1 Microbubble Concentration

Haemocytometers are fixed volume microscope compatible measurement devices to determine the concentration of micron scale particles (Vembadi et al., 2019). Concentration measurements were commonly taken in this work because many characterization methods have specific concentration requirements. Samples were tested following manufacturer protocols where: 1) 10 μ l of sample and 10 μ l of a diluted version is pipetted into respective haemocytometer chambers to be examined, 2) Once the concentration was at a level where particles can be easily differentiated 48 to 64 images of the chamber's subdivisions were taken, 3) Images were then processed in ImageJ and particles were manually counted or with an automated ImageJ extension, 4) The previous step was repeated for each block containing 16 subdivision having the mean count value taken over 3 to 4 sets of blocks, 5) Mean count was finally converted to concentration by accounting for dilution and multiplying by 10^4 following the haemocytometer principle.

2.2.2 Microbubble Size Distribution

The principle behind dynamic light scattering is that Brownian motion contributes to intensity fluctuations within the scattered light from a particle hit by a monochromatic source (Stetefeld et al., 2016). Where, the intensity of the fluctuations is related to a translational diffusion term relating to the viscosity of the solution, temperature and the hydrodynamic radius of the particle. Thus, the size of a particle can be obtained through DLS given a known temperature and viscosity. This coefficient D_τ is described by the Stokes-Einstein-Sutherland Equation:

$$D_\tau = \frac{k_B T}{6\pi\eta R_h}$$

with Boltzmann coefficient k_B , absolute temperature T , viscosity η , and hydrodynamic radius R_h .

Testing protocol was as follows: 1) MB concentration was diluted with PBS, 2) A cuvette was thoroughly rinsed and filled to level with a diluted sample, 3) sample was tested at a given dilution and repeated at a further dilution if appropriate. It is noted that DLS cannot differentiate the contents of a micron/submicron particle and will only determine the size. Given the high fraction of liposomes found alongside agitated MBs, excess coating liposomes will typically skew DLS measurements to submicron scale despite the presence of microbubbles. With the above in mind, DLS measurements were taken strictly after centrifuge washing. Recent technologies such as the resonant mass measurement method can differentiate between the contents of these particles. Alternatively, other cell/bacteria targeted sizing methods such as direct measurement on microscopes or Coulter Counters can also be used as a substitute.

The specific DLS device used was a common-use Malvern Nanosizer from Professor Liu Juewen's Lab Fig. One key point of experience was that results from the device were reported in the number weighted count value when isolated bubbles were examined. Intensity weighted counts have some value because of a key difference in the scattering dynamics of liquid-liquid and liquid-gas interfaces and can be useful when looking at a mixed population such as a non-size selected sample. The refractive index of the particle core material is also important to consider here in order compensate for changes in backscatter intensity.

However, since most literature on decafluorobutane's refractive index is in the liquid state, the refractive index of decafluorobutane gas was assumed to be equivalent to air while in the gas state.



Figure 2.7. A collection of characterization equipment used in this work from Prof. Juewen Liu's Lab in the Chemical Engineering Department at the University of Waterloo: a) Malvern Zetasizer Nano ZS Dynamic Light Scattering device, b) Agilent Cary Eclipse fluorescence spectrophotometer, c) Tecan Spark multimode microplate reader.

Chapter 3

3.1 Introduction

Ultrasound localization microscopy (ULM) is a super-resolution imaging technique based on contrast echo localization (Betzig et al., 2006), where the accumulated image of spatially isolated microbubble (MB) contrast signals over multiple frames enables the resolution of vascular features smaller than the diffraction limit. This technique facilitates the visualization of complex sub-millimeter scale vascular features (J. R. McCall et al., 2022; S. Lei et al., 2022) and may provide velocity information within these features (B. Huang et al., 2023; Opacic et al., 2018). Visualization of microvessels is after all important because it may provide insight on pathologies such as cancer and atherosclerosis (Lin, Shelton, et al., 2017; Yu et al., 2018). It may specifically help to elucidate microvascular development during disease progression, and it opens the possibility of tracking treatment response via vessel recession (Fukumura & Jain, 2007; Mulligan-Kehoe, 2010; Yu et al., 2018). Microvascular imaging of human subjects has also shown experimental success for breast cancer and lower limb vascular visualization (Opacic et al., 2018; S. Harput et al., 2018). Nevertheless, in humans, ULM image quality is known to be affected by various parameters including contrast delivery concentration (Hingot et al., 2019; K. Christensen-Jeffries et al., 2019) and contrast agent selection (Ghosh et al., 2017; Lin, Tsuruta, et al., 2017).

In designing a ULM algorithm, one experimental question that needs to be addressed is how to methodically characterize the algorithm's microvascular resolving power. Present in vivo testing methodology typically involves a comparison between the ULM-resolved vascular features and the corresponding optical microscopy images at the same site (Christensen-Jeffries et al., 2020). Yet, such a comparative analysis is challenging to perform when examining vascular features that are deeper than the penetration limit of optical microscopy. Some studies have performed post-hoc comparisons of the effects of novel algorithm implementations (J. R. McCall et al., 2022) and testing methodology (Hingot et al., 2019) on localization outcomes, but they are inherently unable to determine a ULM technique's resolving power due to the lack of ground truth knowledge on the microvascular anatomy. Alternatively, long duration

acquisition studies show promise in generating across reference for accuracy assessment. Nonetheless, for this approach, practical hurdles are present in vivo because, over the duration (up to 40 min) required to form a reference image comparable to the resolution of optical microscopy ($\sim 5 \mu\text{m}$), motion artifacts are prone to arise even for well-controlled anesthetic conditions (Hingot et al., 2019).

Considering the challenges behind native in vivo studies, it is appropriate to perform in vitro phantom validation of ULM algorithms prior to in vivo experimentation (Kanoulas et al., 2019; Lin, Shelton, et al., 2017). However, there is currently a lack of a compelling in vitro test protocol that is dedicated to ULM performance evaluation. Some studies used single-tube models for performance calibration, but they inherently do not allow for the assessment of channel resolvability in normal (Viessmann et al., 2013; W. Gu et al., 2023) or modified imaging environments (O'Reilly & Hynynen, 2013). Although a few crossed micro-tube configurations have been reported (S. Harput et al., 2020; Z. Dong et al., 2023; G. Zhang et al., 2018) they after all involved separate flow channels and lacked resemblance of microvascular branching characteristics. Attempts using lithography to produce straight (Desailly et al., 2015) or bifurcating ULM phantoms (Desailly et al., 2013) have also been made, but they are limited in their representation of soft tissue because their build material typically has acoustic properties (acoustic speed, attenuation) that are different from those for soft tissue (Lai et al., 2013; Tsang et al., 2015). Efforts to devise 3-D-printed ULM phantoms with ultrasound-compatible materials also suffer from mediocre print stability and positioning errors (Ommen et al., 2021). A suitable ULM phantom with matched acoustic properties and vascular branching features has yet to be developed.

In this section, we present the design and development of a new framework for creating an acoustically compatible microvascular phantom with uniformly increasing flow channel separation that serves well as a calibration feature for ULM performance testing. Our design is guided by two propositions: 1) bifurcating flow features commonly found in capillaries can be approximated as a forked lumen geometry that is cast within a tissue mimicking slab and 2) the rate of channel separation in the forked lumen geometry can be precisely controlled by defining the bifurcation angle of the forked lumen, as can be realized using

computer aided design (CAD) tools. Accordingly, we have devised a microvascular phantom design protocol that involves CAD design, additive manufacturing, and lost-core casting to develop an in vitro testing platform that is representative of microvascular flow. As will be shown in this article, when connected to a microflow circuit, our phantom's forked lumen can effectively mimic microvascular flow characteristics that are useful for ULM performance analysis.

3.2 Methods

3.2.1 Phantom Design Protocol

3.2.1.1 Design of the Forked Microvascular Lumen Core

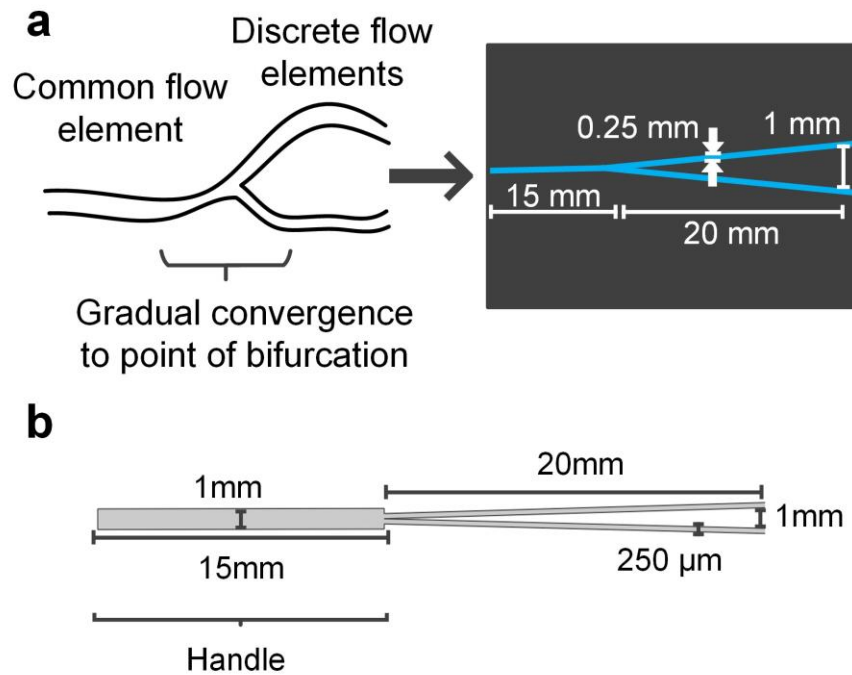


Figure 3.1. Forked microvascular lumen core. (a) The bifurcating feature of a microvessel segment is mimicked using a Y-shaped channel design with an initial edge-to-edge spacing of 1 mm converging into a single channel 20 mm into the phantom. (b) CAD draft of the forked lumen core that is used to create flow channels (250 μ m width, 2 mm height) within a tissue mimic cast.

As shown in Fig. 1(a), our phantom's forked lumen comprised two sub-segments: 1) a straight-tube common inlet (15 mm length) and 2) a V-shaped flow channel pair (20 mm length span). This lumen geometry can be considered as an analog to a microvascular bifurcation segment with common and discrete

channel regions. From trigonometry principles, it is well known that the post-bifurcation separation gradient is linear for this V-shaped flow channel pair. This design feature is important to the testing of an ULM method's resolving power in distinguishing two adjacent flow channels with similar flow dynamics but with linearly varying separation distance.

The forked lumen core was drafted in SolidWorks (Dassault, Systemes, Waltham, MA, USA). As shown in Fig. 1(b), the outlets of the V-shaped flow channel pair are axially spaced at 1 mm apart (measured edge to edge). For this pair of bifurcating flow channels, the corresponding flow channel separation increased at a rate of 50 $\mu\text{m}/\text{mm}$. The corresponding bifurcation angle was 2.9°. The flow channel diameter was set to 250 μm , which corresponds to the smallest size that could be consistently fabricated with a powder-bed fusion system. Note that, as explained in Section V-B, although this flow channel diameter is larger than typical capillary sizes, it is not a critical consideration in the context of using the forked lumen for ULM resolving power analysis. To facilitate the fabrication of a physical replicate of the forked lumen core via additive manufacturing, this geometry was extended in the azimuthal direction by 2 mm in SolidWorks. As shown in Fig. 1(b), to enable subsequent connection of the forked lumen to the flow circuit, the core's common flow inlet segment was expanded to a 1 mm-width rectangular handle.

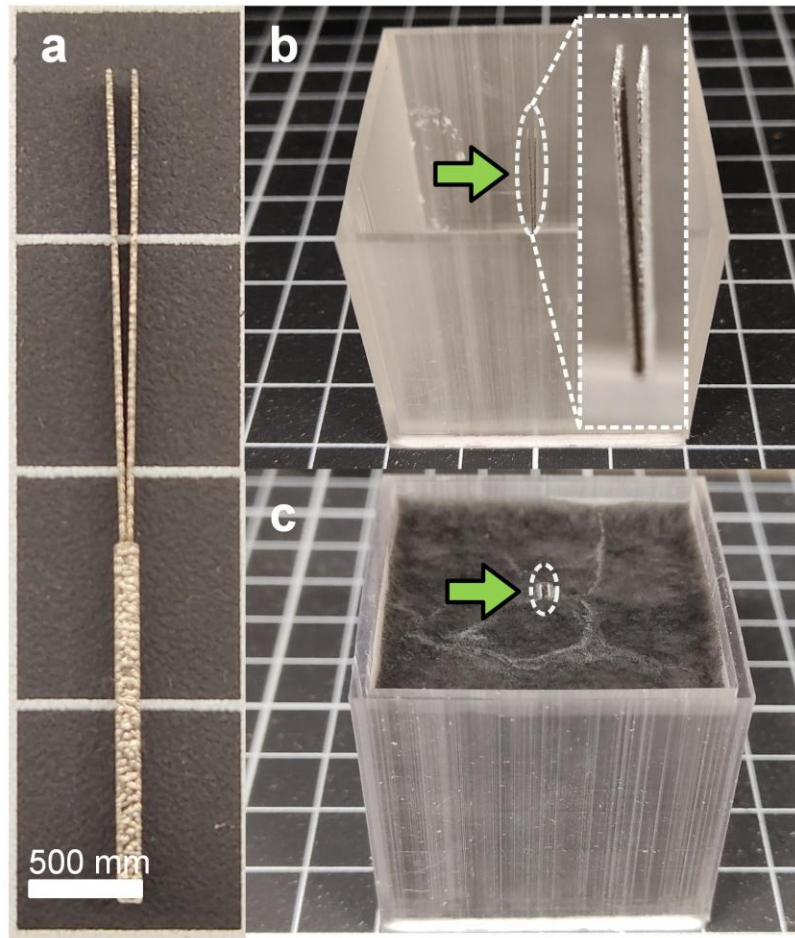


Figure 3.2. Phantom fabrication process. (a) Image of the microvascular lumen core's physical replicate. (b) The Lumen core (circled gray) was positioned in a vertical orientation within a sacrificial container using hot melt adhesive prior to casting PVA tissue mimic in the box (Inset: microscope image of the core overlooking both channels). (c) Sacrificial container partially filled with PVA to the intended level.

3.2.1.2 Fabrication Procedure

The drafted lumen core was exported and transferred to an external facility (Multi-Scale Additive Manufacturing Laboratory, Kitchener, ON, Canada) for fabrication. Physical replicas, a photograph of which is shown in Fig. 2(a), were created via powder-bed fusion additive manufacturing system (AM400; Renishaw, Wotton-under-Edge, Gloucestershire, U.K.), with 80 W power, 70 μ s exposure time, and 90 μ m

point distance. Inconel 625 powder (15–45 μm particle size; Renishaw) was used as the additive

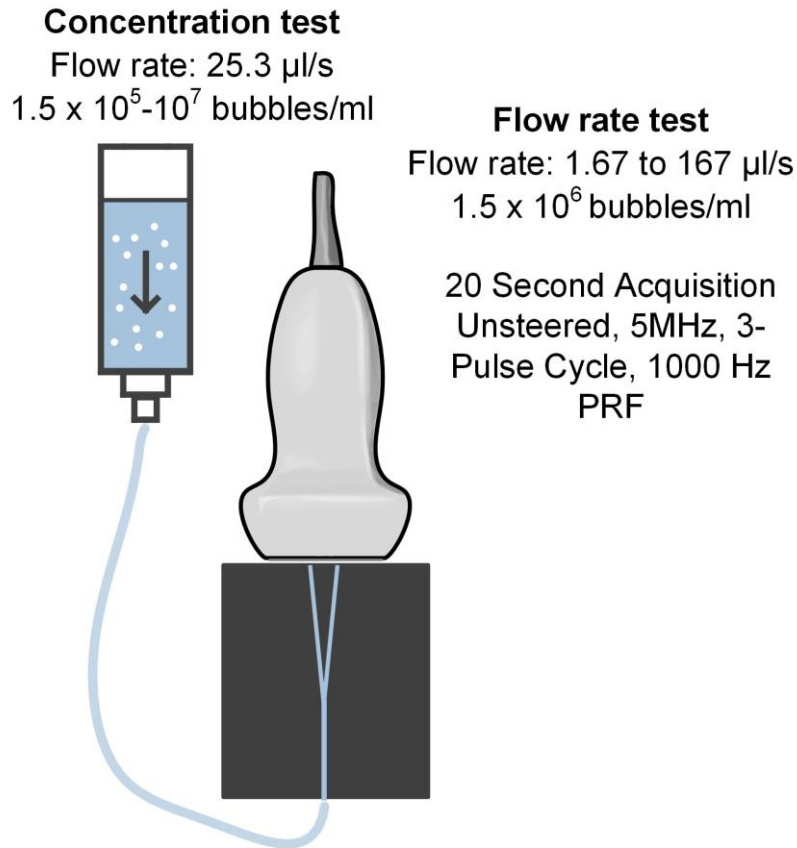


Figure 3.3. Illustration of the flow circuit used to infuse MBs through the forked microvascular phantom. Flow was fed at rates ranging from 1.67 to 167 $\mu\text{L/s}$ through a connection to the common channel of the phantom. The transducer was positioned directly at the top surface of the phantom above the feature to cut across both channels.

manufacturing material since it was known for its high strength properties as a nickel-chromium alloy.

Modifying from our earlier protocol (C. K. Ho et al., 2017), we coated the forked lumen core in petrolatum and mounted it in an upright orientation within a sacrificial casting container using hot melt adhesive shown in **Figure 3.2 (b)** to facilitate tissue mimic casting. The casting container, also drafted using SolidWorks and fabricated with a resin 3-D printer (Saturn; Elegoo, Shenzhen, China), had interior dimensions of $40 \times 40 \times 40$ mm (length $\times$ width $\times$ height). A polyvinyl alcohol (PVA)-based tissue mimicking mixture was then poured into the casting container, as shown in **Figure 2 (c)**, until the mixture height was slightly below the prongs of the branched region.

The PVA formula comprised 88.7% (wt%) degassed water, 10% PVA (341584; Sigma-Aldrich, St. Louis, MO, USA), 1% graphite as acoustic scatterers (282863; Sigma-Aldrich), and 0.3% potassium sorbate (85520; Sigma-Aldrich) (C. K. Ho et al., 2017). Prior to casting, the PVA mixture was magnetically stirred at 90 °C for 1 h and was allowed to degas overnight at room temperature.

The PVA-loaded container with the forked lumen core was subjected to a 24 h freeze cycle at –20 °C followed by a 24-h thaw cycle at 4 °C to allow the slab to congeal. This freeze and-thaw cycle was repeated three times. The corresponding speed of sound and attenuation coefficient of the congealed PVA slab are respectively 1535 m/s and 0.229 dB/(cm·MHz) as previously reported (C. K. Ho et al., 2017). Phantom density was approximately 1016 kg/m³ using volume obtained through displacement.

After the freeze-and-thaw cycles, the walls of the container were removed, while the base of the container was extracted with the phantom. Subsequently, the V-shaped flow channel pair was formed by withdrawing the cast core from the PVA slab. To prevent residual PVA from accumulating on the extracted core, the core removal step was realized by gently pulling on the handle of the cast core with pliers.

3.2.1.3 Flow Circuitry and Introduction of Contrast

The flow channel of the PVA slab was oriented in an upright configuration as shown in **Figure 3.3**. Its common flow inlet at the bottom was connected to a 20 mL syringe via silicone tubing (1 mm inner diameter; 2 mm outer diameter). The flow channel was first washed with degassed water. After that, Definity-like MBs, fabricated using an in-house sonication protocol (Schneider et al., 2012), were added to the syringe in a diluted form with degassed water in different concentrations ranging from 1.5×10^5 to 10^7 bubbles/mL. The syringe was stirred to mix the solution. Flow was then introduced at different flow rates ranging from 1.67 to 167 μ L/s. The contents were perfused for 30 s prior to imaging experiments.

3.2.2 Experimental Methods

3.2.2.1 Measurement of Phantom Core Replicate Geometry

Prior to PVA casting, the forked lumen core that was fabricated using additive manufacturing was imaged using an optical microscope (Celestron Handheld Digital Microscope; Celestron, Torrance, CA, USA) to obtain reference values for flow channel dimensions. These optical images were obtained under 55 $\times$ magnification with a reference scale attached. The acquired images were imported into ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA) to obtain measurements of flow channel width and separation. After the casting process, each extracted core was reexamined under microscopy to check their shape.

3.2.2.2 ULM Data Acquisition Setup

Ultrasound contrast imaging data was collected using unsteered plane wave transmissions from a SonixTouch ultrasound scanner (Analogic Ultrasound, Peabody, MA, USA) with a 128-element linear array (L14-5; Analogic Ultrasound) that was attached to a mechanical micropositioner. The transducer was aligned to the image across both channels in the flow parallel axis. Imaging was conducted with three-cycle, 5 MHz ultrasound pulses, a pulse repetition frequency of 1000 Hz, and an acquisition period up to 20 s. Raw radio frequency (RF) channel data was acquired using a SonixDAQ attachment (Analogic Ultrasound) with a sample rate of 40 MHz (C. C. P. Cheung et al., 2012).

3.2.2.3 Localization Processing Framework

RF channel data were transferred and processed on a GPU workstation using MATLAB (2016a, Mathworks, Natick, MA, USA). A 30th-order finite-impulse-response (FIR) bandpass filter (3–7 MHz passband) was applied to the channel data to suppress out-of-band white noise before beamforming. Data were processed to generate images using a delay-and-sum beamformer as previously presented (B. Y. S. Yiu et al., 2011). A single B-mode image was beamformed with dimensions of 38 $\times$ 30 mm² (lateral $\times$ axial). An area of interest containing contrast signals denoted by high signal intensity was manually identified. All 20 000 frames were then beamformed using a smaller 4 $\times$ 12 mm² (400 $\times$ 600 pixels, 10 μ m lateral pixel size, 20 μ m axial pixel size) window at the area of interest to reduce processing time. A 134th-order FIR

high-pass filter (cutoff at 50 Hz) was applied on the slow-time axis to remove stationary tissue clutter. Localization imaging was achieved through the application of a bubble detection and deconvolution-based framework similar to what was previously reported (S. Lei et al., 2022). The filtered image was cross-correlated with the scanner's point spread function to detect MB positions, and output threshold was set to 50% of the maximum cross correlation value to suppress non-contrast signal. To pinpoint the spatial location of the bubble signal, the cross correlated images were deconvolved using the Richardson-Lucy deconvolution method (Lucy, 1974; Richardson, 1972). The mean of the 20 000-frame stack was taken to form ULM images.

3.2.2.4 Lumen Width and Flow Channel Separation Measurements

Lumen center and width were determined by Gaussian fitting to the cross-sectional profile of the resolved lumens and extracting the full-width-at-half-maximum (FWHM) of the fits. The separation distance between the two post-bifurcation flow channels (edge-to-edge) was obtained by subtracting half of the widths of both lumens from the center-to-center distance. Five measurements were taken for ULM images formed from 10 s of acquisition with a 2 s sliding window between each set.

3.3 Results

3.3.1 Optical Imaging Confirms Dimensions of the Fabricated Forked Lumen Core

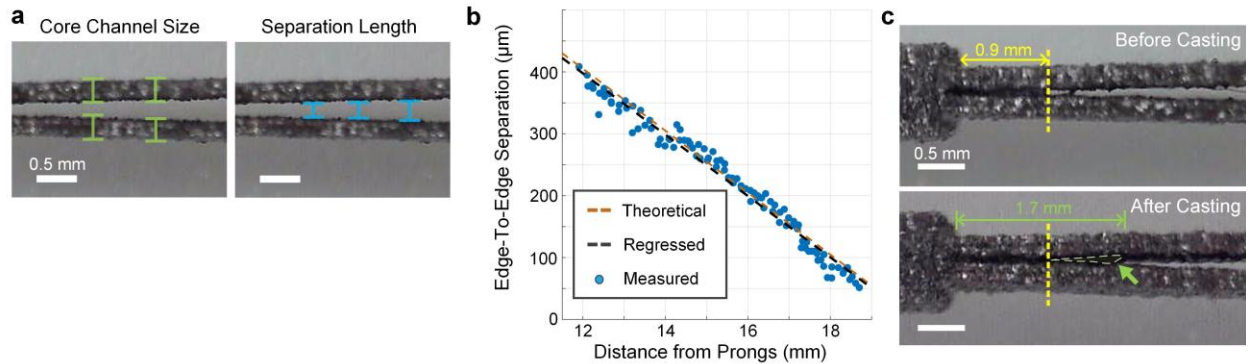


Figure 3.4. Optical images and size measurements of the fabricated forked lumen core. (a) Using ImageJ, the channel width (green) and channel-to-channel separation distance (blue) were measured at different sections of the lumen core. (b) The plot of the measured inner edge separation (blue dots), corresponding regression line (black-dashed), and theoretical separation gradient (orange-dashed). (c) Image comparison between a precast core that was brushed with petrolatum (unremovable excess at yellow line) to reduce surface roughness and a post-cast core containing PVA pullout (indicated by the green arrow). (orange) separation gradient derived from the CAD drawing. The measured channel separation gradient was found to be $50.9 \mu\text{m/mm}$, which is 1.8% different from the theoretical separation rate of $50 \mu\text{m/mm}$.

The forked lumen core fabricated using additive manufacturing was found to have dimensions that are close to the design dimensions. **Figure 3.4 (a)** shows the lumen core's channel width, post-bifurcation flow channel separation, and separation gradient measurements observed under an optical microscope. The cross-sectional width of the lumen core was measured at points perpendicular to the axis of symmetry with examples shown in green markers of the left inset in **Figure 3.4 (a)**. The mean width of the core measured under optical microscopy was $252 \pm 15 \mu\text{m}$ (mean $\pm$ s.d.), which corresponds to a mean error of 0.8% with respect to the design diameter of $250 \mu\text{m}$.

Optical microscopy measurements for the edge-to-edge separation at a given distance away from the bifurcation point are illustrated as blue markers of the right inset of **Figure 3.4 (a)**. The measured separation points are depicted in **Figure 3.4 (b)** together with the regression line (black) for the separation gradient taken from optical measurements and the theoretical CAD measurement.

As shown in **Figure 3.4 (c)**, the fabricated lumen core was found to remain intact in shape during the casting process. Nonetheless, in the separation space between the two diverging channels, there was some excess petrolatum coating and residual PVA near the bifurcation point, as indicated by the yellow dashed line and the green arrow in **Figure 3.4 (c)**. The axial length of this excess zone was measured to be 1.7 mm (of which 0.9 mm was attributed to the excess petrolatum coating). As such, the new phantom's V-shaped flow channel pair had effectively merged at an axial distance of 18.3 mm away from the two flow outlets.

3.3.2 Flow Channels in the Forked Microvascular Phantom Can Be Consistently Visualized Using ULM

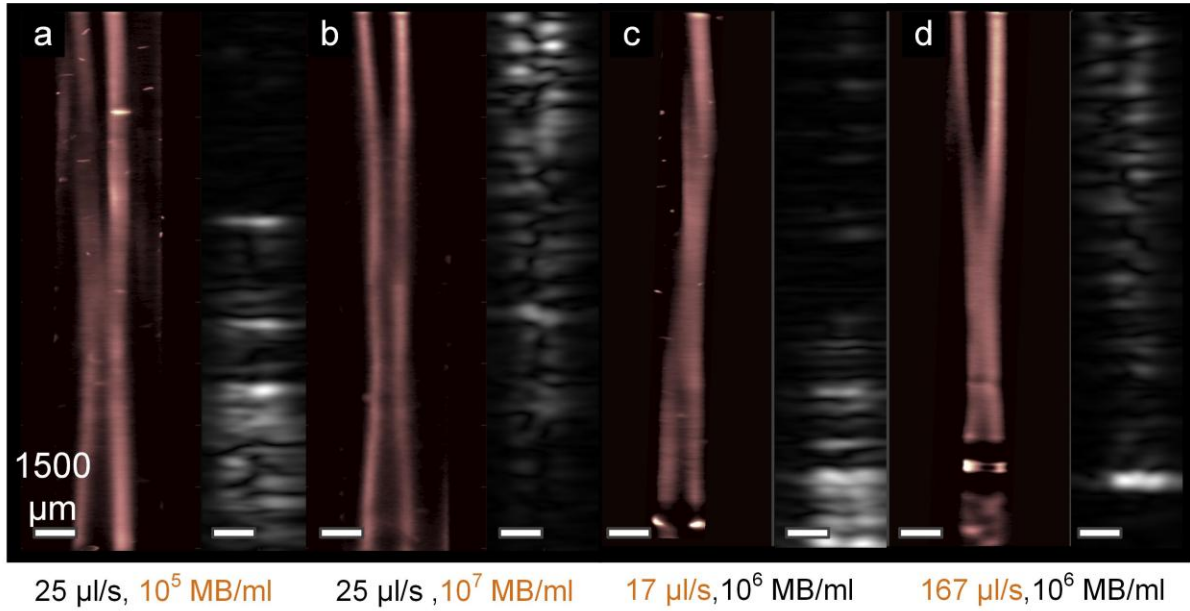


Figure 3.5. ULM images of the new phantom's microflow channels for the following flow rates and MB concentrations (a) 25.3 $\mu\text{L/s}$, 1.5×10^5 MB/mL; (b) 25.3 $\mu\text{L/s}$, 1.5×10^7 MB/mL; (c) 16.7 $\mu\text{L/s}$, 1.5×10^6 MB/mL; and (d) 167 $\mu\text{L/s}$, 1.5×10^6 MB/mL. Note that in (c), the left-side channel is missing because of insufficient flow to that area. Corresponding B-mode image frames are also shown as reference.

When ULM is performed on the new phantom, the V-shaped flow channel pair can generally be detected if appropriate ULM parameters are used. **Figure 3.5** shows a set of ULM images for a 20 s acquisition period, different flow rates (16.7, 25.3, and 167 $\mu\text{L/s}$), and MB concentrations (1.5×10^5 , 1.5×10^6 , and 1.5×10^7 MB/mL). For reference, a corresponding B-mode image for each parameter combination is shown in

parallel. Three observations can be noted from this figure. First, as shown in **Figure 3.5 (d)**, for a 20-s acquisition period, the V-shaped channel is better visualized with a flow rate of 167 $\mu\text{L/s}$ and an MB concentration of 1.5×10^6 MB/mL. Second, as illustrated in **Figure 3.5 (a) and (b)**, at a slower flow rate of 25.3 $\mu\text{L/s}$, the use of a higher MB concentration (1.5×10^7 instead of 1.5×10^5 MB/mL) has improved the resulting visualization of the V-shaped channel. Third, as shown in **Figure 3.5 (c)**, when the flow rate was further lowered to 16.7 $\mu\text{L/s}$, the V-shaped channel cannot be consistently visualized because of insufficient flow to one of the branches. In all these cases, the V-shaped flow channel pair is not visible in the corresponding B-mode images.

3.3.3 ULM Visualization of the Forked Microvascular Phantom at Different Acquisition Times

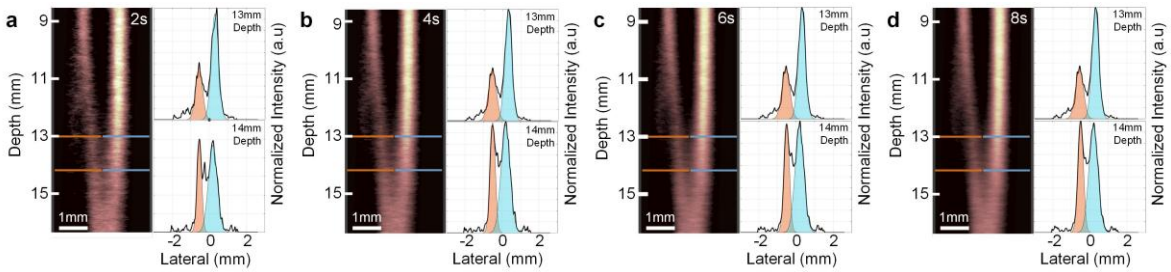


Figure 3.6. Cross section analysis of ULM images of the forked microvascular phantom at increasing acquisition times of (a) 2 s, (b) 4 s, (c) 6 s, and (d) 8 s at depths of 13 and 14 mm. FWHM of each channel was determined by Gaussian fits illustrated in orange and blue for left and right channels respectively. Bubbles were infused at 167 $\mu\text{L/s}$ with concentration of 1.5×10^6 MB/mL.

As a further investigation of ULM's microvascular imaging performance, **Figure 3.6** shows a series of ULM images (left inset of each subfigure) at four acquisition times of the same experiment run (from 2 to 8 s). It can be observed that visualization of the phantom's bifurcating lumen gradually improved as the acquisition time is progressively lengthened from 2 to 8 s. Also shown in **Figure 3.6** are the corresponding cross section profiles at two locations downstream from the bifurcation point (the two right insets of each subfigure). Channel widths and centers, as determined via Gaussian fitting, are depicted in the cross-section profiles where the orange and blue overlays in **Figure 3.6 (a)–(d)** are, respectively, the left (orange) and right (blue) lumen regions of the corresponding cross sections. As can be observed, these cross-section

profiles progressively become less noisy for longer acquisition times. It is also interesting to note that, at all four acquisition times, both branches of the forked phantom have been depicted in the cross-section profiles, even though the left and right flow channels have different intensity levels due to image plane misalignment during ULM data acquisition.

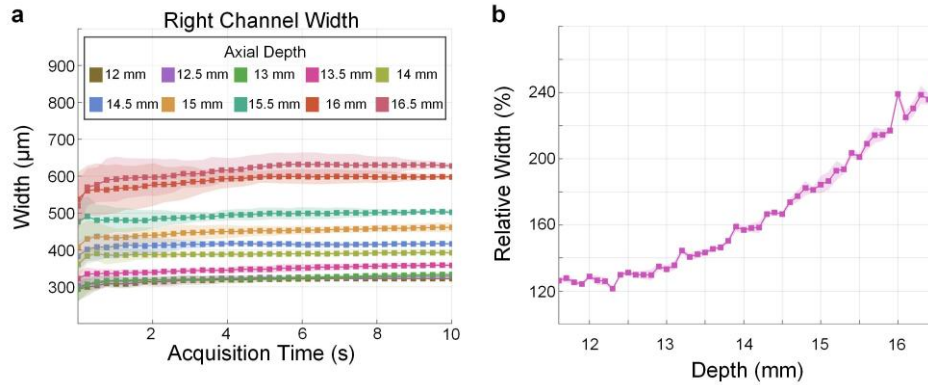


Figure 3.7. ULM-measured width of the right flow channel in the forked microvascular phantom. (a) Average Gaussian-fit FWHM ($N = 5$) measured at different axial depths from the flow outlet as a function of acquisition time (the shaded zone of each line depicts standard deviation). (b) Relative flow channel width (with reference to the optically measured mean width of $252 \mu m$) at different axial depths from the flow outlet.

Quantitative analysis of ULM-measured flow channel widths revealed that ULM's resolving power became stabilized when the acquisition time approached 10 s. Corresponding data are provided in **Figure 3.7 (a)** which shows the Gaussian-fit FWHM of the right channel (mean $\pm$ s.d.; $N = 5$) at different imaging depths (from 12 to 16.5 mm; in 0.5 mm increments) as a function of acquisition time (from 0 to 10 s; 0.25 s intervals).

As can be noted, at greater axial depths that are closer to the bifurcation point (e.g., 16 and 16.5 mm), the estimated flow channel width exhibited high standard deviation when the acquisition time was only a few seconds. Such an estimation variance was progressively reduced as acquisition time was lengthened. In contrast, at shallower depths where the flow channels are more separated, the variance of the estimated flow channel width remained low even at short acquisition times.

3.3.4 Flow Channel Width and Separation Distance Derived From ULM Measurements

Another noteworthy trend of **Figure 3.7 (a)** is that ULM-measured flow channel widths are larger than the optically measured mean width of $252\ \mu\text{m}$ (as mentioned in Section IV-A). Specifically, at 12 mm depth where the two flow channels are in theory separated by $400\ \mu\text{m}$, the estimated flow channel width was $320\ \mu\text{m}$. At depths near the forked phantom's bifurcation point, the estimated flow channel width was found to be even larger and, correspondingly, ULM's resolving power was lower for these imaging depths. In particular, at 16.5 mm depth, the Gaussian-fit FWHM of the right flow channel was $630\ \mu\text{m}$.

To further characterize ULM's resolving power, **Figure 3.7 (b)** plots, as a function of imaging depth, the relative ULM-measured flow channel width with respect to the mean width of $252\ \mu\text{m}$ measured using optical microscopy as presented in Section IV-A. In general, ULM was found to yield flow channel width measurements that were larger than the optically measured width of the forked lumen core. Also, an increase trend in the relative ULM-measured flow channel width can be observed as the imaging depth increases (i.e., as we approach the forked phantom's bifurcation point). At 12 mm depth, the relative ULM-measured flow channel width was 127% of the optically measured width; in contrast, at 16.5 mm, this relative width was increased to 240%.

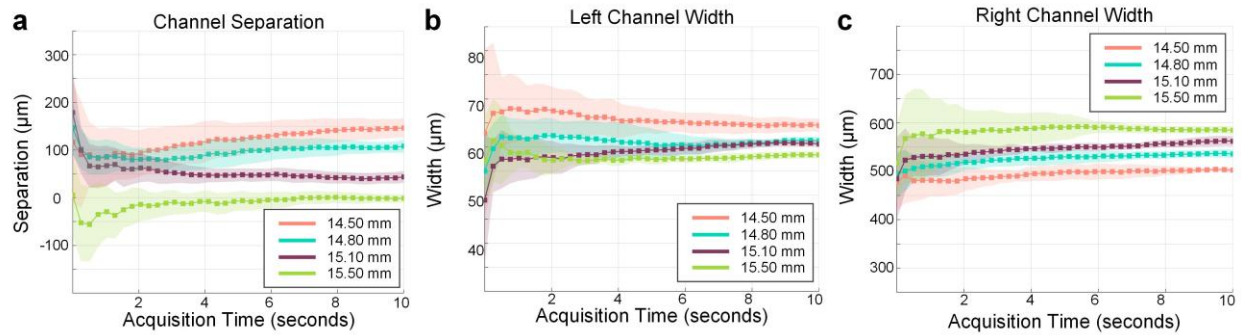


Figure 3.8. ULM-approximated channel-to-channel separation distance a) in the forked microvascular phantom shown as a function of acquisition time for four axial depths: 14.5 mm (orange), 14.8 mm (blue), 15.10 mm (purple), and 15.50 mm (green). Separation distance a) was calculated using the distance between peak intensity of each channel in the ULM image subtracting the width estimates of the left b) and right c) channels.

As a more detailed analysis, **Figure 3.8** plots the ULM-measured flow channel separation distance of the two post-bifurcation flow channels as a function of data acquisition time from 0 to 10 s (with 0.25 s intervals). Results are shown for four imaging depths: 14.5 mm (orange), 14.8 mm (cyan), 15.1 mm (purple), and 15.5 mm (green). As can be observed, at these four depths, the corresponding edge-to-edge separation distance of the two flow channels converged to 150, 110, 50, and 0 μm (at 10 s acquisition period). These separation distance measurements converged when the acquisition time was 8 s or longer.

3.4 Discussion

3.4.1 Summary of Methodological Contributions

As a new microvascular mapping technique, ULM has garnered significant attention in recent years. To facilitate this technique's performance analysis, simulation-based approaches have been adopted to synthesize ULM data for different vascular geometries (J. R. McCall et al., 2022; X. Chen et al., 2022). Nevertheless, these *in silico* solutions inherently cannot be used to evaluate the experimental performance of ULM setups that are subjected to hardware noise and that use real contrast agents. In contrast, this article has presented a new forked microvascular phantom that can be used for *in vitro* assessment of an ULM method's resolving power. Its lumen was designed as a bifurcation geometry with a linear flow channel separation gradient downstream from the bifurcation (see **Figure 3.1**). It was fabricated using additive manufacturing and was used to form a wall-less forked microvascular phantom via lost-core casting principles (see **Figure 3.2**). The dimensions of the fabricated forked lumen core were closely aligned with the design dimensions (see **Figure 3.4**).

The key technical merit of our new phantom is that it is the first ULM performance calibration phantom with: 1) a pair of flow channels that have a uniformly increasing separation and that lie on the same plane and 2) an ultrasound-compatible tissue mimicking slab that encompasses the microvascular lumen. The first feature is critical in determining what is the minimum flow channel separation distance that can be detected by ULM. It is seemingly more expedient to use our new phantom for such an analysis as opposed to devising multiple phantoms involving parallel flow channel pairs with different separation

distances. The second feature is important too as it can effectively avoid aberration (due to speed of sound mismatch) that may hamper contrast echo localization performance and provide background noise to simulate live tissue.

3.4.2 Application of New Phantom in ULM Experiments

When our new phantom was used to perform ULM experiments (see **Figure 3.3**), it was found to be useful in characterizing the resolving power of a previously reported ULM algorithm (see **Figure 3.5**). It also facilitated systematic analysis of the impact of acquisition time on ULM image quality and its resolvability of adjacent flow channels. In general, results have confirmed that longer acquisition times are important to generate high-quality ULM images of the forked lumen geometry (see **Figure 3.6**). Note that the intensity of the left and right flow channels may not be identical depending on the image plane placement during ULM data acquisition. This issue is after all inevitable because the phantom's forked lumen is after all just 250 μm in size and thus it is not possible to use B-mode imaging for navigation and precise alignment of the image plane to the forked lumen. Nevertheless, the two flow channels can be distinguishably visualized and did not affect our phantom's ability to facilitate testing of ULM's microvascular resolving power.

In our ULM characterization analyses, overestimation was found in the measured flow channel widths (see **Figure 3.7**). This issue was more significant at greater axial depths near the lumen's bifurcation point because the narrow channel-to-channel separation distance has made it challenging for the ULM algorithm to properly discern between the two flow channels. Correspondingly, the ULM-measured channel-to-channel separation distance was underestimated and became zero at an axial depth of 15.5 mm (see **Figure 3.8**) that supposedly had a separation distance of 229 μm [for a measured separation gradient of 50.9 $\mu\text{m}/\text{mm}$ as per **Figure 3.4 (b)**]. These findings generally prompt the need to enhance ULM's resolving power through the design of more advanced algorithms. One such improvement is to improve the clutter filter used to separate non-MB signal from tissue clutter before MB localization. Singular Value Decomposition (SVD) is a popular alternative for high-pass filtering for microvascular imaging (N. Zhang

et al., 2020). Briefly, SVD breaks down an ultrasound signal input into its individual scatterer components that are also weighted to its contribution to the combined signal input. Filtering using SVD in blood/MB detected assumes that clutter, blood and noise signal can be differentiated by characteristics unique to each type and selecting an upper and lower cutoff to exclude the weighted components associated with non-blood signal (Riemer et al., 2023). High-pass filters do struggle to separate slow moving flow from tissue clutter in comparison to SVD (Lok et al., 2020). Thus, it may be possible for the high-pass filter to have removed some MB signal and may be worth revisiting this dataset using the SVD method.

3.4.3 Choice of Material for Phantom Fabrication

The focus of this work has been to establish the methodology of preparing a forked microvascular phantom. In this process, we have used an established PVA mixture formula (C. K. Ho et al., 2017) to form our tissue-mimicking material. One potential concern of such a phantom fabrication approach is that the corresponding freeze-thaw process, which naturally leads to mild physical contraction and expansion of the hydrogel slab to foster PVA crosslinking, might unintentionally deform the forked lumen core. To circumvent this issue, we have devised a lumen core fabrication protocol that is based on additive manufacturing using a metallic, mechanically tough nickel-chromium alloy material (Inconel 625 powder). Accordingly, the lumen of our developed phantom has largely maintained its V-shaped geometry. Note that, in our preliminary work, we initially attempted to build the forked lumen core via 3-D printing of polylactic acid (PLA) filaments. We found that, when these 3-D printed lumen cores were used for lost-core casting, the resulting V-shaped flow channel pair was slightly bent near the two flow outlets due to the lighter weight and lower rigidity of the PLA material.

Our PVA mixture formula was based on the use of graphite as acoustic scatterers (C. K. Ho et al., 2017), so the phantom's corresponding tissue-mimicking slab was optically dark. In the future, it is worth investigating the use of other formulations for tissue-mimicking materials (McGarry et al., 2020). One direction worth pursuing is the development of a transparent tissue-mimicking material, since the current

use of graphite in our formula has naturally made the tissue slab opaque and, in turn, has inhibited any optical validation of the flow channels.

Alternatively, it may be helpful to perform microcomputed tomography (Huang et al., 2020) scans to obtain the necessary ground truth needed to validate our phantom's fidelity in resembling the forked lumen that we have drafted using CAD. Another related topic that is worth examining further is how different acoustic scatterer concentrations would affect the background tissue signal and, in turn, influence the microvascular resolving power of a ULM algorithm. This study can be performed by developing a library of forked microvascular phantoms with different tissue-mimicking material compositions and using them to test ULM algorithms.

3.4.4 Limitations of Phantom Fabrication Protocol

It should be noted that the minimum flow channel diameter that can be supported in our phantom design protocol is currently constrained by the print resolution of our additive manufacturing system. In this work, we have demonstrated that it is possible to fabricate forked lumen cores with widths near the design dimension of 250 μm and a designed separation rate of 50 $\mu\text{m}/\text{mm}$. Although this flow channel diameter is larger than typical capillary diameters (which can be down to a few microns), it is fortunately not a critical drawback because ULM's resolving power can be analyzed by leveraging the phantom's V-shaped flow channel pair (with uniformly increasing post-bifurcation separation). Yet, as a future endeavor, it would still be beneficial to devise forked lumen cores with smaller diameters through the use of more advanced systems that have fingerprint resolution than the one used in this investigation.

Another nonideality of our phantom design protocol is that, during the lumen core pullout step that served to form the V-shaped flow channel pair, some congealed PVA remained attached to the crevice at the branching point [see **Figure 3.4 (c)**]. This issue is unavoidable due to the acute angle at the branch point of the forked lumen core. Nevertheless, the extent of this problem is limited because the attachment zone has only axially spanned 1.7 mm from the bifurcation point. The corresponding V-shaped flow channel pair has still spanned an axial length of 18.3 mm between the two flow outlets and the effective merge point

(i.e., 1.7 mm axial distance away from the theoretical bifurcation point). Accordingly, the minimum channel-to-channel separation of our phantom (i.e., adjacent to the effective merge point) was deduced to be equal to 86.5 μm based on the channel separation gradient shown in **Figure 3.4 (b)**.

3.5 Conclusion

In recent years, ULM and super-resolution ultrasound imaging solutions have been actively developed for microvascular visualization applications. However, these algorithmic innovation endeavors have yet to be matched with representative in vitro models for performance calibration and testing prior to in vivo application. Here, we have devised a forked microvascular phantom that was modeled after a bifurcation point. With known design dimensions and its V-shaped flow channel pair feature, our new phantom can be used to quantitatively analyze the microvascular resolving power of ULM methods. Overall, this in vitro test platform will help translate new ULM techniques toward practical use to improve clinical visualization of microvascular pathologies.

Chapter 4

4.1 Introduction

Venous Thromboembolisms (VTE) is a set of disease conditions where unwanted blood clotting occurs in the veins including deep vein thrombosis and pulmonary embolism. VTEs may be pharmaceutically treated by either slowing accumulation of the fibrin molecule using anti-coagulants or directly dissolving the fibrin clot using fibrinolytics.

These specific treatment choices are made based on expert panel clinical guidelines devised to balance bleeding risk with the certainty of treatment effectiveness (Ortel et al., 2020). However, many recommendations pertaining to fibrinolytic or recurrent clot treatment are made conditionally with low-certainty of evidence. Indeed, current radiographic imaging technology offer little evidence on both the true effectiveness of fibrinolytic treatment or the indefinite administration of anti-coagulants during the recurrent prevention period (Ortel et al., 2020; Saha et al., 2016). As such, to enhance general clinical management of VTE, there is a practical need to improve the quality of evidence for efficacy that clinicians have access to. On this topic, magnetic resonance imaging and positron emission tomography have been applied to image VTE via fibrin tagging (Andia et al., 2014; Oliveira & Caravan, 2017). However, they are heavyweight imaging modalities that are not readily accessible. In contrast, with its point-of-care applicability, ultrasound (US) has been used as a workhorse imaging modality for VTE diagnosis (Lim et al., 2018). Yet, currently implemented US technology in VTE diagnosis is limited to flow detection in Duplex Doppler Mode or compressibility/echogenicity of veins along the extremities in B-Mode (Bhatt et al., 2020; Saha et al., 2016; Stone et al., 2017).

Ultrasound contrast microbubbles (MB) have potential to expand US's role in VTE diagnosis. Certainly, MBs have been demonstrated to increase blood-born signals in color doppler to elicit new insights in VTE diagnosis within research (Bucek et al., 2001; Guenther et al., 2013; Smith et al., 2016). MBs are

readily functionalizable for applications such as magnetically guided therapy (Dwivedi et al., 2020; Langeveld et al., 2021), and cancer cell targeting (X. Liu et al., 2018; Slagle et al., 2018). A bevy of fibrin clot targeting options exist for echogenic contrast. This include leveraging targeting peptides (Gormley et al., 2019; Li et al., 2022), repurposed thrombolytics (Klegerman et al., 2008; Z. Wang et al., 2021), among other methods specific to fibrin not yet implemented with ultrasound (Risser et al., 2022). Much of MB clot research are application based towards therapeutic directions despite room for innovation. This is seen with sonothrombolysis (Bader et al., 2016; Goel et al., 2021; Schleicher et al., 2016; F. Wang et al., 2019a; B. Zhang et al., 2019), where the existence of placing a cavitation core near the clot is a greater priority than the specific molecules involved. Yet there are sure directions for these designs to advance imaging applications. Focusing on ligand conjugation, sulfhydryl-based and avidin based conjugation methods respectively struggle with unwanted cleavage at physiological pH (Browning et al., 2021), and immunogenicity (Jain & Cheng, 2017). Introducing these elements onto a MB may cause unwanted biointeractions to complicate the detection of clots imaging. Thus, having a MB design using an abiotic linkage may be the direction to go with VTE.

We hypothesize the use of ultrasound molecular imaging using microbubble (MB) contrast may enhance the role of ultrasound in VTE diagnosis to improve the quality of evidence required for clinical decision making. To achieve this, we report the design of fibrin specific targeting contrast agents for ultrasound and demonstrate that these bubbles successfully adhered to a fibrin coated surface to significantly enhance fibrin detection under ultrasound. This work will leverage the strain-promoted azide-alkyne click reaction (SPAAC) for conjugation in combination with deactivated tPA acting as the fibrin targeting molecule. tPA was specifically chosen to demonstrate functionalization of an applicable but untreated fibrin targeting protein for click chemistry. In summary this will include 1) Deactivation of plasminogen activation of tPA, 2) Functionalization of tPA with an azide group, 3) Attachment of azido-tPA to DBCO-MBs via click reaction. tPA-MBs will be tested *in vitro* using fluorescence microscopy for their ability to adhere to a fibrin surface and supporting data will be provided to validate each reaction step.

4.2 Methods

4.2.1 Microbubble Preparation

4.2.1.1 Liposome pre-cursor preparation

Dibenzocyclooctyne (DBCO) was incorporated into the shell of the microbubble by including pre-synthesized phospholipid containing DBCO into the initial phospholipid mixture. The following lipids were taken from -20°C, opened at room temperature after equilibration, and measured: 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC; Avanti Polar Lipids, Prod. No. 850365P; Alabaster, AL, USA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy(polyethylene glycol)-2000] (DSPE-PEG2k-PE; Avanti Polar Lipids, Prod. No. 880120P), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[dibenzocyclooctyl(polyethylene glycol)-2000] (DSPE-PEG2k-DBCO; Avanti Polar Lipids, Prod. No. 880229P) at a 18:1:1 (DSPC: DSPE-PEG2k-PE: DSPE-PEG2k-DBCO) molar ratio. To form an initial lipid film for hydration, the powder mixture was dissolved in 1ml of chloroform, placed under nitrogen flow to evaporate the solvent and moved into vacuum for 2 hours to remove any excess chloroform. To form a liposome solution, a solution of 7:2:1 (DI Water: Glycerol (Fisher Chemicals, Cata. No. G331; Canada): Propylene glycol (Sigma Aldrich, Prod. No. P4347)) was added to the lipids and the solution was then bath sonicated at 55-60°C for 15 minutes. The mixture was tip sonicated (Branson SFX250, Emerson Electric; St. Louis, MO, USA) at 10% amplitude pulsed for 5s:10s:11min (On: Off: Total). The liposome mixture was allowed to cool to room temperature and stored. 0.1% of total lipid moles of lipophilic dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, perchlorate (DiD'; ThermoFisher Scientific, Cata. No. D307) in ethanol solution was transferred into an empty container and the solvent was evaporated under nitrogen stream.

4.2.1.2 Fluorescent Microbubble Formation

To label the lipids with lipophilic dye, stock lipid solution was transferred into the DiD' container and tip sonicated under similar conditions as previously described for the stock lipid solution. Stock lipid contents were placed into aliquots within headspace vials. Microbubbles were formed by mechanical

agitation, the headspace was replaced with decafluorobutane (PFB; Synquest Laboratories, 1100-2-06; Alachua, FL, USA) and shaken at 4.2kHz with a dental amalgamator. Microbubble size distribution was measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Pananalytical; Malvern, Great Britain) and concentration was obtained using a hemacytometer (Hausser Scientific; Horsham, PA, USA).

4.2.1.3 PPACK Mediated Inhibition of tPA

tPA (Sigma Aldrich, Prod. No. 612200; St. Louis, MO, USA) was reconstituted from powder at a 1mg/ml concentration by manufacturer recommendations using sterile DI and placed into aliquots for storage at -80°C. tPA was removed from storage and allowed to thaw at room temperature prior to reaction. To deactivate thrombolytic qualities of tPA, N-(2-amino-3-phenylpropanoyl)-1-[1-chloro-6-(diaminomethylideneamino)-2-oxohexan-3-yl] pyrrolidine-2-carboxamide (PPACK; Sigma Aldrich, Prod. No. 520222) at a 1:1 molar ratio to tPA was added into a pH 7.2 50mM phosphate buffer (Sigma Aldrich, Prod. No. P3288) solution with 20mM l-arginine (Sigma Aldrich, Prod. No. A5006) and 1% Tween-20 (Sigma Aldrich, Prod. No. P1379). This mixture was reacted in the dark for 1 hour at room temperature on a tube revolver at 10 Hz (Tube Revolver, ThermoFisher Scientific, Cata. No. 88881001; Canada). To isolate the tPA after reaction, the solution was transferred into 30kDa centrifugal filters (Ultracel-30, Sigma Aldrich, Prod. No. UFC5030) and washed (Centrifuge 5910 R, Eppendorf AG; Germany) at least 6 times against 1x phosphate buffered saline (PBS) at 10-minute cycles of 14000g.

4.2.1.4 Inhibited tPA Ligand Azide Functionalization

Azide and DBCO groups were respectively functionalized to tPA or MB to perform click conjugation. Deactivated tPA was functionalized with an azide group for SPAAC through the reaction between N-hydroxysuccinimide (NHS-Ester) and a primary amine on tPA. A similar reaction was reported by Klegerman to enable a sulfhydryl conjugation of tPA to eLIPs, where tPA's fibrin binding ability was maintained despite modification. Deactivated tPA was transferred into a solution of 1x PBS at 7.4 pH and 30x molar excess Azido-PEG4-NHS Ester (TCI America, Prod. No. A2388; Montgomeryville, PA, USA) and reacted for 90 minutes at room temperature on a tube revolver. The reacted protein was isolated

following a similar 6+ wash cycle using centrifuge filters against 1x PBS and the isolated protein was stored at -80°C until use.

4.2.1.5 Microbubble Conjugation Reaction

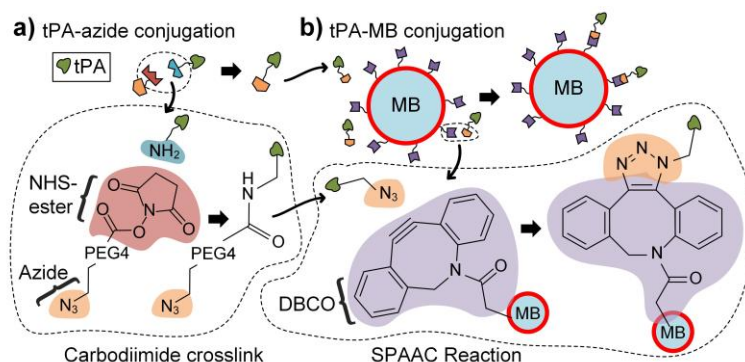


Figure 4.1. Overview of functionalization steps for MB preparation: (a) to impart azide group ($-N_3$) onto tPA by reaction with primary amine ($-NH_2$ blue) and NHS-ester (red), and (b) click-conjugation reaction between ligand and MB through reaction between azide (yellow) and microbubble bound DBCO (purple).

Theory

MBs conjugation reactions typically use biocompatible reaction methods, or more practically, the methods associated with what is available on Sigma Aldrich. Some potential alternatives include direct amine-NHS ester linkage, maleimide-cysteine, and biotin-avidin conjugates.

MBs were differentially centrifuged at 15g at 120s and 137g for 300s. Isolated MBs were then suspended in 1ml of 1x PBS at 7.4pH. Azido-tPA was added to the solution at a 10x molar excess in relation to the estimated DSPE-PEG2k-DBCO content at the current bubble concentration. The reactants were mixed on a tube revolver for 90 minutes at room temperature. Reacted MBs were diluted to 1ml then washed 2 times of unreacted azido-tPA by centrifugation at 77g for 300s and replacing 400 μ l of supernatant with fresh PBS. The bubbles were concentrated with a final 77g cycle for 300s at the syringe plunger and removing as much solution as possible. The above reaction is summarized in the cartoon of **Figure 4.1**.

4.2.1.6 Microbubble and Reagent Characterization

To confirm that DBCO was incorporated onto the MBs shell, DBCO-MBs were reacted with fluorescein azide (FITC Azide; Sigma Aldrich, Prod. No. 910147) at 10x molar excess in 7.4 pH 1xPBS for 90 minutes and imaged under fluorescence microscope under 488nm LED. To confirm azide functionalization, Azido-tPA was reacted with dibenzocyclooctyne-PEG4-Fluor 545 (DBCO-PEG4-Fluor 545, Sigma Aldrich), washed 6x against 1x PBS at 14000g with a 30kDa centrifuge filter, and analyzed using fluorescence spectrometry (Cary Eclipse Fluorescence Spectrometer, Agilent; Santa Clara, CA, USA) between 500nm and 650nm. This was repeated for a control reacting DBCO-PEG4-Fluor 545 with PBS.

The 10x molar excess was selected to manage the reagent cost of tPA with experimental observations of fluorescent signal saturation. An azide functionalized dye (Alexa Fluor 488, ThermoFisher

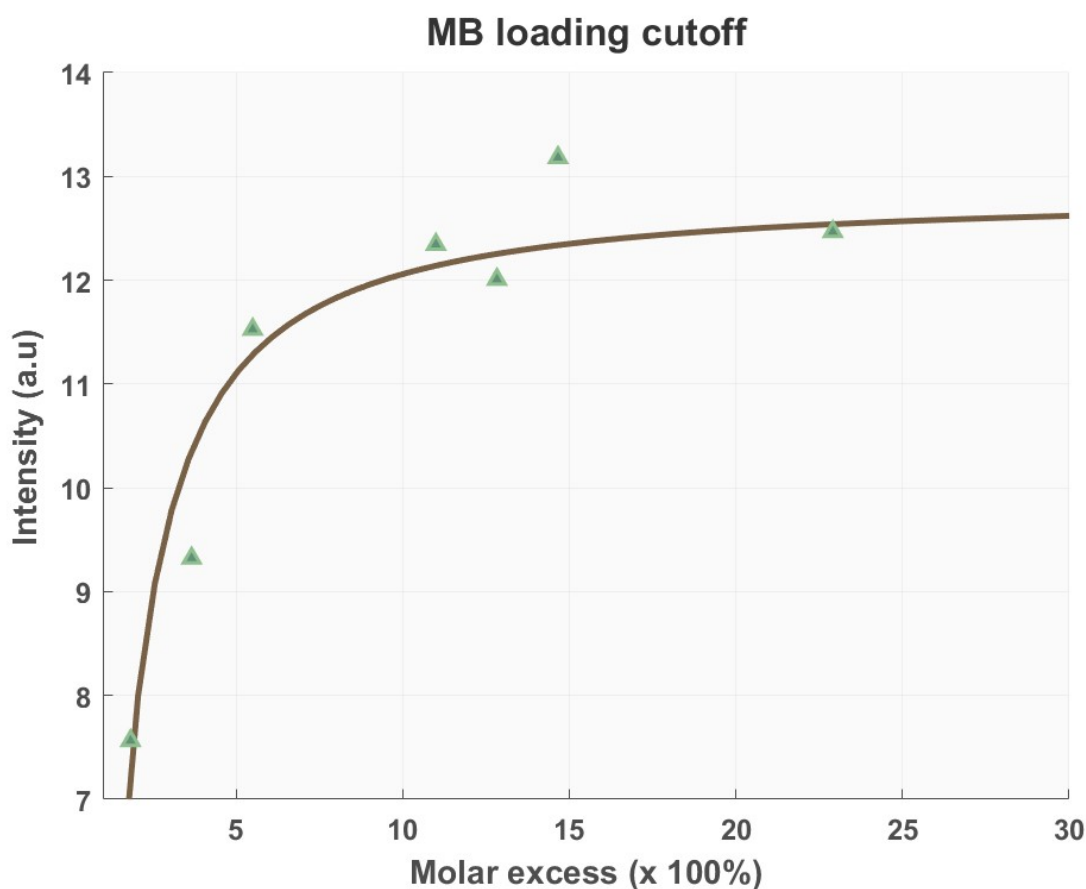


Figure 4.2. Fluorescent intensity of multiple washed DBCO-MB samples reacted with azide functionalized fluorescent dye to estimate a working molar excess for the reaction. Fluorescence intensity values approach a saturation after an inflection before 10x molar excess.

Scientific, Cata. No. A10266) was reacted with increasing molar excess to size selected DBCO-MBs. Based on observations shown in **Figure 4.2**, we can see that fluorescence intensity values begin to approach saturation with an inflection approximately before a point of 10x molar excess. Fluorescent intensity at the 10x excess was found to be with 10% of the estimated intensity plateau.

The rinse cycles needed to rinse out excess reagent was determined by progressively centrifuging a sample containing DBCO-PEG4-Fluor reacting with NHS ester-PEG4-Azide and analyzed under fluorescence microscopy. Intensity was normalized to pre-wash baseline values. It was found that the normalized intensity of samples approached a plateau after 6 washes (**Figure 4.3**).

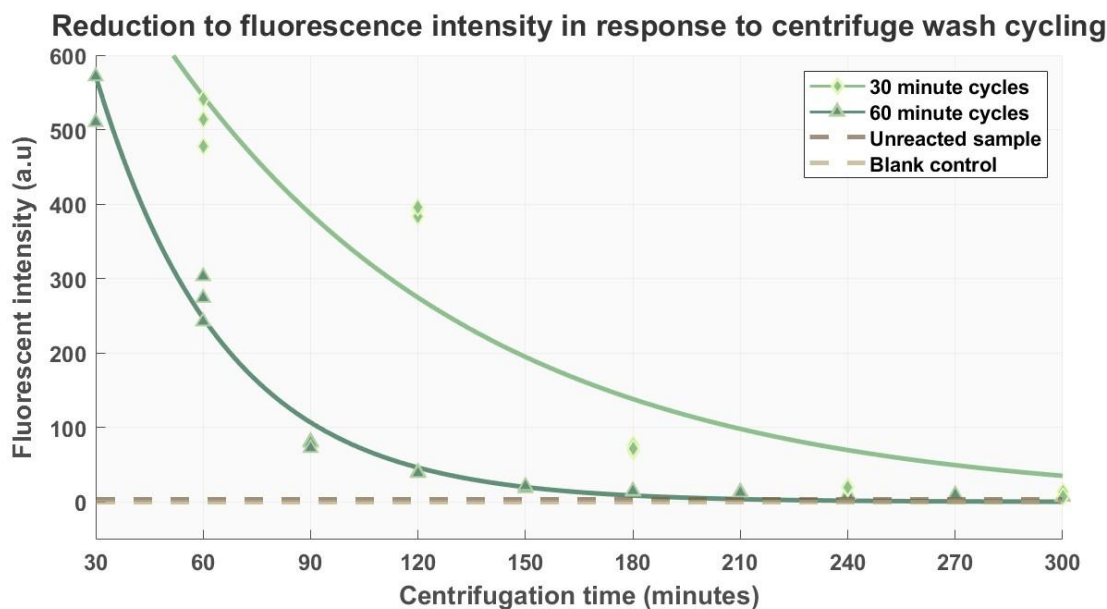


Figure 4.3. Fluorescent intensity of a dye analog to assess impact of wash cycling on remain low MW reagents (~1000 Da). A fluorescent dye MW ~ 1kDa was used as an analog for similar sized molecules. We observed that replacing the solution more frequently per unit time improved resulted in a greater decrease of fluorescence in the sample. 6 wash cycles at 30 minutes resulted in a value within 90% of the fluorescent plateau of the blank control samples. Where successive washes had diminishing returns.

4.2.2 Experimental Methods

4.2.2.1 Microscopy container set-up

A schematic of the testing frame is shown in (**Figure 4.5 (a)**). A frame for the test assembly was drafted using computer aided design software (SOLIDWORKS 2021, Dassault Systemes; Waltham, MA, USA) and exported into the stereo-lithography (.STL) format. The file was converted into 3D printing

instructions using slicer software (KISSlicer; Version 1.5) and exported into a 3D printer (Model DX-V2; CreatBot 3-D Printer, Zhengzhou, China) to prototype the model using poly-lactic acid filament (PLA 3D filament; Esun, Shenzhen, China). To construct the bottom of the container 22x22 mm cover slips were cleaned and sealed to the frame with vacuum grease (Dow Corning; Midland, MI, USA).

4.2.2.2 Fibrin clot sample preparation

Fibrin clots were created as reported by Acconcia et. al. (Acconcia et al., 2014), the coverslip was coated in fibrin through the crosslinking between fibrinogen and thrombin. Human fibrinogen (Fibrinogen-plasminogen depleted from human plasma, Sigma Aldrich, Prod. No. 341578) was mixed with 20 LU/mL human fXIII (Human Factor XIII, Enzyme Research, Prod. No. 5810A; South Bend, IN, USA) and fluorescently dyed human fibrinogen (ThermoFisher Scientific, Cata. No. F13191) in a 7.2 pH 50mM Tris (Trizma base, Sigma Aldrich, Prod. No. T6066), 100mM sodium chloride (Sigma Aldrich, K51865804007), 1mg/ml bovine serum albumin solution (Sigma Aldrich, Prod. No. A9418) at concentrations of 3mg/ml fibrinogens in a 9:1 mass ratio (non-labelled: labelled) and stored at -80°C. Fluorescent fibrin was chosen to help distinguish fibrin within the image. Human thrombin (Sigma Aldrich, Prod. No. T8885) was reconstituted from powder at 0.5 NIH U/ml in tris-HCl solution and stored at -20°C, 20mM calcium chloride was added (Sigma Aldrich, Prod. No. A1396782919). All reagents were transferred from freezer to 4°C for 24 hours prior to experiment. To create the fibrin layer, 100µl of both the fibrinogen and thrombin solutions were sampled into a temporary container and briefly aspirated with the pipette. The contents were then pipetted onto the framed coverslip and swirled for an even coating onto the glass, these steps were performed in under 30s. The frames were then transferred into a petri dish and elevated from the bottom of the dish to mitigate fogging. The dish was then incubated at 37°C for 3 hours. Prior to experimentation, the fibrin layer was washed 10 times with 500ml 1x PBS and filled to the top of the frame.

Other attempts before this human based clot model were explored using porcine or ovine whole blood as initially proposed. Previously, blood from a slaughtered animal was transferred into vacutainer tubes and incubated at the species' body temperature for 180 minutes and stored at 4°C to allow for retracted

before manually sectioning for analysis (**Figure 4.4**). Several factors resulted in the final decision for the choice towards a fibrin clot model: 1) The opacity of red blood cells limits the analysis of attached clots to the edges of a whole blood clot, 2) The similar size of blood particles made it difficult to differentiate between constituents and microbubbles, 3) Human tPA may not interact ideally with non-human blood products.

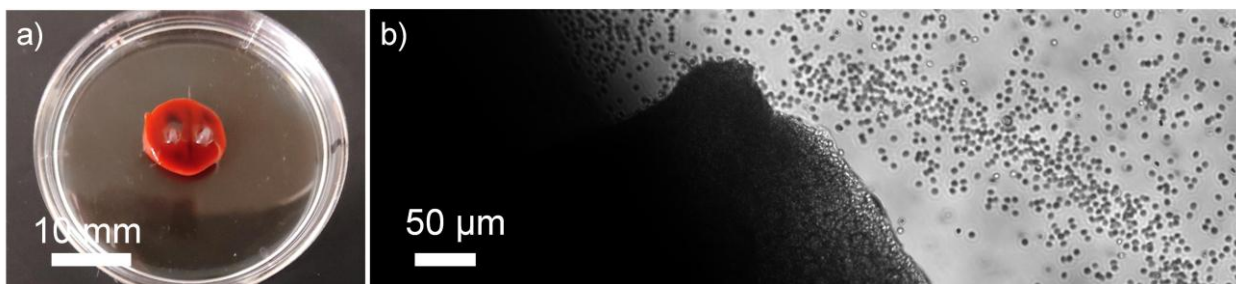


Figure 4.4. An example of a ovine whole blood clot section a) skewered onto a modified 35 x 10 mm petri, b) when viewed under brightfield microscope after initial rinse under PBS. Excess whole blood constituents can be seen polluting the sample. The opacity of red blood cells in the clot limits analysis on the edges of the clot seen in b).

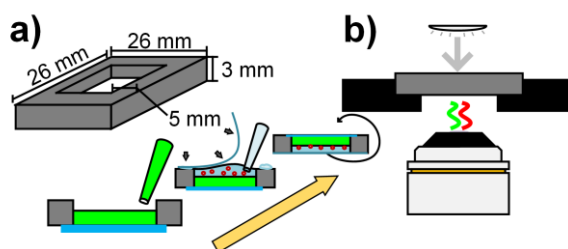


Figure 4.5. a) i) FITC bound fibrin precursors were used to form a fluorescing fibrin sample, DiD' tagged MBs were sealed and incubated with the fibrin sample before b) microscopy.

4.2.2.3 Fibrin Sample Preparation for Fluorescent Microscopy

The slide preparation and sample loading process were summarized in **Figure 4.5**. Vacuum grease was spread on the frame to create a temporary seal. The targeted bubble solution was mixed and a 50 μ l sample (10^8 bubbles) was gently pipetted into the enclosure and sealing it with clear polyester film (PET film, 0.003" Thickness Sheet, McMaster Carr, Prod. No. 8857K32; Elmhurst, IL, USA). The frame was inverted and held for 120s before reverting upright and left for 120s. The PET film was peeled off and the frame was mounted noting the location of the notch on the frame and imaged for the pre-wash acquisition

on a wide-field fluorescence microscope (Zeiss AxioObserver, Carl Zeiss AG; Oberkochen, Germany). Samples were exposed to bright field, 488nm and 647nm LEDs with 35ms, 200ms, and 400ms, exposure time respectively. After the acquisition, the enclosure was washed with 500mL of 1x PBS 20 times, mounted again and imaged again at the same sites for comparison.

Acquired images were exported into ImageJ (FIJI ImageJ, NIH LOCI University of Wisconsin; Madison, WI, USA) for particle counting. The RGB data of the 647nm fluorescent channel corresponding to labelled microbubble signal was converted to gray scale to eliminate noise then thresholded to exclude particles below the lower size cutoff of the microbubbles and counted. Automated counting was cross checked by manual counting of the raw channel image.

4.2.2.4 Ultrasound fibrin sample preparation

A 20mg/ml fibrinogen solution and a 25 U/ml thrombin solution were each prepared in pH 7.0 Tris-HCl mixing overnight on an orbital mixer at 80 rpm. Calcium chloride was added to the fibrin precursors for a final concentration of 20mM. Both mixtures were placed in an orbital mixer for 30 minutes at room temperature before use. 2% agar powder (Sigma Aldrich, Prod. No. A1296) was magnetically mixed in PBS heated to 85°C and allowed to cool to 65°C after the agar was fully dissolved. A 50mm petri dish was then filled with the agar, covered, and chilled at 4°C for 3 minutes. 80 µl of fibrinogen solution, followed by 40 µl of thrombin solution were transferred onto to the agar. The fibrin was incubated at room temperature for 30 minutes where the agar was then allowed to set at 4°C for 3 hours.

Agar's weight fraction was selected through empirical observations as a balance between fibrin adhesion behavior and the handleability of the phantoms. Higher weight fraction agar >2.5 wt% tended to rapidly solidify causing the fibrin pad to easily delaminate from the surface of the sample after preparation. Lowering the agar weight fraction generally improved the incorporation of the fibrin pad into the agar slab. However, the manipulability of lower weight fraction agar <1.5 wt% at room temperature was considerably delicate as the weight fraction decreases.

Clots samples were found to be sensitive to air exposure during handling. An older iteration of a whole fibrin clot is shown in **Figure 4.6** where bubbles formed from exposure to air are seen prior to the introduction of targeted MBs. It was necessary to degas the clots overnight at 4°C to mitigate the presence of air bubbles.

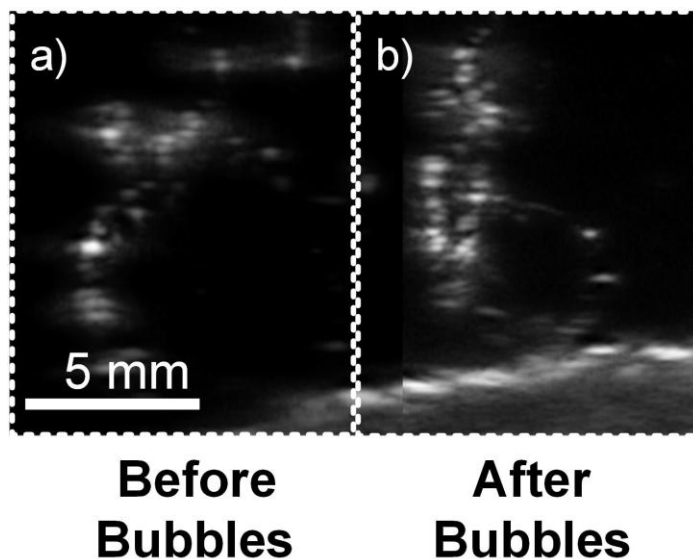


Figure 4.6. Harmonic mode ultrasound images taken using a clinical scanner of a previous iteration of a fibrin clot model shown: a) before the introduction of targeting MBs, b) after incubating with targeting MBs. The higher intensity harmonic signal in a) was because of a large presence of natural air bubbles gathered in the fibrin clot during handling and transfer that also made it the sample float towards the transducer unless secured. Fibrin clot was mostly anechoic in outside without the influence of air bubbles.

4.2.2.5 Ultrasound Imaging and Acquisition

Contrast ultrasound imaging parameters are summarized in **Table 1**. B-Mode images were acquired using a GE Venue Go (Venue Go; GE Healthcare, Mississauga, Ontario, Canada) clinical scanner with a L-4-12t Linear array probe using harmonic mode at 4.5MHz and MI of 0.16. We used acoustic outputs corresponding to a Mechanical Index of 0.16 to minimize bubble loss due imaging. Prior to testing, the treated agar samples were removed from petri dishes and immersed in PBS overnight. Similarly, imaging was performed using a custom reflection reducing stage soaked in PBS for 30 minutes before imaging. Agar samples were manually positioned, and at least 5 images were taken per slice of the sample. MBs concentrated at 77 RCF were reconstituted in 1mL PBS at approximately 1×10^8 bubbles/mL and gently

mixed. 200 μ l of MB solution was pipetted onto the fibrin pad. The samples were wet with 200 μ l PBS over 30 minutes to ensure it remained moist.

ImageJ's Region of Interest (RoI) analysis tool was used to compare area normalized mean intensity of the harmonic ultrasound image. Briefly, 1) A lasso RoI was drawn around the fibrin surface, 2) Repeated at least 5 images per sample before MB loading and after washing, 3) Results were averaged for each sample and compared for the change in mean intensity.

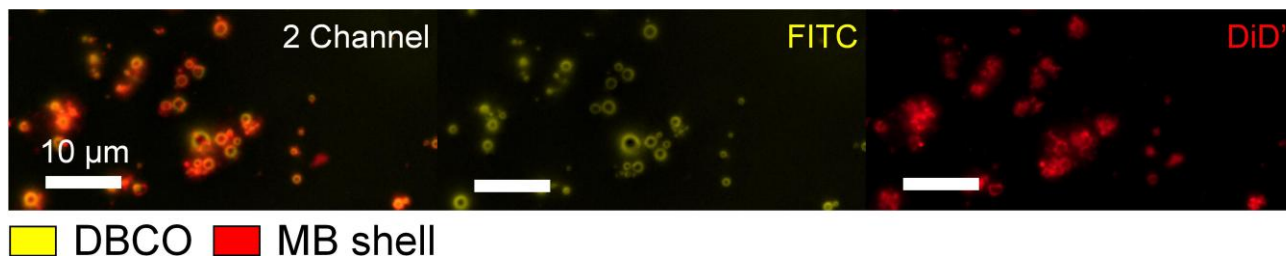


Figure 4.7. MBs dyed with DiD' membrane dye (red) tagged with FITC-azide (yellow) to mark DBCO located on the monolayer, the presence of both dyes on the MB in the two-channel overlay was used to confirm that DBCO was successfully incorporated onto the MB to enable the click reaction.

4.3 Results

4.3.1 Successful Conjugation Behavior Observed through Characterization

DBCO MB dyed with the lipophilic dye, DiD', click reacted with FITC-azide were imaged under fluorescence microscopy. MBs in **Figure 4.7** are fluorescent under both FITC and DiD' channels in the 2 channel overlay. tPA-azide was reacted with DBCO-PEG4-Fluor 545 and analyzed using fluorescence spectrometry in comparison to a blank PBS control. Shown in **Figure 4.8** the washed tPA-azide solution remained fluorescent when compared to unreacted dye and blank control. The average MB diameter

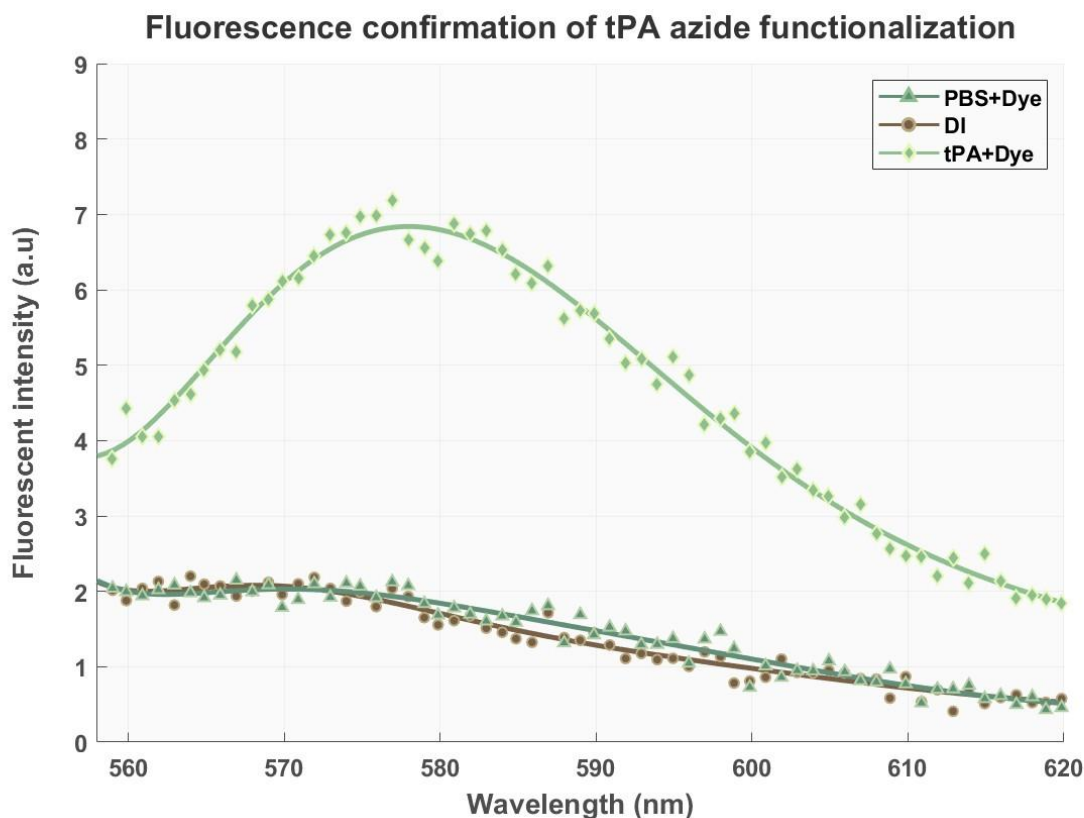


Figure 4.8. tPA inhibited by PPACK and undergone the azide functionalization process was tagged with DBCO-Fluor 545 and after passed over a 30 kDa centrifuge filter to isolate the tagged protein. A comparison of the fluorescence treated tPA samples against a DI water blank and a sample containing unreacted DBCO-Fluor 545 sees an increase in fluorescent intensity of samples retained above the 30 kDa filter which was attributed to the successful SPAAC reaction between the azide functionalized tPA and the DBCO marked dye. determined by dynamic light scattering as 2 μ m pre-reaction with an increase in size for both tMB and MB after reaction handling, **Figure. 4.9**.

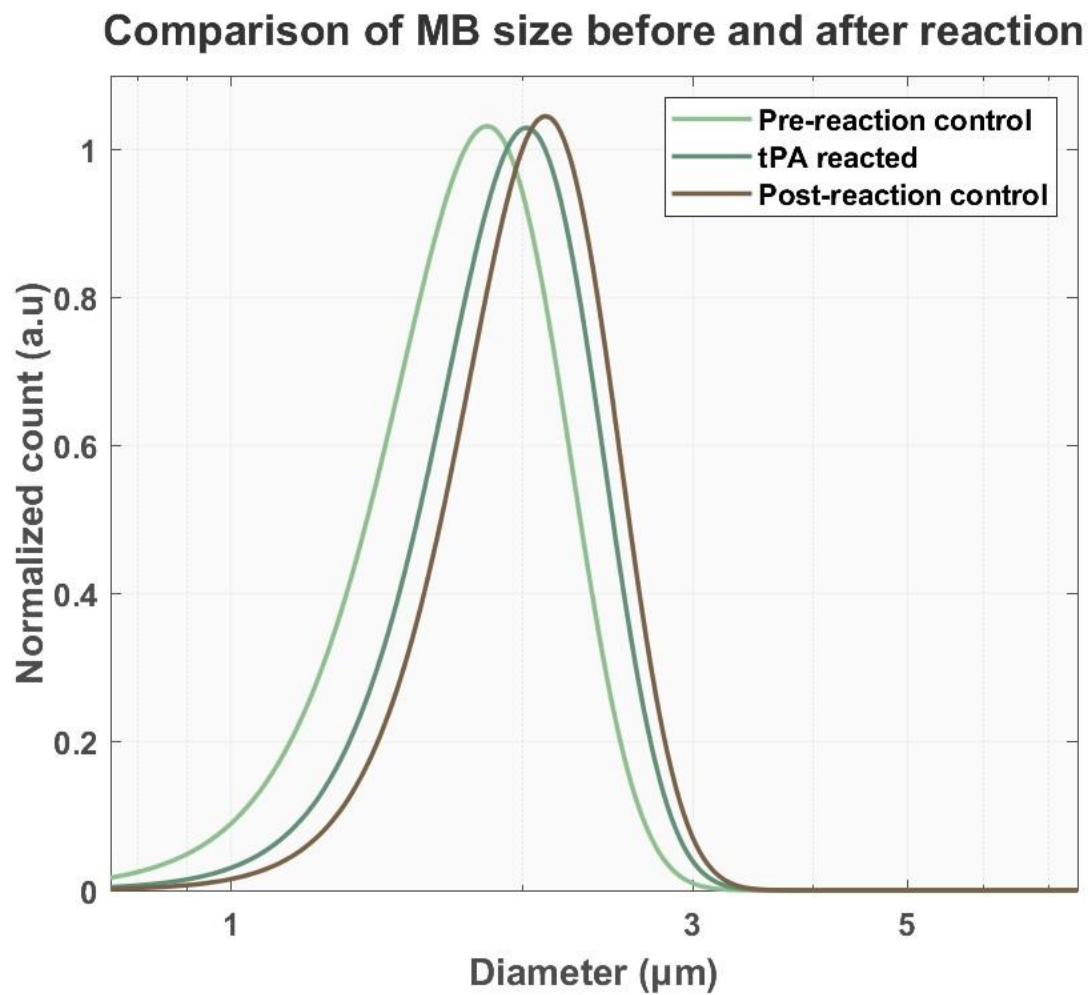


Figure 4.9. Microbubbles were sized using DLS to examine the effect that incubation over a 2-hour reaction period has on their size. DBCO MBs incubated with tPA azide and with a normal blank solution observed an expected increase in size over the incubation period.

4.3.2 tPA Ligand Conjugation Improves Retention of MBs on Fibrin Surface

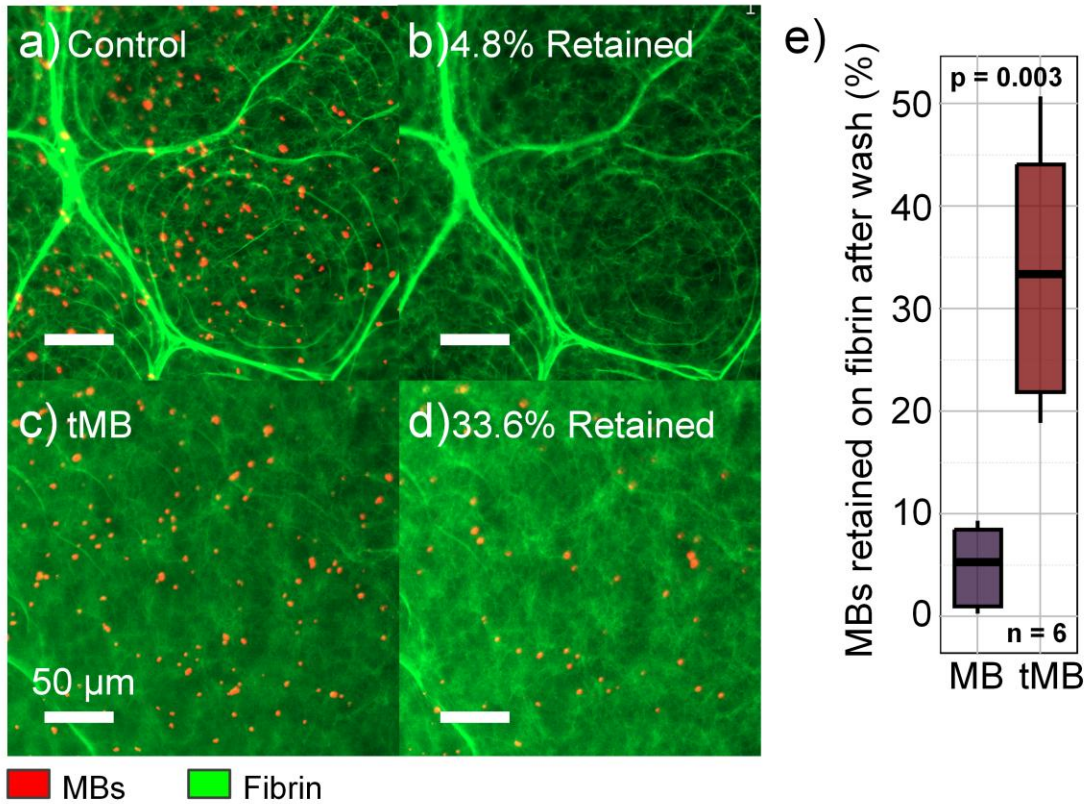


Figure 4.10. Fluorescence microscopy images of fluorescent fibrin (green) coated glass surface and labelled MBs (red). Presented are fibrin surfaces loaded with labelled MBs without tPA conjugation before (a), and after (b) rinsing; and tPA conjugated MBs before (c) and after (d) rinsing, a 33.6% MB retention was observed in the targeted sample (e) (n=6).

MBs conjugated with tPA were loaded onto a fibrin coated coverslip and compared against unreacted controls to establish the pre-washed image (**Figure 4.10 (a), (c)**). MBs were then washed 6 times with PBS and the post-washed sites were imaged for a comparison (**Figure 4.10 (b), (d)**). MBs were observed in both the control and tMB images after loading (**Figure. 4.10 (a), (c)**). Both groups (**Figure. 4.10 (b)** [control], **Figure. 4.10 (d)** [tMB]) were seen to have reduced MB counts after rinsing. Pre-washed and post-washed sites were compared using the ImageJ Particle Counter Addon. Briefly, MBs in the DiD⁺ channel were thresholded. A $4.8\% \pm 4.2$ MB retention was observed with undecorated MBs on the fibrin pad compared to the $33.6\% \pm 13.5$ of

the tMB samples (n=6).

4.3.3 tPA-MBs Improves Fibrin Surface Identification Under Ultrasound

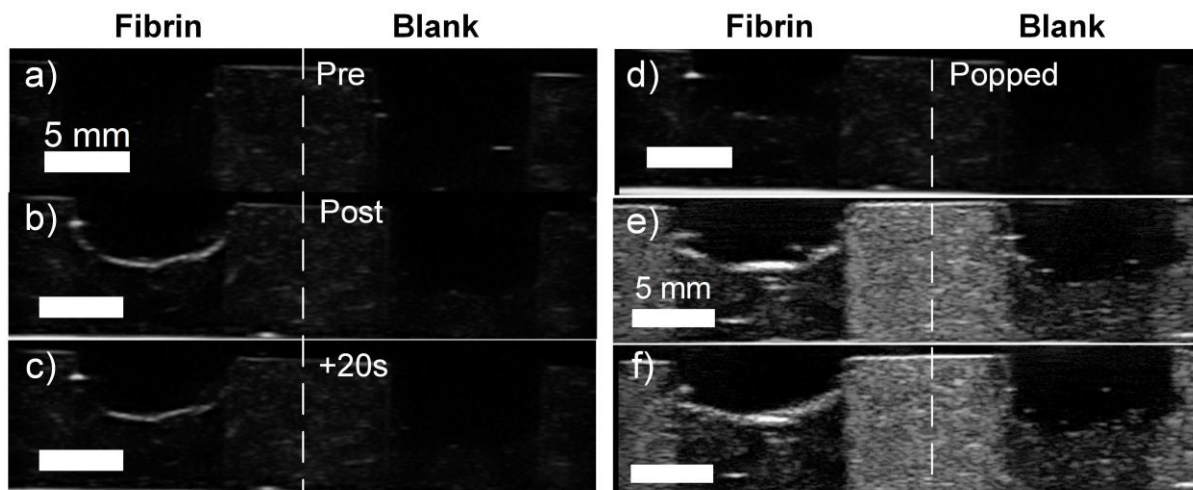


Figure 4.11. Preliminary images of a fibrin surface on agar well site taken using a BK Medical SonixTouch and Orcheo Sonoscanner. a) Harmonic image of MB loading site prior to loading taken at -10 Power on the SonixTouch depicting minimal signal, b) specific enhancement of fibrin surface after loading and wash, c) site after 20s of continuous imaging depicting some loss of bubble signal, d) site after imaging at 0 Power (Maximum) following bubble destruction, e) and f) before and after loading of a fibrin surface on the Sonoscanner with some air contamination seen in e).

Figure 4.11 depict preliminary trial images taken using a BK Medical SonixTouch **a-d**, and an Orcheo LITE Sonoscanner **Figure 4.11 (e), Figure 4.11 (f)**. **Figure 4.11 (a)** and **Figure 4.11 (b)** depicting before and after wash observes similar enhancement to local harmonic image pixel intensity at the fibrin surface. The initial ultrasound trial for this study using an agar supported well plate coated to enable the simultaneous imaging of a fibrin coated and untreated agar surface for a direct comparison in the same imaging plane. **Figure 4.11 (c)** loss of harmonic enhancement was observed after 20s of continuous imaging at minimum power $\sim$ MI 0.16 whereas imaging at base power $\sim$ MI 1.96 resulted in complete signal loss. **Figure 4.11 (e)** and **Figure 4.11 (f)** depict images taken on a portable scanner showing some enhancement in signal intensity at the fibrin coated well surface after incubation **Figure 4.11 (f)**.

B-mode US images of the fibrin containing site are shown in **Figure 4.12** taken using the GE Venue Go (Venue Go; GE Healthcare, Mississauga, Ontario, Canada) after experience equipment malfunction or

lack of availability for the scanners used in **Figure 4.11**. The fibrin region is circled in purple in **Figure 4.12 (a)** atop the agar pad. All samples were immersed in PBS for 24 hours before imaging to reduce bubble signal from air bubbles. Both fibrin film and agar pad produced ultrasound signal prior to contrast introduction (**Figure 4.12 (b), (c)**). MBs were loaded to the surface, allowed to incubate for 30 minutes, and rinsed 6 times before imaging. 5 different cross-sectional images were averaged for each sample to account for realigning the sample after washing. Post-wash fibrin samples loaded with tMB were observed to have brighter fibrin region in comparison to the MB control after both were washed **Figure 6(d), (e)**. Post-wash observations are summarized in **Figure 4.12 (f)**. Images taken of tMBs post-wash saw increased local log-compressed signal intensity at the fibrin film by $94.9\% \pm 16.1$, in contrast the MB control only saw an increase of $27.6\% \pm 10.0$ ($n=4$, $p= 0.004$).

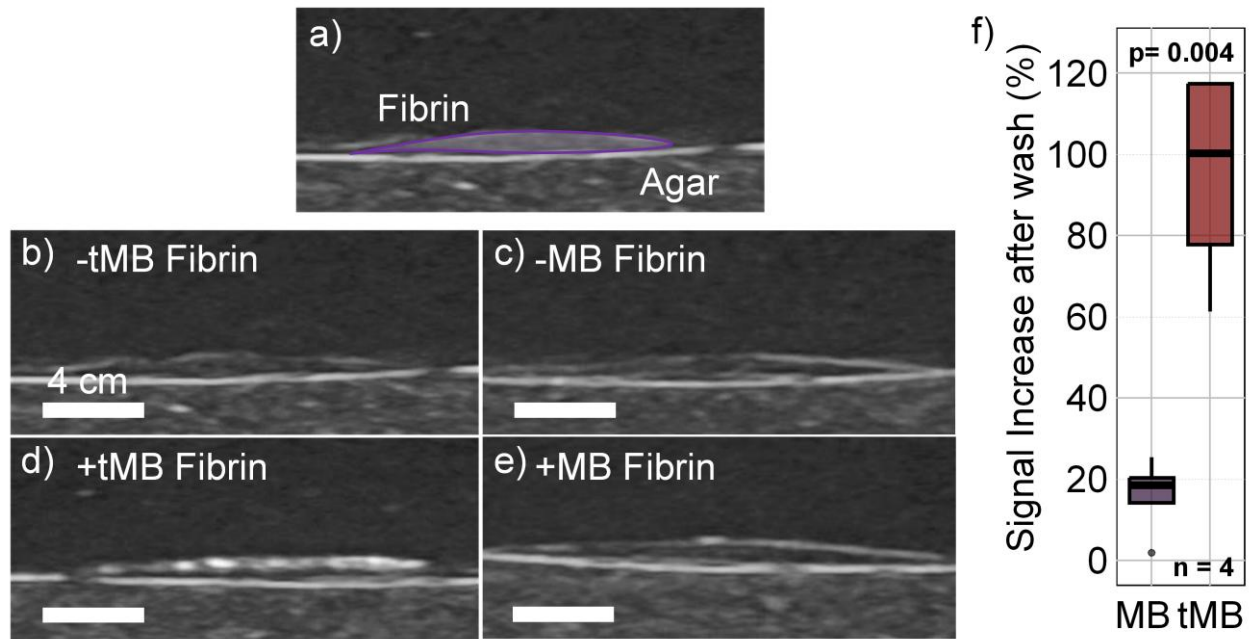


Figure 4.12. Representative harmonic images showing fibrin clot loaded onto an agar sample (a), with (b), (c) before MB introduction, corresponding images of the washed fibrin clot after tMB (d), and (e) MB introduction show 94.9% intensity increase in tMB shown in (f).

4.4 Discussion

Fibrin molecules are an important biomarker in VTE management but are inaccessible by conventional US. Thus, we have devised a contrast platform that attaches to fibrin surfaces to highlight fibrin regions under US. As such, we combined phospholipid stabilized microbubbles with fibrin specific inactivated tPA using a click chemistry framework [Figure 4.1] supported with analysis of reactions at key stages [Figure 4.7], [Figure 4.8], [Figure 4.9]. The targeting characteristics of these microbubbles were then demonstrated [Figure 4.5] [Figure 4.10] in epi fluorescence microscopy. Our observations under harmonic-mode ultrasound imaging using a clinical-research scanner [Figure 4.12], yielded similar findings where we encountered a 94.9% increase in signal on our fibrin coated surface in contrast to a non-fibrin substrate. In general, our results reported successful processing of the reaction intermediates using our methods and the expected functional outputs in the final product.

The focus of this study was to present the design of click chemistry enabled targeted US contrast to enhance ultrasound detection of fibrin. We used a chemically inactivated tPA-ligand click conjugated to a contrast microbubble to achieve this. While the modifications for click chemistry were straightforward, the caveat with this design comes through the choice of the tPA-ligand. A practical concern *in vivo* is that the targeting ability of tPA may be diminished by its therapeutic effects. Specifically, it may be possible for activated plasmin to dissolve fibrin that MBs are attached to causing US enhanced clusters of fibrin to be lost. Since the *in-vitro* reagents did not contain plasminogen to convert to plasmin, we were unlikely to have seen thrombolytic effects in our test.

Second, the concentration of microbubble used for both the microscopy and ultrasound image testing is notably high at 10^8 MB/ml. This concentration was chosen to be consistent between the microscopy and ultrasound-based tests with an emphasis on loading MBs onto the fibrin surface. One possible consequence of using this higher contrast agent concentration is the acoustic shadowing of features found at greater depths when imaged under ultrasound which is further exacerbated with imaging at lower power output (Fetzer et al., 2023). It is noted that the ultrasound samples in the quantitative tests were

imaged before MB introduction and after MB wash-off where free floating MB concentration was significantly lower than the concentration used for loading. Thus, the possible effects of acoustic shadowing caused by the large concentration used in loading was not initially considered here. After noting the above, one main concern would be that the retained loaded MBs were of sufficient concentration to cause shadowing. Yet this effect was not qualitatively observed to a degree warranting inquiry here with reference to the representative image depicted in **Figure 4.12 (d)**. Regardless, one possible method of assessment with the test model here would be to examine the area normalized intensity difference between a RoI in the agar region beneath a loaded tMB and control MB segment akin to **Figure 4.12 (f)**. Naturally, the effects of shadowing would be of greater consideration when pivoting towards more realistic models such as a the case where more than one fibrin surface is interrogated by the incident acoustic pulse in the axial direction. For further thought is that targeting MBs were also observed to not penetrate past the fibrin surface likely owing to the large physical size of the contrast compared to pores within fibrin clot. It may be possible that shadowing is unlikely from a combination of low binding efficiency as well as the limitation of only binding to the surface when operating much lower contrast concentrations. Overall, the effects of shadowing do require more investigation in the future.

Third, the functionalization of the ligand was made through reaction between a bridge molecule with NHS-ester to endogenous primary amine. Since primary amines are abundant on the tPA peptide and it is possible that more than one primary amine on a single tPA protein was functionalized in the process. Possible observable outcomes from this include clustering of bubbles around a core tPA protein containing more than one azide functional group. Also, instances of tPA proteins anchored by more than one DBCO-PEG-lipid on a single bubble may inhibit lipid pullout during shear flow. Since the latter effect has functional implications on how adherent the bubble will be under flow conditions quantifying the amount of functionalized azide groups on a tPA molecule may be worthwhile. Overall, it should be recognized that tPA may be better suited for therapeutic applications. However, it can be treated as an analog for other ligands with accessible primary amines using this process. One promising direction that can be explored

for fibrin targeting peptides comes from activatable proteins that are sensitive to clotting enzymes in the local environment. A non-specific strategy was developed by Lux et. Al (Lux et al., 2017) using thrombin cleavable ligands to modify the surface charge of a conjugated MB to electrostatically target positively charged red blood cells in a whole blood clot. This has shown promise recently where a thrombin activated peptide has seen use as a release mechanism for a separate fibrin targeting peptide in photoacoustic applications (Yang et al., 2020).

In this work we demonstrated the creation and use of fibrin targeting microbubbles for ultrasound molecular imaging shown under both microscopy and ultrasound imaging under *in vitro* set up. Although both experiments were under static conditions unlike that of realistic *in vivo* flow settings. In this case it was sufficient in showing that the bubbles do stick to the fibrin surface to demonstrate that the modifications to the ligand did not affect fibrin binding characteristics of the ligand. Thus, the logical future endeavor would be to determine if the adherent bubbles are still functional under flow conditions.

4.5 Conclusion

Fibrin targeting technologies sees steady development in recent years to address a clinical need for fibrin clot detection. However, current conjugation methods used in fibrin targeted US contrast designs have yet to catch up to the state-of-the-art method for clinical translation. Here, we have produced a fibrin targeting ultrasound contrast agent leveraging a biorthogonal conjugation scheme and the native fibrin targeting capabilities of tPA. With the improved binding to fibrin, our new contrast can be used to enhance B-mode signals of fibrin surfaces to aid in its detection on clinical scanners. Overall, this more robust preparation method eases the translation of fibrin visualization technologies into clinical venous clot diagnosis.

Chapter 5

5.1 Introduction

Pathological formation of blood clots within veins make up an umbrella of disease conditions including deep vein thrombosis and pulmonary embolism. These diseases may be pharmaceutically managed through inhibiting fibrin accumulation using anti-coagulants or directly destroyed through fibrinolytics. The choice of treatment pathways are made by consultation to clinical guidelines devised to balance bleeding risk with the certainty of treatment effectiveness (Ortel et al., 2020). However the low-certainty of evidence behind many fibrinolytic or recurrent clot treatment methods results in conditional approaches in treatment selection. Currently, clinical imaging technology offer little evidence on both the true effectiveness of fibrinolytic treatment or in the indefinite administration of anti-coagulants during the recurrent prevention period (Ortel et al., 2020; Saha et al., 2016). With both methods having an associated risk to the patient. There is a practical need to improve the value added of medical imaging to enhance the quality of indications made for clot management. This need is reflected in pushes for contrast agents to fulfill a role of clot labellers in magnetic resonance imaging or positron emission tomography (Andia et al., 2014; Oliveira & Caravan, 2017). Ultrasound (US), as the workhorse point of care modality in this field (Lim et al., 2018), also shows potential in the development of novel contrast enhanced therapeutic strategies and imaging investigations (Bader et al., 2016; Bucek et al., 2001; Smith et al., 2016). Yet a translational barrier for all contrast agents comes from their classification as a pharmaceutical instead of medical device (Navarro-Becerra & Borden, 2023). Consequently, it is prudent to understand the practical implications of the ligands used to enable clot targeting for contrast ultrasound.

Ligand choice is key, venous clot targeting exploits the high fibrin content of venous events and arterial clot targeting focuses on elevated presence of platelets mirroring clinical treatment objectives (S. Liu et al., 2025). Briefly, MB targeting in arterial processes leverage arg-gly-asp-ser to bind to glycoprotein 2b/3a receptors specific to platelets (Hua et al., 2014; Nederhoed et al., 2017). Outside of these specific markers, marker agnostic strategies also exist as seen with a ligand solution intended to detect acute clots

through thrombin activated electrostatic targeting to negatively charged red blood cells (Lux et al., 2017). In the context of venous clot specific targeting in fibrin, the predominant ligands in study use small peptides adapted from other applications including: modified EP2104-R, a fibrin specific peptide agent with multimodal potential via gadolinium chelation (Mohammed et al., 2024; Oliveira & Caravan, 2017; Overoye-Chan et al., 2008; Yang et al., 2020); CREKA, a pentapeptide (cys-arg-glu-lys-ala) used for specific targeting of fibrin-fibronectin complexes (Gormley et al., 2019; Stefanelli & Barker, 2015; Y. Zhong et al., 2019); and recombinant human tissue plasminogen activator (rtPA, tPA) leveraged for therapeutic applications (Klegerman et al., 2008; Z. Wang et al., 2021). Currently, the research emphasis comes from a therapeutic angle of enhancing existing treatment options or as an alternative to high risk indications (Goel et al., 2021; Schleicher et al., 2016; F. Wang et al., 2019b; B. Zhang et al., 2019). We previously documented a MB based contrast agent using an inhibited rtPA ligand as a robust imaging agent. However in considering current contrast ultrasound's trend toward treatment, the drug classification placing greater emphasis on safety, and tPA's status as a high-risk clinical indication we must investigate the value of using the inhibited form as ligand for imaging.

We hypothesize that PPACK inhibited tPA will experience key differences in function within the presence of plasminogen compared to peptide based ligands. To demonstrate this, we tested a tPA based microbubble contrast agent against a similar peptide based MB within in vitro conditions in a scaled microfluidic chamber. In summary this will include 1) Binding comparison of both ligands outside the presence of plasminogen under aggressive washout conditions, 2) A similar comparison of both ligand within the presence of plasminogen, 3) A demonstration of both ligand-MBs under continuous flow conditions. tPA-MBs will be tested *in vitro* using fluorescence microscopy for their ability to adhere to a fibrin surface and characterization data will be provided to support finding in microscopy.

5.2 Methods

5.2.1 Targeted Microbubble Preparation

5.2.1.1 Ligand Preparation

tPA (Sigma Aldrich, Prod. No. 612200; St. Louis, MO, USA) was reconstituted from powder at a 1mg/ml concentration by manufacturer recommendations using sterile DI and placed into aliquots for storage at -80°C. tPA was removed from storage and allowed to thaw at room temperature prior to reaction. To deactivate thrombolytic qualities of tPA, N-(2-amino-3-phenylpropanoyl)-1-[1-chloro-6-(diaminomethylideneamino)-2-oxohexan-3-yl] pyrrolidine-2-carboxamide (PPACK; Sigma Aldrich, Prod. No. 520222) at a 1:1 molar ratio to tPA was added into a pH 7.2 50mM phosphate buffer (Sigma Aldrich, Prod. No. P3288) solution with 20mM l-arginine (Sigma Aldrich, Prod. No. A5006) and 1% Tween-20 (Sigma Aldrich, Prod. No. P1379). tPA was reacted in the dark for 1 hour at room temperature on a tube revolver at 10 Hz (Tube Revolver, ThermoFisher Scientific, Cata. No. 88881001; Canada) then isolated by centrifugation with 30kDa centrifugal filters (Ultracel-30, Sigma Aldrich, Prod. No. UFC5030) and washed (Centrifuge 5910 R, Eppendorf AG; Germany) at least 6 times against 1x phosphate buffered saline (PBS) at 10-minute cycles of 14000g. The washed protein was functionalized with NHS-ester – PEG4 – Azide (TCI America, Prod. No. A2388; Montgomeryville, PA, USA) for 90 minutes at room temperature on a tube revolver and similarly washed 6x times by centrifugation. CREKA-PEG4-Azide pentapeptide was acquired through Thermofisher's protein synthesis service.

5.2.1.2 Base Microbubble Preparation

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC; Avanti Polar Lipids, Prod. No. 850365P; Alabaster, AL, USA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy(polyethylene glycol)-2000] (DSPE-PEG2k-PE; Avanti Polar Lipids, Prod. No. 880120P), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[dibenzocyclooctyl(polyethylene glycol)-2000] (DSPE-PEG2k-DBCO; Avanti Polar Lipids, Prod. No. 880229P) were measured at a 18:1:1 (DSPC: DSPE-PEG2k-PE: DSPE-PEG2k-DBCO) molar ratio. The mixture was dissolved in 1ml of chloroform, placed under nitrogen flow to evaporate the solvent and moved into vacuum for 2 hours to remove excess chloroform. A solution of 7:2:1

(DI Water: Glycerol (Fisher Chemicals, Cata. No. G331; Canada): Propylene glycol (Sigma Aldrich, Prod. No. P4347)) was added to the lipids and the solution was then bath sonicated at 55-60°C for 15 minutes, then tip sonicated (Branson SFX250, Emerson Electric; St. Louis, MO, USA) at 10% amplitude pulsed for 5s:10s:11m (On: Off: Total). The liposome mixture was allowed to cool to room temperature and stored. 0.1% of total lipid molarity of lipophilic dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, perchlorate (DiD'; ThermoFisher Scientific, Cata. No. D307) in 5 mg/ml ethanol solution was transferred into an empty container and the solvent was evaporated under nitrogen stream. To label the lipids with lipophilic dye, stock lipid solution was transferred into the DiD' container and tip sonicated under similar conditions as previously described for the stock lipid solution. Stock lipid contents were placed into aliquots within headspace vials. Microbubbles were formed by mechanical agitation, the headspace was replaced with decafluorobutane (PFB; Synquest Laboratories, 1100-2-06; Alachua, FL, USA) and shaken at 4.2kHz with a dental amalgamator.

5.2.1.3 Microbubble Conjugation Reaction

MBs were size selected by differential centrifugation at 15g at 120s and 137g for 300s and suspended in 1ml of 1x PBS at 7.4pH. Azido-tPA was added to the solution at a 10x (tPA) or 30x (CREKA) molar excess in relation to the estimated DSPE-PEG2k-DBCO content at the current MB concentration. The reagents were mixed on a tube revolver for 90 minutes at room temperature. Reacted MBs were diluted to 1ml then washed 2 times of unreacted azido-tPA by centrifugation at 77g for 300s and replacing 400µl of supernatant with fresh PBS. The bubbles were concentrated with a final 77g cycle for 300s.

5.2.2 Experimental Methods

5.2.2.1 Microbubble and Reagent Characterization

We used an in vitro flow chamber to control the delivery of MBs. Briefly, MBs were first delivered by syringe pump into a commercial cell culture to interact with the fibrin surface, afterwards, excess bubbles were flushed using PBS and the surface was imaged by fluorescence microscopy where the full preparation pipeline is shown in **Figure 5.1**.

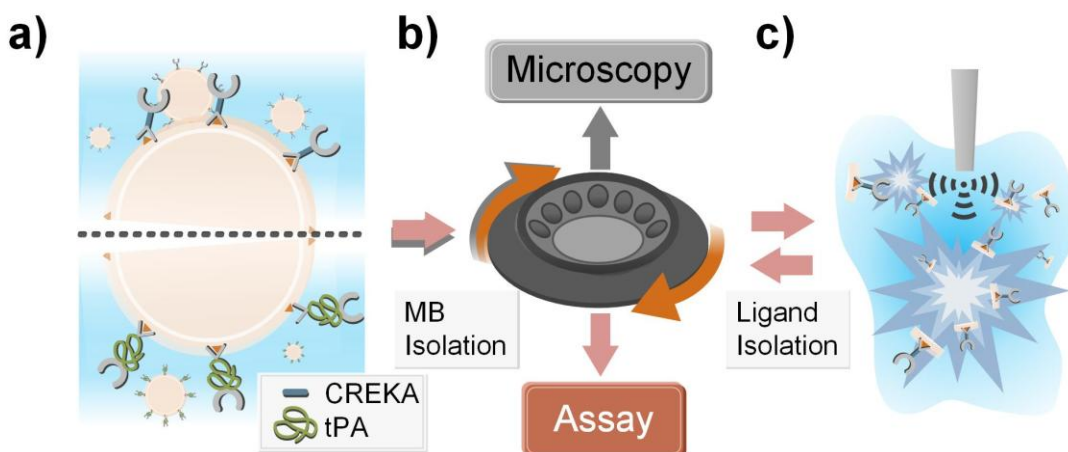


Figure 5.1. Overview of the MB processing and analysis pipeline: a) size selected DBCO-MBs were reacted with a common fibrin targeting pentapeptide (CREKA) or the inhibited fibrin targeted thrombolytic (tPA), b) unreacted targeting proteins were then washed by serial differential centrifugation where upon MBs were used for either microscopy experiments or c) destroyed by cavitation via horn sonication followed by b) centrifuge filtering to isolate the reacted phospholipids and used in assays.

We examined attachment of MBs in different modes, a comparison was made between the number of retained MBs on the surface before and after incubation followed by PBS rinse. Fibrin sample preparation and microscopy are summarized in **Figure 5.2**.

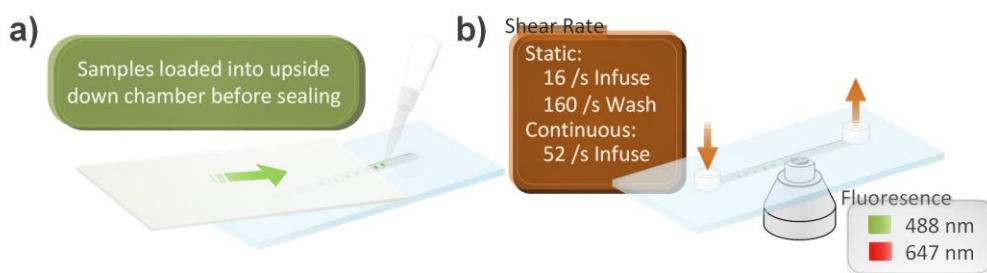


Figure 5.2. (a) FITC bound fibrin precursors were used to form a fluorescing fibrin sample, DiD' tagged MBs were infused into a sealed chamber and incubated with the fibrin sample before b) microscopy.

5.2.2.2 Microbubble loading and imaging set-up

A schematic of the testing frame is shown in (Figure 2a). A frame for the test assembly was drafted using computer aided design software (SOLIDWORKS 2021, Dassault Systemes; Waltham, MA, USA) and exported into the stereo-lithography (.STL) format. The file was converted into 3D printing instructions

using slicer software (KISSlicer; Version 1.5) and exported into a 3D printer (Model DX-V2; CreatBot 3-D Printer, Zhengzhou, China) to prototype the model using poly-lactic acid filament (PLA 3D filament; Esun, Shenzhen, China). To construct the bottom of the container 22x22 mm cover slips were cleaned and sealed to the frame with vacuum grease (Dow Corning; Midland, MI, USA).

5.2.2.3 Ligand concentration characterization

Bound CREKA concentration was determined using a free thiol (-SH on the cysteine residue) quantification assay (Free Thiol Assay Kit; abcam, Prod. No. ab112158) using a multiplate reader (Tecan Spark, Tecan Group Ltd., Männedorf, Switzerland) at 490 nm. Unreacted CREKA samples were reconstituted in PBS as received and diluted to the manufacturer recommended concentration for testing. Reacted MBs were first destroyed by bath sonication for 5 minutes, centrifuge for six (6) 15 minutes cycles at 14000 RCF over a 3kDa filter, and diluted to a working concentration before being handled as recommended. Bound tPA concentration was similarly determined using an ELISA kit (Human tPA ELISA Kit; ThermoFisher Scientific, Catalog # BMS258-2) following manufacturer recommendations at 450 nm. Reacted MBs were similarly prepared but filtered over 30kDa centrifuge filter.

A detailed overview of the free-thiol assay is as follows:

1) Concentrated lipids were serial diluted in duplicated according to manufacturer recommendations reproduced below for a standard 96-well plate:

STANDARD [μM (mol^{-6}/L)]	STANDARD [μM (mol^{-6}/L)]	CREKA Azide [μM (mol^{-6}/L)]	tMB 1 [μM (mol^{-6}/L)]	tMB 2 [μM (mol^{-6}/L)]	tMB 3 [μM (mol^{-6}/L)]
30	30	1.875	1.48	1.48	1.48
7.5	7.5	0.46875	0.37	0.37	0.37
1.875	1.875	0.117188	0.093	0.093	0.093
0.46875	0.46875	0.0292875	0.023	0.023	0.023

0.117188	0.117188	7.5	1.48	1.48	1.48
0.0292969	0.0292969	1.875	0.37	0.37	0.37
0.0073242	0.0073242	0.46875	0.093	0.093	0.093
BLANK	BLANK	0.117188	0.023	0.023	0.023

Table 5.1. Free thiol quantification experiment tracker sample. Standard curve was performed in duplicate following manufacturer recommendations. As received CREKA azide sample was analyzed in duplicate to account as a before-reaction point, three separate MB samples harvested post reaction were performed in duplicate to produce the after-reaction results. Sample concentrations used were working estimates based off of serial dilution calculations.

2) Sample preparation as recommended by abcam are as follows: a) a 1 mM (mol⁻³/L) glutathione (GSH, single free thiol group) stock standard solution was prepared using MilliQ water with excess aliquoted and stored at -20°C, b) a 100X stock solution of thiol green indicator stock was diluted using dimethyl sulfoxide with excess aliquoted and stored at -20°C, c) A working indicator solution was prepared by adding 50µl of the 100X thiol green indicator into 5mL of the provided assay buffer, d) A working GSH standard solution was prepared from 30 µl of GSH standard and 970 µl of assay buffer for a 30 µM working concentration, e) 200 µl of the working GSH standard was used for serial dilution at a 1:3 ratio using the assay buffer, f) samples were also serial diluted using assay buffer, g) 50 µl of standard or sample were added to the 96-well microplate and 50 µl of working thiol green indicator was added to each well by multichannel pipette and aspirated, h) samples were incubated at room temperature for 10 minutes to 1 hour with protection from light and monitored at 5 minute intervals under 490/520 nm excitation/emission.

Data analysis was performed through interpolative fitting of sample data on a linear regression curve. Data processing was performed in MATLAB. To calculate loading efficiency, control and loaded samples were respectively normalized by dilution before final outputs and efficiency was calculated by difference in molar concentration before and after loading.

A detailed overview of the human tPA ELISA protocol is as provided:

STANDARD [pg/ ml]	STANDARD [pg/ ml]	tPA-Azide [pg/ ml]	tMB 1 [pg/ ml]	tMB 2 [pg/ ml]	tMB 3 [pg/ ml]
1000	1000	6630	8000	8000	8000
500	500	3315	4000	4000	4000
250	250	1657	2000	2000	2000
125	125	829	1000	1000	1000
62.5	62.5	6630	8000	8000	8000
31.3	31.3	3315	4000	4000	4000
15.6	15.6	1657	2000	2000	2000
BLANK	BLANK	829	1000	1000	1000

Table 5.2. tPA ELISA experiment sample calculations of Test Standard, PPACK inhibited tPA azide, and harvested lipids after reaction with MBs. *Not shown, in a calibration trial, as received tPA was tested following the standard serial dilution range and found to roughly follow the expected standard. Dilution estimates for the inhibited tPA azide and tMB harvested samples were modified from manufacturer recommendations after inhibited tPA azide measurements were found to deviate from untreated tPA results. We hypothesize that the inhibition and functionalization processes changed epitopes targeted by the anti-bodies used for the assay causing a reduction in amplification in this test. Additionally, a Thermofisher Technical Support Representative responded to inquiry that they did not perform epitope mapping for the test at the time.

Briefly: 1) Packaged assay solutions were diluted to their stock or working concentrations following manufacturer recommendations 2) The working standard was serial diluted at 1:2 serial dilutions in microcentrifuge tubes, 3) Microwell strips were separated and washed using the packaged wash buffer solution aspirated by multichannel micropipette between washes and placed upside down on a wet absorbent, 4) Standard dilutions for all concentrations were added to the standard wells in duplicates in the layout shown in *Table 2* and mixed by aspiration and ejection by multichannel pipette, 5) Blank wells were filled using 100 μ l of sample diluent, 6) 90 μ l of sample diluent as placed in each “sample” well followed

by 10 μ l of sample solution in their corresponding wells, 7) 50 μ l of working horseradish peroxidase (HRP) was transferred into each well from a multichannel reservoir, 8) The plate was then covered in an adhesive cover and aluminum foil and incubated for 2 hours on a microplate shaker, 9) After 2 hours, the covers were removed and washed 6 times following recommended instructions, 10) 100 μ l of substrate solution was transferred from a reservoir to all wells with the plate covered to protect samples from light exposure to incubate for 10 minutes at room temperature, 11) Specifically, as recommended by the manufacturer, color development was monitored using reference absorbance measurements at 620 nm and stop solution was added as soon as the solution reached an O.D. value of 0.9 to 0.95, 12) Finally, the absorbance readings of each well at 450 nm was measured immediately after adding the stop solution.

Briefly, a standard curve was formed through a Four Parameter Logistic Regression with slight modification from the manufacturer's recommended Five Parameter Model. Where the loss in the additional parameter will fail to account for asymmetrical sigmoidal datasets. The concentration of sample was determined through interpolation by the inverse function. Data processing was performed using MATLAB with an open-source code (Cardillo, 2025).

5.2.2.4 tPA enzymatic activity characterization

tPA enzymatic activity was measured by colorimetric assay following manufacturer recommendations (Tissue type Plasminogen Activator Activity Assay Kit; abcam, Prod. No. ab108905) using a multiplate reader at 405 nm. Briefly, untreated tPA control, PPACK treated azido-tPA, and an unreacted azide-PEG4-NHS ester were handled as described by the recommendations. Reacted MBs were first destroyed by bath sonication for 5 minutes, isolated by centrifuge for six (6) 15 minutes cycles at 14000 RCF over a 30kDa filter and diluted to a working concentration before being handled as recommended.

To describe the multiplate sample preparation in greater detail: 1) Harvested sample were serially diluted under the manufacturer recommended standard reproduced as follows:

Standard Point	Dilution	[Tissue type Plasminogen
----------------	----------	-----------------------------

		Activator] (IU/ml)
1	1:3 (Standard 160 IU/ml: Diluent)	40
2	1:3 (Point 1: Diluent)	10
3	1:3 (Point 2: Diluent)	2.5
4	1:3 (Point 3: Diluent)	0.625
5	1:3 (Point 4: Diluent)	0.156
6	Assay Diluent	0

Table 5.3. tPA activity colorimetry assay dilution instructions. Reproduced from Abcam Copyright © 2024

Detailed procedure are as follows: 1) Kit provided reagents are prepared following manufacturer specifications, 2) A tPA standard solution was reconstituted with 0.8 ml assay diluent for a 160 IU/ml stock concentration, 3) Triplicate standard points were prepared by serial dilution and for 40, 10, 2.5, 0.625, 0.156 IU/ml solutions using assay diluent with a diluent only solution as the blank where remaining standard solutions were aliquot to store at -20°C, 4) Reactions for each well were organized using: 60 µl diluents, 10 µl plasminogen, 10 µl plasmin substrate labelled as the “Assay Mix” and added to each well, 5) 20 µl of standard, blank or sample were added to their corresponding wells and gently aspirated by multichannel pipette. 6) An absorbance reading was taken at 0 minutes to establish a background O.D., 7) Subsequent 405nm absorbance readings were taken at 1 hour intervals with focus on the tPA standard and as received tPA samples as “High Tissue type Plasminogen Activator samples” defined by the manufacturer, 8) All samples were also monitored at hourly intervals 20 hours after the background measurement following instructions by the manufacturer for “Low Tissue type Plasminogen Activator samples” in the case of PPACK inhibited tPA samples.

Similar to previous assays, initial untreated tPA control trials were made using manufacturer estimates of 300 KU/mg or 300000 IU/mg and adjusted to be within the activity testing range of the assay kit's standard. A similar molar concentration of PPACK treated tPA was used for its own activity assay. An overview of data analysis was shown in **Figure 5.4** and was processed by Four Parameter Logistic Regression of assay standard of the 8hour time point to form a standard curve and interpolation of "High Tissue type Plasminogen Activator sample" data onto the curve. This process was repeated with the standard measurements at 20 hours for the "Low tPA" sample data corresponding to PPACK treated tPA samples.

5.2.3 Microscopy

5.2.3.1 Fibrin flow slide setup

An open cell culture flow channel (Ibidi μ -sticky-Slide I Luer 0.4mm, Ibidi GmbH., Gräfelfing, Germany) was coated with fibrin in discrete segments through the crosslinking between fibrinogen and thrombin. Human fibrinogen (Fibrinogen-plasminogen depleted from human plasma, Sigma Aldrich, Prod. No. 341578) was mixed with 20 LU/mL human fXIII (Human Factor XIII, Enzyme Research, Prod. No. 5810A; South Bend, IN, USA) and fluorescently dyed human fibrinogen (ThermoFisher Scientific, Cata. No. F13191) in a 7.2 pH 50mM Tris (Trizma base, Sigma Aldrich, Prod. No. T6066), 100mM sodium chloride (Sigma Aldrich, K51865804007), 1mg/ml bovine serum albumin solution (Sigma Aldrich, Prod. No. A9418) to a working concentration of 3mg/ml fibrinogens in a 9:1 mass ratio (non-labelled: labelled). Fluorescent fibrin was chosen to help distinguish fibrin within the image. Human thrombin (Sigma Aldrich, Prod. No. T8885) was reconstituted from powder to a working concentration of 0.5 NIH U/ml in tris-HCl solution and 20mM calcium chloride was added (Sigma Aldrich, Prod. No. A1396782919). Precursors were stored as stock solution aliquots: 27 mg/ml fibrinogen, 15 mg/ml Alexa 488 fibrinogen, and 25 IU/ml thrombin. All stock aliquots were transferred from freezer to 4°C for 24 hours before diluting to working concentrations. To create the fibrin layer, 1.5 μ l of the fibrinogen working solution was pipetted on discrete spots on the channel. 1.5 μ l of thrombin working solution was pipetted into a fibrinogen spot and briefly aspirated with the pipette before repeating this procedure with the subsequent spot. The flow chips were then transferred into a moisten petri dish and incubated at 37°C for 3 hours. The channels were flooded with

PBS to reduce the formation of air bubbles and closed with a glass cover slip against the chip's adhesive backing shown in **Figure 5.2 (a)**. In order to fully seal the chamber, a UV-cured resin (Top Gelcoat X, Prod. No. APTGCX-15, Applied Lacquer Industries; City of Industry, CA, USA) was applied to the edges of the coverslip, an aluminum foil strip was used to cover the channel of the chip to protect the sample from photobleaching, and finally the exposed gel was cured under 350W UV lamp for 30s. The chip was then stored overnight at 4°C to degas.

A key difference between this model and Chapter 4's example was that having flow introduce either the MB or wash out fluid necessitated a more permanent test vessel sealing method. First, while sealed Ibidi chips were on market, we were not able to find a practical method of consistently generating clots inside sealed chambers. Simply put we did not have a good precursor delivery method. Rubber needle catheter kits did work with the larger lumen size sealed chips e.g. Ibidi μ Slide I Luers with heights 0.6 and 0.8 mm. However, there was a real chance of cross contamination between catheter needle tips containing the fibrin precursors given the rapid formation of the fibrin clot after thrombin's addition. Further, catheter lines typically operated with a larger fluid overhead meaning that a large amount precursor was required for commercial lines to be usable because of the overall longer bits of tubing used. With the above, having readily consumed rubber needles with a customized shorter line would have been a godsend when using sealed chips. Naturally, given the hassle of working with pre-sealed chips we also considered the open sticky backed slide chambers to just pipette the precursors onto the slides.

Unfortunately, the sticky slide had their own problems. As received, the sticky Ibidi slides left gaps between a cover slip and the Ibidi chip which allowed fluid to escape while being infused with the syringe pump. Vacuum grease did not do much to prevent this and in the worst cases the cover slips delaminated in their entirety. However, sealing solutions were notably easier to find than customizing needles with some like vaseline-lanolin-paraffin (VALAP) mixes being a staple in microscopy. Of the options within literature, UV cured gel nail polish worked surprisingly well as a solution here given the lab had already had infrastructure for UV curing 3D-printed resin models and its opacity under fluorescent imaging. A

comparative example between wicked glass sections of 3D-printing UV resin (AnyCubic, Hong Kong) and the gel polish (GelX Topcoat ApresNail; Applied Lacquer Industries, Rowland Height, CA, United States) in seen in **Figure 5.3** showing a clear difference in visibility under fluorescence at 647nm.

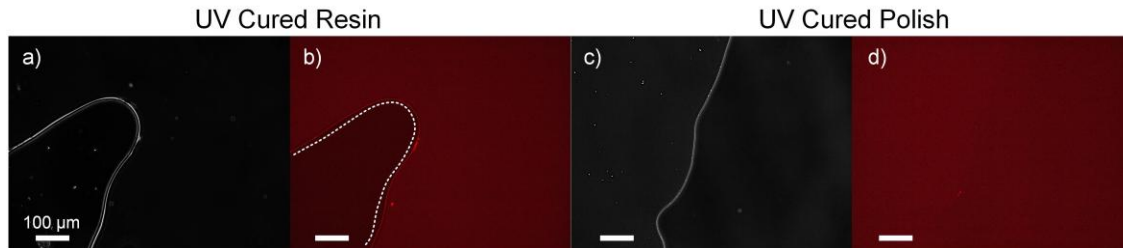


Figure 5.3. Brightfield a), c) and accompanying fluorescence b), d) images emitted at 647 nm taken of the same site comparing UV cured resin section with UV cured gel polish section. A distinct region outlined in b) separates a non-resin wicked horn section with a surrounding wicked region. Due to native fluorescence of the cured

5.2.3.2 Fluorescence Imaging

The flow chamber sample was first checked on each fibrin site on a wide field fluorescence microscope with 488 nm LED (Zeiss AxioObserver, Carl Zeiss AG; Oberkochen, Germany). A microbubble sample was diluted to 10^6 bubbles and continuously infused into the flow device at a rate of 1.5ml/min (Standard Infuse/Withdraw PHD Ultra Syringe Pump, Harvard Apparatus, Holliston, MA, USA) with care taken to not introduce air bubbles. The pump was stopped after 120s infusions to allow MBs to incubate with the surface for 15 minutes. The bubble solution was flushed from the chip by first inverting the device then infusing PBS at a rate of 1.5ml/min for 360 seconds. Samples were imaged with exposure to bright field, 488nm and 647nm LEDs for 72ms, 72ms, and 250ms respectively. An initial pre-wash acquisition was made followed by a wash of PBS at 15ml/min for 4 minutes. The volume flow rate that was used approximates to flow within the central femoral vein and the chip was inverted during the process to force non-attached buoyant bubbles away from the fibrin surface. The wash step was followed by a post wash acquisition at each site.

5.2.3.3 Plasminogen Incubation

The basic imaging sequence was altered when assessing the presence of plasminogen (Plasminogen from Human Plasma, Sigma Aldrich, Prod. No. 528175) on MB binding. Plasminogen at a 100 IU/ml

concentration was introduced after the pre-wash imaging step was completed and allowed to incubate on the sample for 30 minutes. The channel and inlet lines were then flushed using PBS at 1.5ml/min for 360s and a post plasminogen infusion image was taken on all sites before the washing at 15ml/min per the basic sequence.

5.2.3.4 Loading Under Continuous Flow

MB samples were reacted when appropriate and reconstituted to a volume of 1 ml. The MB concentration of the sample was measured by haemocytometer before 0.3 ml of the solution was diluted to 50 ml with PBS. A spot on the edge of a fibrin blob was then chosen for the video location. A flow shear rate of 52 s^{-1} (approx 500 $\mu\text{l}/\text{min}$) was used to introduce and wash away MBs to mimic shear rates found in the CFV whilst accounting for viscosity difference between PBS and blood. Shear rate estimates were made based on manufacturer recommendation for the 0.4 mm flow chamber dimensions.

5.2.3.5 Image Analysis

Acquired images were exported into the ImageJ Particle Counter (FIJI ImageJ, NIH LOCI University of Wisconsin; Madison, WI, USA). The RGB data of the 647nm fluorescent channel corresponding to labelled microbubble signal was converted to gray scale to eliminate noise then thresholded to exclude particles below the lower size cutoff of the microbubbles and counted.

5.3 Results

5.3.1 PPACK treatment reduced plasminogen activation activity of tPA

DBCO-MBs were reacted with ligands and washed of excess reagents through differential centrifugation, where MBs were then destroyed by sonication and ligands were isolated for characterization. tPA azide reacted at 10x molar excess was found by ELISA to have 75% of input ligand remaining in the isolate solution. Whereas CREKA-azide reacted at 30x excess was found at 7.5% of input. In **Figure 5.4**, tPA after PPACK treatment and reaction with azide-PEG-NHS ester exhibited a 98% decrease in plasminogen activation activity in comparison to untreated control.

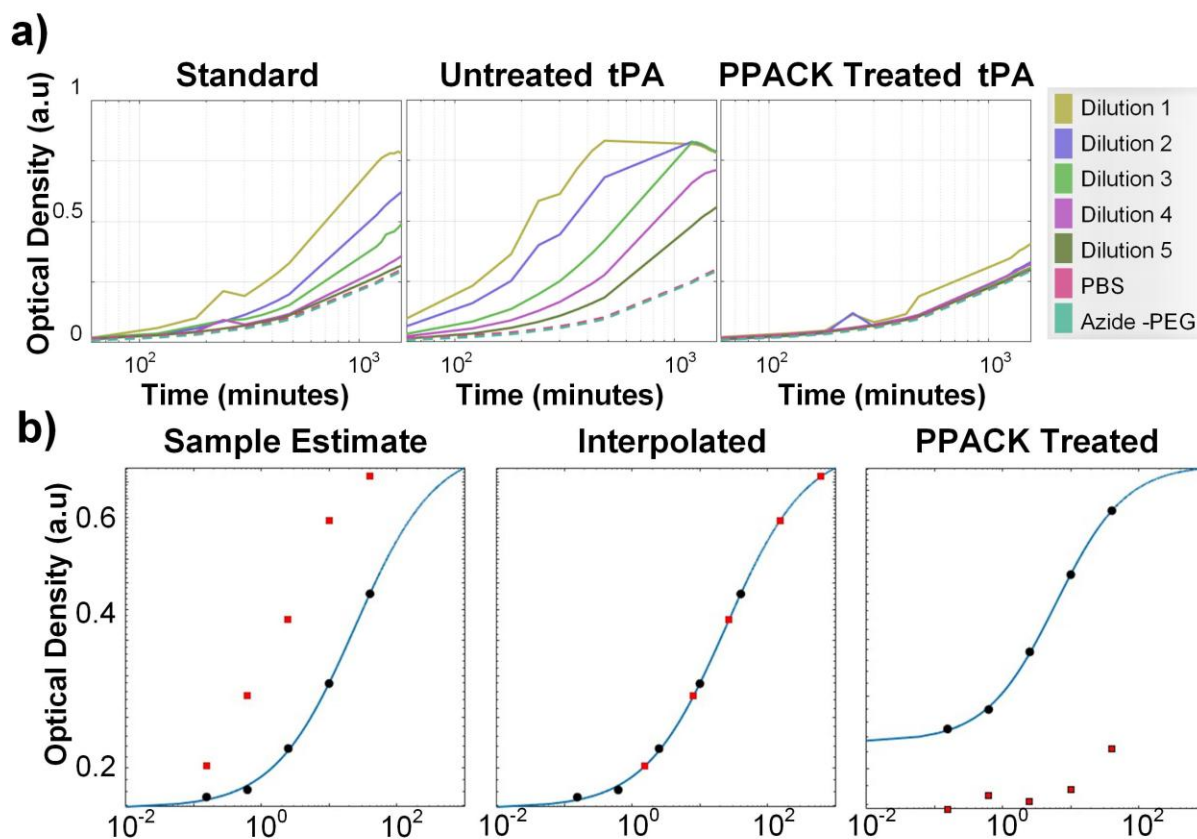


Figure 5.4. Enzymatic activity assays were performed on tPA Standard, tPA as-received, PPACK treated azide-tPA ligand. A) Untreated samples were serially diluted following manufacturer recommendations with reference to standard Dilutions 1 to 5 (40, 10, 2.5, 0.625 and 0.156 UI/ml), treated tPA followed similar dilution scheme. A PBS solution and PBS solution containing azide-PEG-NHS ester were prepared as controls. B) Data analysis was performed through interpolation of sample data onto a Four Parameter Logistic regression curve at 8 and 20 hours of observation for treated and untreated samples respectively. Dilutions concentrations were adjusted for tests outside of the interpolation range seen with the PPACK treated sample for that dataset. A decrease of ~98% in tPA activity was observed in the PPACK treated sample following treatment.

5.3.2 Both conjugated ligands significantly improved MB retention onto a fibrin surface

MBs carrying tPA-type and peptide binding ligand were loaded under static conditions and washed with PBS at high shear rate. Representative images found before and after wash-off for the respective

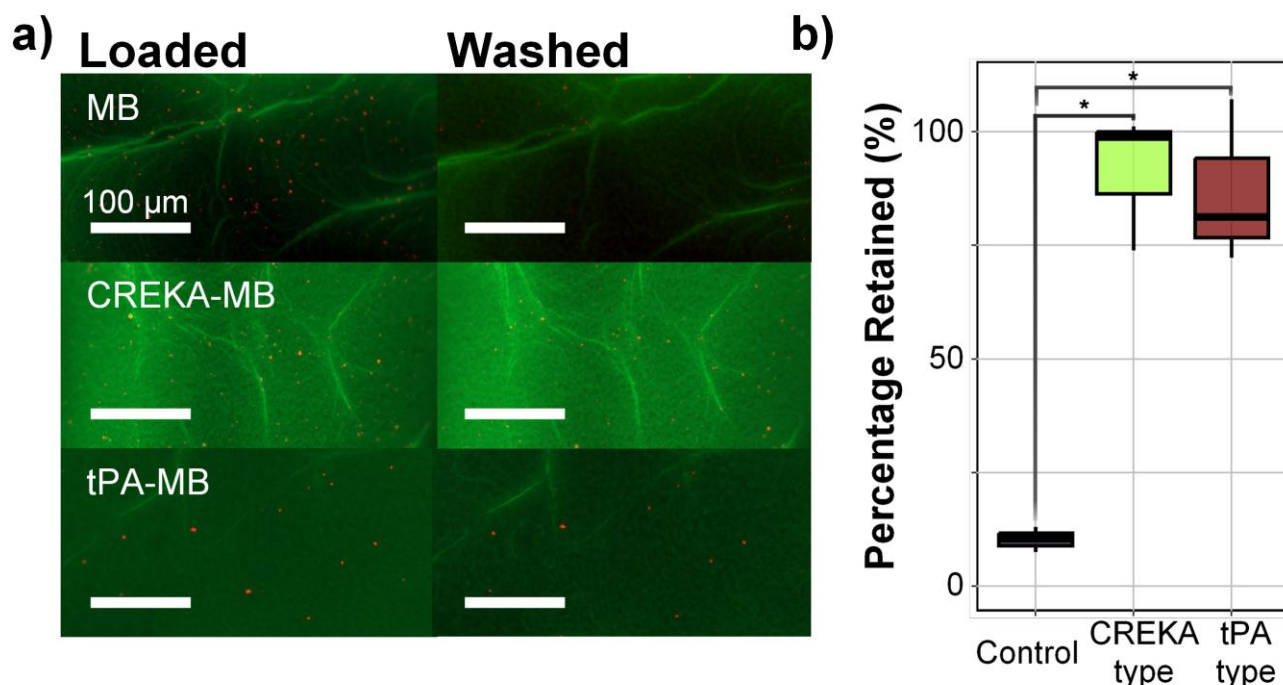


Figure 5.5. Representative images of fibrin sites after loading with MBs (a) with some qualitative difference between unreacted DBCO-MB with ligand loaded MBs, the percentage of retained bubbles are summarized in (b) where a significant increase in retention is observed for both CREKA peptide-type MB and tPA type MB compared to the unreacted control (n=3).

ligands and an unreacted DBCO-MB control are seen in **Figure. 5.5 (a)**. A quantitative summary of MB retention after wash is provided in **Figure 5.5 (b)**. Mean retention for the peptide type ligand was 91.25% (± 15.12 , n = 3), and 86.87% (± 18.13 , n = 3) for the tPA-type. Both values observed a significant difference from the unreacted control retention of 10.33% (± 2.76 , n = 3) where $p < 0.05$.

5.3.3 Presence of plasminogen on tPA-MB loaded surface sees significant decrease in retention under flow

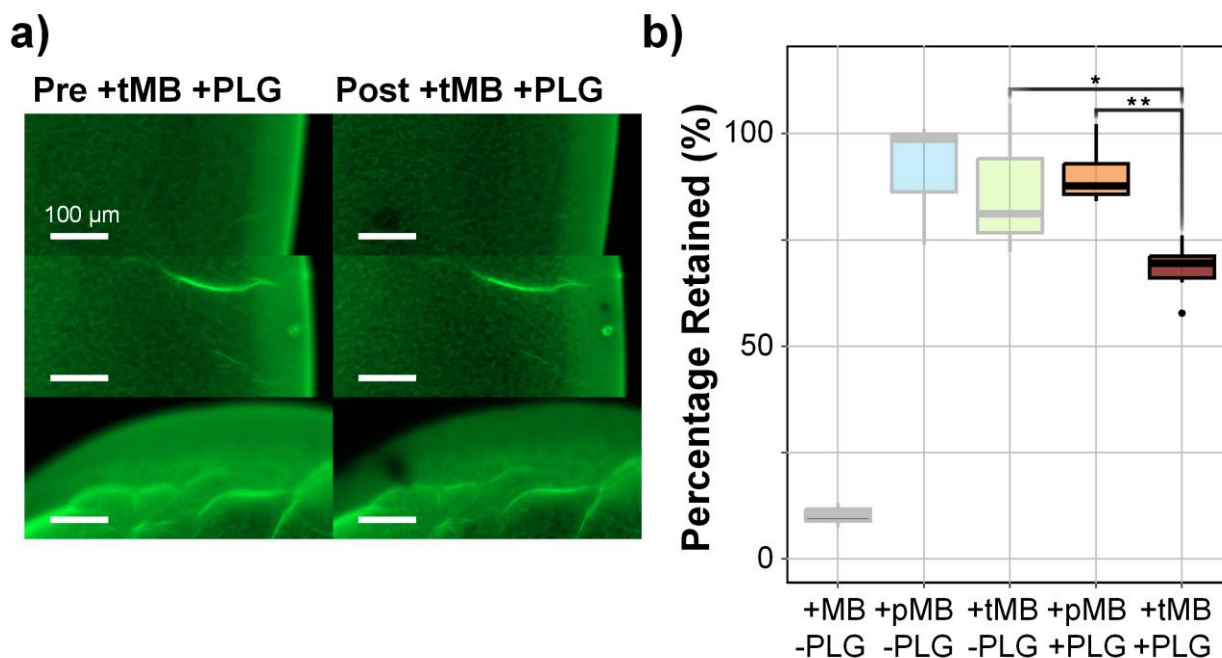


Figure 5.6. Microscopy images comparing fibrin site before tMB introduction and after the introduction of tMBs + incubation with plasminogen a) black pits were exclusively observed around sites with tPA MBs after plasminogen incubation, b) tPA MBs saw a significant decrease in retention in the presence of plasminogen compared to its CREKA MB counterpart and in the absence of plasminogen.

Potentially unwanted therapeutic effects of the treated tPA during imaging was examined in the presence of plasminogen. Here, fibrin samples that were loaded with targeted MBs were further incubated with plasminogen prior to wash off for follow-up images. Pits were uniquely observed on fibrin loaded with tPA-MB in these follow-ups (**Figure 5.6 (a)**). Further analysis of this observation is shown in **Figure 5.6 (b)**. While there was no significant difference in retention of peptide type MBs after plasminogen incubation 90.30% (± 6.87 , $n = 6$), tPA type MBs were found to have significantly lower post-wash retention 68.28% (± 6.25 , $n = 6$, $p < 0.01$) than both the peptide type MB in plasminogen, and tPA type MBs in the absence

of plasminogen ($p < 0.05$).

5.3.4 Effects of gravity observed when MBs fed at physiological flow rate

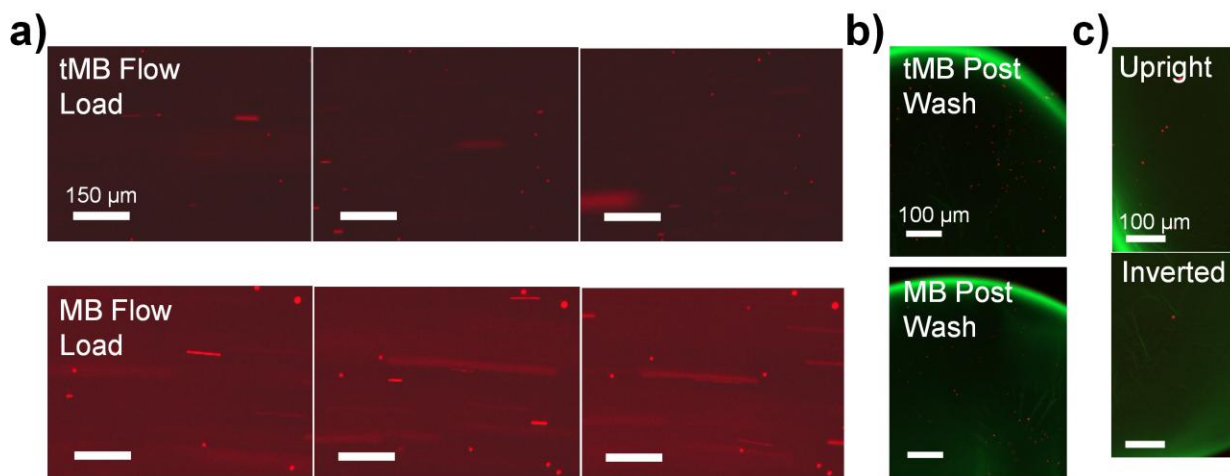


Figure 5.7. Fluorescence Microscopy images of the DiD' channel showing accumulation of MBs over the imaged site over 5s of continuous flow at 50 /s shear over three frames (a) video in supplementary materials, (b) MBs at the same site after a washout at 50 /s for ligand conjugate and unreacted MBs, (c) observed effects of buoyancy on continuous loading of control MBs.

Ligand loaded MBs were continuously introduced by syringe pump at a shear rate approximate to the central femoral vein (CFV) followed by a washout of PBS at the same flow rate performed in the absence of plasminogen as an initial assessment of live loading under flow conditions. Videos captured of untreated and peptide-type ligands MBs are provided under Supplemental Materials and brief frames over 5s of flow care are shown in **Figure 5.7 (a)**. Within snapshots of 5s, we observed similar post loading images for both control and ligand conjugated MBs when continuously loaded in an upright position where final loading results after rinsing are seen in **Figure 5.7 (b)**. Exploring further, a qualitative difference is seen with representative images of fibrin surfaces loaded continuously with untreated MBs loaded in buoyancy favored or unfavored positions in **Figure 5.7 (c)**.

5.4 Discussion

The targeting of fibrin using MB contrast offers new opportunities for ultrasound's imaging and therapy capabilities. Given that various strategies do exist to achieve fibrin binding with contrast agents, it is also crucial to also understand the key advantages that a ligand offers when using them for targeted

imaging. In this work we compared the modified therapeutic agent tPA and a small peptide CREKA to gain more insight on the merits of these differing approaches in targeting fibrin in acknowledgement of tPA's existing therapeutic capabilities. For this comparison, tPA's enzymatic activity was first chemically inhibited through reaction with PPACK [Figure 5.4] before click conjugation to the MB carrier. The retention of tPA-MBs were compared with peptide conjugated CREKA-MBs where both variants were found to significantly improve MB retention on a fibrin surface after a wash off [Figure 5.5]. Moreover, the presence of plasminogen in the flow chamber resulted in a significant decrease in tPA-MB retention in comparison to both tPA-MB retention in absence of plasminogen as well as CREKA-MB with plasminogen presence [Figure 5.6]. In addition to these findings, we examined the behaviour of MBs loaded under physiological shear rates and some effects of buoyancy on under the tested conditions [Figure 5.7]. Overall, we were able to identify a possible difference in functionality between the modified tPA and CREKA conjugate MBs.

This work's focus is to identify functional differences between a inhibited therapeutic with targeting ability (PPACK treated tPA) and a pharmacologically inert fibrin targeting peptide (CREKA). Naturally, having an active therapeutic breaking down the very molecule that one is looking to image is not ideal for imaging applications. Fortunately, CREKA-MB retention was not affected by the addition of plasminogen in the test chamber [Figure 5.6]. However, the decreased retention of inhibited tPA-MBs in the presence of plasminogen as well as the presence of pits in the fibrin sample is a result of plasminogen activation within the sample. It is noted that the activity assay of inhibited tPA was diluted to similar mass concentrations to that of the untreated tPA sample as seen in Figure 1. Additionally, the concentration of ligand used for the loaded targeting study is several orders of magnitude higher than the allowable testing range of the assay. It is posited that the sheer quantity of inhibited ligand loaded onto the MBs caused some activation of plasmin resulting the creation of pits and decrease in retention seen in Figure 5.6.

MBs introduced at physiological shear rates at the veins were observed to be affected by buoyancy. Moreover, while we were able to simulate flow through similar shear rates within our model, representing

the true length scale of a venous model remains a limitation. Namely: 1) the chamber height of 0.4mm is at least ten times smaller in height than most veins, 2) the MBs produced were around 1 to 3 μm and are comparatively larger with respect to the chamber lumen than *in vivo* settings. It should also be mentioned that the loading surface of the fibrin clots can be positioned on the coverslip in **Figure 5.2 (a)** to be less affected by buoyancy during continuous loading. In mind of these factors for this model it may be necessary to further investigate these variables in future study.

Lastly, the loading of proteins onto a MB surface does have functional impact on the MB. From a structural standpoint, incorporating large molecules onto MBs can alter the cavitation and resonance behaviour (Dicker et al., 2013). It is noted that neither MB dynamics nor acoustic characterization were performed in this work and thus the acoustic response of these MBs is currently unknown. With the above in mind, it would be a prudent next step to measure the acoustic characteristics of these MBs. Further, the loading of ligands would also influence the surface chemistry of these contrast agents to which impacts their stability in solution. The above warrant further look into changes in the surface chemistry of these agents in response to ligand loading in analyzing their zeta potential as an example.

In this work, we found a functional difference between a tPA based and a custom peptide based targeting system for contrast ultrasound. It was demonstrated that PPACK inhibited tPA's activity at the quantity loaded onto the MBs significantly decreased its retention onto a fibrin surface in the presence of plasminogen when compared to a non-therapeutic fibrin targeting peptide. This study opens possible lines of inquiry: 1) For therapy, using inhibited tPA-MBs as an image guided therapy tool via sonoporation where some plasmin activation is possible while not fully impeding targeted sonoporation of a fibrin clot, 2) For imaging, improving the specificity of fibrin specific peptide MBs through modification such as having a function of only binding to fibrin in the presence of other clot biomarkers.

5.5 Conclusion

The trend towards theranostics in ultrasound has propelled r-tPA to be a strong player as a fibrin targeting ligand for targeted contrast with the added benefit of being able to chemically inhibit its plasmin

activating function. However, the reputation of thrombolytics like tPA within clinical settings and the absence of a full deactivation of its enzyme activity does merit additional investigation into its efficacy when used as a targeted contrast enabler. Here, we compare inhibited r-tPA against a fibrin targeting peptide to investigate the efficacy of tPA loaded MBs in greater detail. We found that while both ligands performed similarly in terms of their retention, inhibited tPA loaded MBs in the presence of plasminogen saw significant reduction in MB retention onto a fibrin surface as well as exhibiting signs of fibrin degradation. Overall, this study helps in defining the role that tPA has when considering it as a targeting ligand and highlights peptide-based solutions as the key to future fibrin visualization strategies.

Chapter 6

6.1. Summary of Contributions

The safe management of venous thrombus is vital for improving patient QoL and optimizing treatment outcomes. Medical imaging methods are essential tools that enable such VTE treatment pipelines to function within the clinic (Chan & Weitz, 2020; Lim et al., 2018). US imaging, with readily available access to flow visualization through power doppler as well as in situ compression testing under B-mode imaging, is an important bridge between less involved VTE screening tests and the less accessible but more intensive imaging modalities. Yet the disease does present diagnostic challenges in the acute and recurrent stages which can impact detection of these clots by a sonographer (Bassi et al., 2013; Stevens, 2020). Fibrin is a key biomarker and treatment target in venous blood clots, however, the direct detection of fibrin by ultrasound is often limited by its low echogenicity. Prevailing strategies to detect fibrin use the more intensive imaging modalities in MRI or PET which do not carry the convenience of ultrasound (Izquierdo-Garcia David et al., 2022).

This dissertation demonstrates the effectiveness of contrast ultrasound for targeted *in vitro* enhancement of fibrin clots under ultrasound imaging as well as their use in the assessment of a starter super resolution algorithm using a novel flow phantom. Here, we leveraged microbubble contrast to devise novel fibrin targeting agents to enable the selective enhance of fibrin surfaces on clinical ultrasound scanners. Standard in-house MBs were used in a novel super-resolution ultrasound flow phantom (**Chapter 2 and 3**) that was used to validate the images formed through a basic ULM algorithm. The localized signal enhancing characteristics of MBs were taken further where we modified existing fibrin targeting tPA protein to be compatible with the state-of-art SPAAC technique for MB loading resulting in significant enhancement of ultrasound signal on fibrin (**Chapter 4**). Lastly, this new tPA-MB was compared against a similarly formulated peptide loaded MB to discover the impact that protease inhibition has on tPA-MB under more realistic *in vitro* conditions (**Chapter 5**).

The general impact of this dissertation is to bring current state-of-the-art techniques in targeted ultrasound agents to the application of using fibrin as a biomarker for venous clot detection within the clinic. At present, fibrin is the primary treatment target for venous clots. The detection of the molecule can be exploited in MRI and PET settings to track clot fibrin content within research literature. One performance hurdle is that the large size of US contrast hinders the visualization of fibrin within the clot. Further, this work aims to translate this technology to US imaging and concurrently tackle potential regulatory concerns in the translation process. First, we specifically introduced a biorthogonal conjugation scheme (**Section 4.2.1.5**) to simultaneously have a stable covalent bridge to the MB as well as limiting biological interactions to the ligands in contrast to current loading methods in fibrin targeting US. Second, recognizing r-tPA's normal role as a high risk thrombolytic, we inhibited the activity of r-tPA (**Section 4.2.1.3**) to only exploit its fibrin targeting capabilities within this imaging setting as well as further analyzing the efficacy of using the inhibited ligand in a separate study (**Chapter 5**). By tackling these factors, this work pushes the current understanding of fibrin targeted ultrasound imaging and facilitates the development of future MBs with greater clinical utility.

In terms of engineering impact, this work makes contributions to contrast ultrasound research. Despite its incompatibility as a clot phantom the microvessel phantom design was one of the first durable acoustically compatible biomimicking flow phantoms for ULM validation (**Section 3.2.1.2**). The validation work (**Section 3.3.1**) was crucial to understand the effects of lost core casting at the size scale that the phantom was produced in. In the process engineering domain, this dissertation makes one of the first demonstrations of modifying r-tPA to be compatible with the SPAAC conjugation scheme (**Section 4.2.1.5**) whilst retaining its fibrin targeting ability. To this end, it was critical to thoroughly characterise the intermediate processes (**Section 5.2.2.1**) before the reaction with the DBCO MBs to ensure the sub-reactions were successful.

Improving fibrin detection under contrast ultrasound is the key clinical contribution affected by this dissertation. In terms of clinical impact, the significant improvement of fibrin detection on a modern clinical

ultrasound scanner using tPA-MB (**Section 4.3.3**) demonstrates clear benefits in fibrin detection with CEUS. These results show that while MB agents are inherently limited by size, the added benefit of outlining fibrin at the surface has potential in detecting fibrin clots. The above in combination with our initial studies performed on two other commercial scanners demonstrates that this strategy is agnostic of the imaging system (**Section 4.3.3**). This work is also one of the first to examine the efficacy of inhibited tPA ligand in ultrasound imaging settings with a direct comparison to protein-based alternatives (**Section 5.3.2** and **Section 5.3.3**). These results provide more insight on the unique utilities of the ligands available to researchers within this field. Overall, these results demonstrate that the novel fibrin targeting MBs are a robust tool to enable fibrin detection within the clinic and holds merit for further research.

6.1.1 Enabling Visualization of Submicron Features within a Tunable Validation Phantom

The primary research contribution in **Chapter 2**, was in the design of a novel flow phantom for validation of super resolution contrast ultrasound algorithms with the following design features: 1) submillimeter to micron scale flow features that can be subject to user design preferences, 2) acoustically compatible medium to mimic the characteristics of tissue under ultrasound.

ULM uses MB contrast as consistent ultrasound scatterers where the temporal summation of the spatial location of isolated scattering events is used to map out the MB containing vessels. To perform *in vitro* validation of such an algorithm a microvessel branch structure was simplified and replicated in a CAD model (**Figure 3.1**). This model was prototyped via additive manufacturing (**Figure 3.2**) for lost core casting making use of the stiffness of the AM material to ensure lumen shape is preserved during handling. The prototyped core was optically validated (**Figure 3.4**) to ensure a good conversion into the physical model shaping the flow vessel. The resulting flow phantom (**Figure 3.3**) was assembled and assessed at varying MB concentration and flow rate.

This design is one of the first reported validation methods to replicate microvascular branching structures within a tissue-like medium for this contrast application. In comparison to its contemporary

solutions, this process offers a more expedient method of testing the resolvability limits of an ULM algorithm while offering a large degree of control over the input parameters prior to a jump into more intensive *in vivo* models. The design also offers a degree of customization via CAD software. ULM's visualization of flow insufficiency within the mock microvessels seen in (Section 3.3.2) and (Figure 3.5) is of possible interest when considering the effects that microvascular thrombi have on local flow. As a connection to fibrin imaging, the *in vitro* targeted imaging of these thrombi would be of interest but likely requires more specialized contrast agents as well as overcoming a hurdle in not delaminating from a tissue mimic's surface.

6.1.2 Demonstrating Selective Ultrasound Signal Enhancement at Fibrin Surface

The main contribution to research in Chapter 4 is through: 1) modification of r-tPA for azide functionality to enable the SPAAC reaction, 2) development of a fibrin targeting agent leveraging the SPAAC reaction scheme.

Fibrin targeting MBs were created through covalent linkage of click chemistry compatible MBs and ligands using the SPAAC conjugation scheme (Section 4.2.1.5 and Figure 4.1). The reaction required the use of MBs stabilized with a coating containing DBCO (Section 4.2.1.1) and r-tPA modified with azide functional groups (Section 4.2.1.4). The intermediates were characterized (Section 4.2.1.6) to ensure modifications were successful (Section 4.3.1). tMBs were incubated with a fibrin surface (Section 4.2.2.3) where a significant increase MB retention was confirmed through the conjugation of r-tPA onto the MBs (Section 4.3.2 and Figure 4.10). For the clinical ultrasound application, these findings were corroborated by significant improvement in ultrasound signal (Figure 4.12) on a fibrin test surface (Section 4.2.2.4-5) when imaged under a clinical ultrasound scanner.

The MB design is the first to introduce the state-of-the-art SPAAC conjugation into fibrin targeting ultrasound contrast study. The biorthogonal nature of SPAAC reagents (DBCO and azide) circumvents quenching reactions necessary of common conjugation schemes used within current literature. Further, the lax solution requirements of the reaction make it better suited for clinical adoption. Understandably, r-tPA

originates as a thrombolytic in the clinic where its activity was inhibited by PPACK in this work (**Section 4.2.1.3**). While this work mainly focuses on imaging application there may be merit in using tPA in its uninhibited state leveraging the MB as a potential drug carrier. Finally, preliminary work was also performed using additional clinical scanners demonstrating that this tool can be readily adopted with a variety of existing clinical equipment. This does come with the caveat that some degree of transducer power control needs to be present to avoid popping MBs.

6.2.2 Understanding the Role of tPA as a Targeting Ligand

The study performed in **Chapter 5** served as a comparison between the dominant ligand types reported within literature for fibrin at the time. Ultimately, the objective was to determine the efficacy of using tPA type ligands over peptide types specifically in an imaging application in the presence of plasminogen.

Both ligands were loaded in the SPAAC scheme (**Section 5.2.1.3**) with tPA ligand preparation performed as seen in (**Chapter 4**). Here, the loading efficiency of treated r-tPA and CREKA were analyzed to both confirm reaction success as well as to quantify the loaded ligand concentration (**Section 5.2.2.3**). The key biological interaction here is the activation of plasminogen into plasmin through its interaction with tPA. Accordingly, assessing activity of tPA after PPACK inhibition was key to understanding (**Section 5.2.2.4**) the effects that the inhibited ligand had within the sample. The retention of ligand bound MBs were tested under a flow chamber mimicking physiological flow rates (**Section 5.2.3.2**). By introducing human plasminogen, we were able to simulate more realistic biochemical conditions found in blood (**Section 5.2.3.3**). It was found that the tPA and CREKA peptide's binding were effectively the same in the absence of plasminogen (**Figure 5.3.2**). Whereas incubation with plasminogen resulted in a significant decrease in inhibited tPA loaded MBs in comparison to CREKA MB and standard MB controls (**Figure 5.3.3**).

The current state of the art within fibrin targeting ultrasound contrast leans towards a therapeutic focus. MBs can indeed function as image guided therapy enablers, acting as both a drug carrier and strong acoustic scatterer. Naturally, the prospect of using an inherently fibrin targeting protein with fibrinolytic

functions is desirable in treatment settings but bring question to its effectiveness in anchoring a contrast agent to the surface. This study was one of the first to compare the MB binding mechanics of tPA against a non-drug derived binding ligand in CREKA. From a mechanistic perspective, this study demonstrates that the lingering effects of plasminogen activation does compete with the goal of binding MBs to fibrin. While this work was performed under microscopy and not ultrasound imaging, the knowledge gained through these findings in combination to the ultrasound findings for tPA in **Chapter 4** can aid in future design considerations of MBs or experiments involving the tPA ligand under ultrasound settings. From a clinical perspective, these results directly identify the limitations that selecting the tPA ligand strategy has for targeting applications.

6.2 Limitations and Future Directions

6.2.1 Progression Towards Multifunctional Ligands

A direction to take this research comes from the versatility of peptide-based targeting ligands. Of the dominant fibrin targeting ligands used in ultrasound research, peptide ligands were shown to be preferable over the tPA based alternative in this dissertation (**Section 5.3.3**). Given the versatility of designer peptides, one possible future direction comes in selecting ligand designs that only target fibrin in the presence of other clotting cascade specific biomarkers to improve the specificity of the contrast agent towards active fibrin clots. This has been demonstrated in literature by exploiting native thrombin to activate an electrostatic targeting method (Lux et al., 2017). Specially designed peptide ligands are also a boon for non-US modalities considering the existing role that US has a gateway to heavier modality in the clinic. EP2104 is an example of a fibrin targeting agent with gadolinium chelating function to enable MRI contrast (Overoye-Chan et al., 2008). The versatility of MBs with the ability to pivot between loaded designer ligands enable the introduction of multimodal diagnostic pipelines which may significantly improve clinical insight for the decision-making process.

6.2.2 New Investigations Using Physiologically Relevant Environments

Currently, sonographer expertise plays a large role in current VTE diagnosis. The fibrin targeting strategy can possibly improve the efficiency of sonographers by visualizing fibrin clots in cases where power doppler or compression testing prove challenging without pulling resources away from more in demand modalities. To bridge these *in vitro* findings to address the above clinical need, more physiologically relevant models should be explored to improve the robustness of these targeting agents. The *in vitro* study in this work (**Chapter 5**) featured a simple biological environment where plasminogen was the only interactive protein and fibrin was the only component of these clots. A more realistic *in vitro* model including whole blood clots, working in plasma or moving to animal models altogether may lead to new insights as well as provide better testing environments for novel ligands with greater specificity to pathology. As the results in this dissertation are obtained through fibrin clots, it is currently unknown if additional blood constituents would interfere with the retention of MBs on a thrombus. While initial trials at animal whole blood clot studies were attempted (**Section 4.2.2.2**), it would be prudent to attempt more thorough study into using more representative *in vitro* or *in vivo* model in a detailed investigation.

6.3 Conclusion

Ultrasound contrast MBs have high potential in specialized imaging applications such as super-resolution or molecular imaging. This dissertation details the design of a novel phantom to assess super resolution ultrasound algorithms as well as the development of state-of-the-art MB agents that enable improved ultrasound detection of fibrin clots. This research is crucial for the development of the next generation ultrasound contrast studies in both the microvascular and targeted imaging domains.

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