In-vitro-sonothrombolysis by using streptokinase-loaded pre-PGS microbubbles for the treatment of Ischemic stroke

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Abstract

Microbubble-mediated sono-thrombolysis is an effective method of thrombus breakdown. The goal of this Invitro study was to determine the efficacy of Invitro sonothrombolysis by using streptokinase-loaded pre-PGS microbubbles. Synthesis of streptokinase loaded microbubbles was carried out by using solvent displacement method and synthesis was confirmed by FTIR and SEM analysis. Invitro blood clot models synthesized by using human blood were subjected to 30 minutes of sonothrombolysis in study groups of US alone, US+MB, US + Drug and US+ Drug loaded Microbubbles. Frequencies used for this purpose were 2.8 MHz and 3.2 MHZ at a Mechanical index of 0.1, 0.3, 0.6 and 0.9. Outcomes of sonothrombolysis were monitored by measuring pre and post weight of clot, mean gray intensity analysis of images captured during 30 minutes of sonothrombolysis by using image. J, histological analysis of clot residues in which fibrin degradation was assessed and spectrophotometric absorbance of hemoglobin present in effluents of blood clots collected after 30 minutes of the procedure. Monitoring outcomes depicted that maximum reduction in mean gray intensity and maximum degradation of fibrin network, and higher spectrophotometric absorption took place by using drug loaded microbubbles in the presence of ultrasound. This Invitro sonothrombolysis by using drug-loaded microbubbles has shown promising results and can be further utilized for evaluating its potential in in vivo studies.

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Abbreviations

tPA	Tissue plasminogen activator
US	Ultrasound
MB	Microbubbles
PFP	perfluropentane
SK	Streptokinase
MI	Mechanical index
PGS	polyglycerol
SEM	Scanning electron microscopy
FTIR	Fourier transform infrared

Chapter 1

1. INTRODUCTION

1.0 Introduction

Stroke is one of the leading causes of disability and mortality throughout the globe. Two third of stroke incidents are Ischemic in nature. Thrombus formation in cerebral blood vessels leads to the occurrence of acute Ischemic stroke (AIS) (Jolugbo and Ariëns,2021). It hinders the blood flow to a certain area of the brain which causes severe damage to neurological functioning. Approximately 70% of stroke incidents are Ischemic in nature. Some damages caused by Ischemic stroke are irreversible while some caused by reduction of blood flow are reversible (Phipps and Cronin,2020). Stroke risk is higher in the older age group but it can take place at any age. Factors associated with the occurrence of stroke are almost similar in different age groups such as diabetes, hypertension, obesity, smoking, hypercholesterolemia, etc. Common symptoms that are associated with stroke include sudden trouble in understanding and speaking, a sudden weakness if legs, arms, or face, sudden loss of balance, difficulty in walking, and sudden loss of vision. These signs may last longer or fade away quickly, in the latter case it is referred to as “mini-strokes" (Randolph,2016).

During thrombus formation blood flow is hindered to vital organs of the body. It occurs due to the formation of blood clots in the blood vessels. There are 2 distinct mechanisms that cause the formation of a thrombus. Firstly, the coagulation cascade consists of the activation of various factors responsible for blood clotting, which are basically protease enzymes that break down proteins, and secondly, hemostasis occurs in order to prevent bleeding (.Chung and Lip, 2003). During this process, platelets are aggregated. Both of these processes have a severe associated risk of myocardial infarction, venous thrombosis, and cerebrovascular disorders. Damage caused to endothelial linings of blood vessels often results in thrombogenic surfaces but it may also take place due to prosthetic heart valves, and vascular grafts that activate coagulation pathways (Casa and Ku,2017).

However, the type of thrombi formed in the different hemodynamic environments depends upon the coagulation process and aggregation of platelets. In deep vein thrombosis coagulated red blood clots are found commonly while in arteries platelets are mainly responsible for the formation of thrombus and it appears differently. These thrombi can be visualized by the naked eye and hence are also known as white clots (Lowe,2003).

Myocardial Infarction is a fatal arterial disease commonly referred to as a heart attack. There is a very high associated risk of ischemic stroke with Myocardial Infarction. It is observed that about 2.5% of patients with myocardial Infarction go through Ischemic stroke within 4 weeks of the onset of the disease since 1980(Kamel,2003). It is affecting 7 million individuals throughout the globe annually with a 17% mortality rate (Kamel and Healey,2017). It generally takes place due to the formation of a blood clot in the epicardia artery that supplies blood to heart tissues. There are two types of myocardial infractions. The first is known as transmural in which three layers of heart tissues (endocardium, myocardium, and epicardium) are completely damaged due to intense blockage of blood flow. While the second type is known as nontransmural in which only one layer of heart tissues is damaged which is known as myocardium.

There are various treatments used for Ischemic stroke such as endovascular retrieval of the clot, and intravenous thrombolysis. These treatments help to restore the blood flow to the occluded vessel and have been found to improve the outcomes (Phipps and Cronin,2020).

1.1 Thrombolytic drugs as a frontline therapy

Stroke has a huge impact on the lives of people because there is a huge risk of loss of lives and disability. Until 2014 there was only one approach that was found to be effective in reversing the neurological deficiencies caused by an acute ischemic stroke which was the administration of an intravenous tissue plasminogen activator. It is administered within 4.5 hours of the onset of symptoms of stroke. Several other approaches have been introduced to tackle this problem which includes mechanical thrombectomy of large occluded vessels but associated benefits are very low. This is due to lack of experts and resources for carrying out the procedure. This method can't be a successful treatment for every incident of ischemic stroke because major case of ischemic stroke are caused by a blockage in cerebral arteries and small size of these arteries is a major hindrance in carrying out mechanical thrombectomy by using catheters (Nikitin et al.,2021).

In order to overcome these issue, various new methods are being introduced but thrombolytic drugs have shown greater promise. It has, been proven effective clinically and can be utilized for stroke management. However, the efficacy of thrombolytic drugs

is low and there is an associated risk of hemorrhage with the use of these drugs hence it leaves room for improvements.

There are different types of thrombolytic drugs available in the market that are as follows:

1.1.1 Tissue plasminogen activator

Tissue plasminogen activator(tPA) was extracted from animal tissues in 1947 and its purification was carried out in 1979.Origianlly Tpa is produced in the form of single chain protein but plasmin has the ability to convert it into two chain form without sacrificing its fibrinolytic efficiency. It is secreted by neurons, glial cells and vascular endothelial cells. It is basically a protease enzyme that converts inactive plasminogen into its active form that is plasmin whose function is to lyse fibrin clots (Angles et al.,1985). This is the only FDA approved drug to be used for fibrin degradation. Many attempts have been made to increase the half-life of tPA which is 6 minutes in plasma but these attempts lead to reduction in fibrinolytic efficacy.

1.1.2 Urokinase

This was found in human urine in 1947. It is secreted by fibroblasts, epithelial cells, smooth muscle cells, endothelial cells, macrophages and different types of cancer cells. This enzyme performs multiple physiological roles such as complement activation, tissue remodeling, cell migration, and healing of wounds. This is cost-effective as compared to that of alteplase and in developing countries it is used for the treatment of ischemic stroke. Urokinase is used to cure deep venous thrombosis in developed countries. FDA has approved it to be used for the catheter-based treatment of pulmonary embolism

1.1.3 Streptokinase

β-hemolytic streptococci is the source of streptokinase. It is indirectly involved in the breakdown of fibrin network during streptococcal infections. It has been approved by FDA to be used for arterial thrombosis, deep vein thrombosis, pulmonary embolism and myocardial infarction.

1.1.4 Staphylokinase

This is also obtained from bacterial sources and act as indirect activator of plasminogen. Administration of staphylokinase for the process of sonothrombolysis is under clinical trials

1.2 USCAs mediated Sonothrombolysis

Microbubbles have large number of applications as contrast agents in imaging by ultrasound. They have small sizes almost similar to that of blood cells and can be used for observing circulation of blood. They play vital role in improving dthe elineation of endocardial border in echocardiography (Saint et al.,2014). They also assist in imaging of larger vessels of blood and also for visualizing blood perfusions in smaller vessels in particular it plays important role in identifying any sort of abnormal behaviors via Doppler imaging. Apart from wide range of diagnostic applications of microbubbles there is an increase in interest towards using them for therapeutic applications. Microbubbles have very high potential for being used as therapeutic agents. Such as they can be used for increasing the permeability of cell membranes and can help in transport of different molecules into the cytoplasm. This property can be utilized for genes or drug delivery and can prove helpful for various disorders (Lindner.,2004). Furthermore, microbubbles also have the potential for altering the permeability of walls of blood vessels and can help in the penetration of drugs into surrounding enbviornment, this approach can be utilized for treating many disorders such as cancer (Ferrara et al.,2007).

There is an emerging interest in use of microbubbles for the process of sonothrombolysis in cases of ischemic stroke. Currently the only FDA approved thrombolytic treatment for clot lysis is the use of tissue plasminogen activators such as Tenecteplase, urokinase, alteplase, streptokinase etc. however there is very high associated risk of hemorrhage and it may prove fatal for the patients. This side effect is major barrier towards effective application of intravenous tissue plasminogen activator. Due to these high risks it is need of the hour to introduce such adjuvant therapies that can enhance the safety and outcomes of thrombolytic treatments by using t-PA. Using microbubbles and US is one of the most promising approach for sonothrombolysis. It has the potential to reduce the dosage requirements of t-PA which ultimately reduce the risk of hemorrhage and it will

also allow continuous monitoring of patient during treatment (Xie at al.,). Microbubbles-mediated sonothrombolysis has exhibited huge potential for improving the outcomes of thrombolysis. Wide range of studies has been conducted both Invitro and In vivo for understanding the role of ultrasound in clot lysis and using microbubbles of different nature along with US have exhibited positive outcomes.

1.3 The acoustic principles of ultrasound contrast agents

Ultrasound contrast agents have been widely accepted as a noninvasive imaging agent. Ultrasound contrast agents(UCA) basically consist of a gas core encapsulated by a polymeric shell. The acoustic properties of any microbubble is determined by the inert gas while the shell is responsible for viscoelastic properties such as the durability and stability of UCA. Size range of these microbubbles lies from 1-10 μm that allows its systemic circulations. Ultrasound contrast agents exhibit best backscattering response that enhances the gray intensity up to 30dbm. This backscattering response is also known as linear motion of microbubbles.

Upon exposure to acoustic pressure, linear vibrations are observed but these patterns shift to nonlinear vibrations with increasing acoustic pressure which is referred as a mechanical index. Oscillations of microbubbles at low mechanical index exhibit harmonic oscillations but by increasing mechanical index leads to instability of USCAs. This occurs because high mechanical index leads to rupturing of microbubbles and this sends the high-energy signal (Ignee et al.,2016).

The microbubbles enhance the contrast by a two-way process, firstly scattering of acoustic waves takes place by bubbles that results in difference if acoustic impedance between surrounding liquid and gas core. Secondly higher compressibility of gas core causes volumetric oscillations as result of ultrasound waves. Due to this phenomenon these bubbles exhibiting oscillations serves as a sound source with scattering responses that have magnitude higher than that of spherical rigid object with same impedance and size(Hilgenfeldt et al. 1998). This property of back scattering and nonlinear behavior has made microbubbles as the most suitable ultrasound contrast agents.

With the increasing importance of microbubbles as contrast agents,an increase in interest in the ososcillating properties of microbubble has also been observed. Because this property can have wide range of theranostic applications. Firstly, nonlinear oscillations of the bubbles will result in transfer of momentum to the surrounding fluid that may result in microstreaming or steady flow which can have an effect on any sort of therapeutic material being used. It may also result in shear stress on biological membranes which might influence the permeability and triggers the therapeutic outcomes. Secondly, microbubbles can also produce localized thermal effects that can be used for different purposes such as for releasing drug from heat-sensitive carrier molecules (versluis et al.,2020).

Currently, available contrast agents are very very fragile and they require very low mechanical index that is defined as the peak negative acoustic pressure divided by the square root of the frequency of ultrasound. The mechanical index is displayed at clinical ultrasound machine and this value represents that how many chances of rupturing of USCAs. Because usually they are stable at low MI. by increasing the MI nonlinear oscillations are observed. Microbubbles can be ruptured immediately with a single pulse by increasing MI closer to the FDA limit of MI that is 1.9 but other factors apart from MI may also influence the response of microbubbles (Chong et al.,2018)s.

Chapter 2

2. LITERATURE REVIEW

2.0 Review of The Literature

2.1 Production of Commercially available USCA as thrombolytic agents throughout the Generations

Use of ultrasound contrast agents for the clinical purpose was initiated in 1960s. Gramiak and Shah were the first one to report that these air bubbles can be utilized as ultrasound contrast agents. They encountered ultrasound contrast agents for the first time while studying the aortic regurgitation of a patient in a laboratory of cardiac catheterization. a strong contrast was observed upon administering of air bubbles along with rapid bolus injection (Gamiak and Shah.,19680, Karamanou et al.,2012). After that observation, many attempts have been made for producing gas or air bubbles that can be utilized as echogenic contrast agents so that they can enhance visibility during ultrasound procedures. For this purpose, in order to get better contrast, many attempts have been made such as altering the speed of injection, shaking contrast before injection, and using different types of injection methods. These procedures were yielding microbubbles with no defined size distribution and roughly of size > 50 μm (Feigenbaum et al.,2005). These bubbles showed contrast up to some extent but it was not good enough and was not good at reproducing the results.

The first generation of microbubbles was synthesized by Bayer and Schering which is a german pharmaceutical industry. These microbubbles were named as levovist. These bubbles are produced from microcrystals of galactose that generates air in the vessel. Galactose powder is dissolved in water in order to produce a suspension. In this suspension, air bubbles stick to the surface of microcrystals that initially maintained a solid state but it later on gets dissolved and these microbubbles are released into blood post-injection (Correas, J. M., & Quay, S. D. (1996). The main purpose of the synthesis of these bubbles was to make the signals stronger. For this purpose, a certain amount of surfactant (Palmitic acid) was added. It was observed that these bubbles remained stable for 1-4 minutes in the blood. An increment of ~20 Db was observed in ultrasound signals. It was observed that it was providing better imaging at the grayscale for major vessels and the heart (Calliad et al.,1998). These bubbles exhibit an average size of 2-5 μm. This

size range is ideal to be used because the microcapillaries of the lungs lies at the depth of 7 μm and this makes the traveling of microparticle more effective and results in improved imaging. The major drawback of using these bubbles was that the air from these levovist bubbles was dissolving in the blood faster while for an ideal contrast agent this should stay longer in order to obtain better imaging. For this purpose, a polymer-based material was used for the synthesis of microbubbles so that their stability can be improved but it should not influence the quality of image.

Ultrasonography made a lot of advances and the second generation of microbubbles was produced. These bubbles were named as Sonovue. They consist of Sulphur hexafluoride that is made stable by the addition of phospholipids. These bubbles are stable for a longer duration. 1ml of sonovue contains 100 to 500 million bubbles. The mean diameter of Sonovue bubbles is 2.5pm. Most of the bubbles have sizes less than that of 8pm. These bubbles are subjected to an increase in pressure post-intravenous administration. The solubility of SF6 is very low in the water and consists of small molecular weight is chosen for these bubbles formation because it was observed that it adds good strength to bubbles and exhibits resistance towards pressure changes taking place in the coronary circulation, left ventricle or pulmonary capillaries (Schneider et al.,1995). The strong echogenic response has been observed by Sonovue due to their favorable small size and high concentration per ml. One of the most important characteristics of these bubbles is that it is stable for a long time even after reconstitution. No changes were observed for the duration of approximately 6 hours but it is recommended that it shouldn't be used after 4 hours of reconstitution. these bubbles have a 1-minute distribution half-life and a 6-minute half-life for elimination. It has been reported that within 11 minutes about 80% of gas is pumped out through the lungs. These bubbles were safe to use for humans and hold the potential to be used for the assessment of microcirculatory abnormalities and myocardial perfusions (Schneider and Michel.,1999)

Third-generation microbubbles are specifically labeled and they tend to be used for molecular imaging of inflammatory disorders and physiological localization. These bubbles provide good signal-to-noise at a lower mechanical index. The air core is replaced by inert gasses that are slightly soluble in blood. These bubbles exhibit a half-

life of more than 15 minutes (Dindyal & Kyriakides.,2011). The main goal for the synthesis of these bubbles was to increase response time and to create the ligand-bound shells of microbubbles that can aggregate and can show target-specific results. These microbubbles can be modified to target specific responses for disorders such as ischemia, inflammation, tumors, and thrombosis.

2.2 Mechanisms of microbubble-enhanced sonothrombolysis

The underlying mechanism of sonothrombolysis by using microbubbles is not completely elucidated yet. There are multiple physical phenomena that are responsible for clot lysis such as heating and acoustic cavitation.

2.2.1 Acoustic cavitational response

When the acoustic field is applied, gas or vapors-filled bubbles start oscillation, this phenomenon is known as acoustic cavitation. There are many pieces of evidence that this cavitation plays a vital role in the process of sonothrombolysis. There are various ways to observe these acoustic cavitations such as actively or passively. Different types of bubbles exhibit different response during oscillations, they might exhibit volumetric osciliations and generate acoustic signals of different frequencies. Bubbles exhibit different types of response depend upon excitation pressures. At low pressure linear oscilliations are observed. Upon increasing the pressure, the symmetry of oscillations is disturbed and they no longer oscillate in linear manner due to increase in amplitude. Stable cavitation produce a frequency spectrum that contains both higher harmonics and ultra and sub harmonic response. Bubbles show this oscillating response up to a certain excitation level, beyond that excitation level they start collapsing and generate signals at wide frequency ranges. This cavitation is known as inertial cavitation. With the type of theoretical framework that is being used, the definition of inertial and stable cavitation varies and exhibits some controversies. Inertial cavitation holds the potential to be used for clinical monitoring of different treatments. Francis and Everbach were the first ones to explain the role of cavitation in sonothrombolysis. They observed the cavitation response in the absence of microbubbles. Datta et al .,2006 were the first to study in detail about the effect of inertial and stable cavitation in sonothrombolysis. It has been observed that by adding microbubbles the acoustic pressure required for cavitation is reduced because

these bubbles show better cavitation as compared to that of the surrounding medium. This phenomenon can serve as evidence for enhancement in lysis in the presence of microbubbles along with the acoustic field (Tachibana and Tachibana.,1995, Prokop et al.,2007) The harmonic and ultra-harmonic response generated by cavitation of microbubbles been previously associated with fibrin degradation.

2.2.1.1 Stable cavitation

Oscillation of microbubbles in a sustained manner in such a way that it momentum is being transferred to the surrounding medium is known as stable cavitation. This movement of microbubbles may encourage erosion of the thrombus surface (Everbach et al.,200). This may also help in the movement of drugs to the thrombus which results in the enhancement of the lytic process (Sakharov et al.,2000). Another property of microbubbles is that they exhibit expansion and contraction and this phenomenon may directly break the fibrin network and contributes to the lytic process (Pfaffenberger et al.,2003). Invitro and ex vivo studies have supported the hypothesis that ultra and subharmonic responses of microbubbles have enhanced the clot lysis in the presence of tissue plasminogen activators. While lack of these fibrinolytic drugs does not have an impact on improvement in the lysis of the clot. This observation is also consistent with the hypothesis that the transport of drug to the clot site is improved by using microbubbles. A study was conducted to understand the effect of microbubbles in sonothrombolysis and it was found that rt-PA penetration was enhanced in the presence of definity microbubbles as compared to that of treatment with ultrasound (US) alone or by using ultrasound and rt-PA in combination in which fluorescently labeled drug was found only at the surface (Datta et al.,2008). Moreover, it was observed that beyond the threshold of ultra-harmonic emissions and rising acoustic pressure very little mass loss takes place

2.2.1.2 Inertial cavitation

During inertial cavitation microbubbles, bursts, and fluid move at a very high speed resulting in breakdown of the fibrin network. It may also release shock waves if excitation pressure is very high and it may cause direct deterioration of the surface of the clot. A correlation is found between the enhancement of fibrin degradation via inertial cavitation as compared to that of us alone or rt-PA and US in combination. A study was conducted

by Kim et al using lipid-based microbubbles and it was found that the volume of the clot was decreased and it was linked to the enhancement in the emission of broadband noises (Kim et al.,2012). Along with that in the target group, it was observed that irregular patterns were being formed at the surface of the clot that was in front of the US transducer. Usually, inertial cavitation is not employed for diagnostic purposes because it may cause damage to endothelium due to collapsing of microbubbles. Therefore, the beneficial outcomes of microbubble-mediated sonothrombolysis must be weighed against the possible side effects of using them and they should also be compared with the outcomes of sonothrombolysis by incorporating stable cavitation instead of inertial cavitation. It should be kept in view that patterns of replenishment of bubbles can affect the cavitation behavior at the clot site, inertial cavitation last for less time because it leads to the destruction of clots while stable cavitation last for a longer period of time (Prokop et al.,2007). However, the exact underlying mechanism of how this cavitation affects the clot lysis is yet unclear. There is the possibility that both of these phenomena coexist depending upon the conditions of excitation.

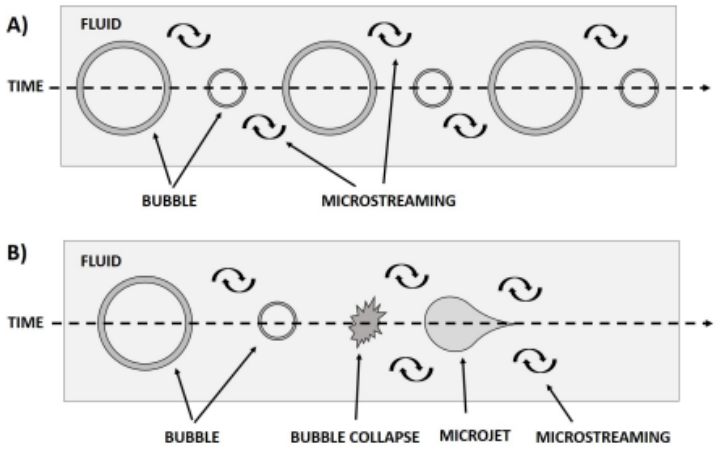

Figure 1:Schematic diagram of stable cavitation(A) and Inertial Cavitation(B) (Goel and Jiang.,2020)

2.3 Thermal Mechanism

The effect of an increase in temperature has also been studied for microbubbles-mediated sonothrombolysis. An increase in temperature at the range that is suitable for

clinical use (25-41C) has been found to be linked with an increase in enzymatic lysis (Yenari et al.,1995). However, when microbubbles are not present in the medium, only the heating effect does not account for a good lytic effect. several studies have been reported in which mild heating of the clot site and the surrounding medium is observed due to absorption of US (Blinc et al.,1998 and Francis et al 1992). Oscillations of microbubbles can further increase the temperature by absorbing the energy from the acoustic field. Due to the presence of friction in the surrounding fluid, repetitive oscillations of microbubbles can further generate the localized heating effect. During the compression of microbubbles heat conduction also takes place and it further contributes to the thermal effect (Stride.,2009). These enhancements in temperature are concerning for diagnostic purposes and can pose a threat to the safety of patients because they may damage the surrounding tissues. However, for therapeutic applications, it is necessary to balance the thermal effect depending on the expected benefits of therapy. There are very limited studies about the thermal effect caused by microbubbles but it has been observed in invivo studies this effect is slightly present (Needlemann.,2010)

2.4 Other mechanisms

Certain cases are reported by authors in which they claimed that sonothrombolysis has been observed in the absence of cavitation or thermal elevation (Prokop et al.,2007 and , Soltani et al.,2008) which suggests that there are additional mechanisms also present. These hypotheses include microbubbles percolation by radiation force through the clot, in which US waves force the movement of microbubbles in the clot (Chuang et al.,2013). The effect of radiation forces in clot lysis has been studied in vitro by Acconia et al (Acconia et al.,2013). US of frequency 1Mhz have been employed to study the effect of microbubble on the fibrin network by using the camera of high frame rate. It was observed that clot surface was being deformed in the presence of microbubbles, the boundary was displaced with microbubbles but no such effect was observed in the absence of microbubbles. Furthermore, it has been observed that penetration of microbubbles occurs in a clot that creates tunnel-like structure in fibrin matrix. By changes in nitric oxide cyle an increase in microcirculation has been observed and this may also enhance the transport of drug to the clot (Suchkova et al.,2002).The leading mechanism for sonothrombolysis is acoustic cavitation but it is not the only parameter. There are multiple

other processes that might play a role In it.optimization of these mechanisms can enhance the efficacy of treatment but it is also necessary for safety of patients in order to maintain the walls of vessels intact.

2.5 Clinical trials of different approaches of Sonothrombolysis

There are huge number of reports about high potential of sonothrombolysis I n pre-clinical studies for the treatment of occlusion in middle carotid artery (MCA). Many studies have been conducted by using t-PA and transcranial Doppler for treatment of MCA in clinical settings as well (Daffertshofer et al., 2005, Alexandrov et al.,2004 and Alexandrov et al.,2019). These trials have exhibited promising outcomes while the major risk associated with this method is development of intracerebral hemorrhage. There is need to conduct more research in order to optimize ways by which this risk can be mitigated. Clinical trials are also conducted by using microbubbles in combination with t-PA by using transcranial Doppler for the treatment of MCA occlusions (Molina et al.,2006, Perren et al.,2008 and Ribo et al.,2008). These trials have shown promising results by decreasing the time of treatment and improved lytic efficiency. Recent advances by using novel contrast agents and loaded and targeted microbubbles have created room for further improvements by reducing the amount of t-PA instead of using increased concentration of bubbles with standard doses of t-PA . many clinical trials have been conducted by using catheter based system for sonothrombolysis and by using intravascular transducers. Outcomes can be improved by optimization of these transducers at low power (Chait et al.,2019, Engelberger et al.,2014 and Tichelaar et al.,2016).

2.5.1 Therapeutic Ultrasound

The major benefit of sonothrombolysis is that it has the potential to eradicate the use of thrombolytic agents that are used for lysis of clot. By implementing the techniques of therapeutic ultrasound such as by histotripsy, high intensity have shown positive outcomes without using thrombolytic agents. The schematic diagram of using these techniques is given below in **figure 2.** Contrast agents are usually not utilized in these techniques therefore higher power outputs and peak negative pressures are required for generating acoustic radiation force and cavitation**.**

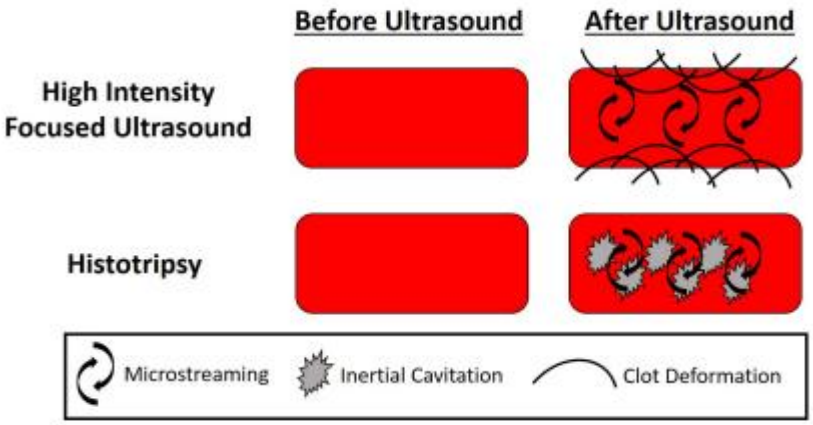

Figure 2: Schematic diagram of High intensity focused ultrasound and histotripsy

In transcranial applications, therapeutic ultrasound has experienced great success by the application of high-intensity focused ultrasound(HIFU) (Hynynen,2010 and Ng and Liu,2002). In conventional applications of HIFU , tissue destruction occurs mainly due to thermal ablation, for applications of sonothrombolysis, a scheme of pulsed ultrasound is utilized which lies below the threshold of thermal ablations. The main process of lysis of clot is displacements of localized tissues , acoustic radiation frce and deformation of clot (Jones et al.,2010, Yang and Zhu,2017 and Stone et al.,2003). Initial reports about HIFU has shown the potential of enhanced sonothrombolysis in the presence of t-PA (Jones et al.,2010 and Stone et al.,2003). According to recent reports, it has been demonstrated both in vitro and in vivo (animal models) that clot lysis can be enhanced by using HIFU in the absence of a thrombolytic agent (Yang and Zhu,2017, Holscher et al.,2013 and Ahadi et al.,2013). Studies have shown that higher peak negative pressures, higher duty cycles and higher pulse repetition frequency can enhance the lysis of clot (Yang and Zhu,2017 and Holscher et al.,2013).

Histotripsy is a method of destruction of tissue by using induced cavitation without thermal ablation. This method has been recently used for the lysis of the thrombus. One of the major advantages of this method is that it does not need a thrombolytic agent for fractionating clots thus it automatically reduce the risks that may occur by using thrombolytic agents (Zhang et al.,2016-Xu et al.,2015 and Shi et al.,2018). Initial studies

have shown good outcomes of histotripsy by adjusting the pulse repletion frequency, duty cycle, and peak negative pressures (Maxwell et al,.2009, Xu et al.,2015 and Maxwell et al.,2011). However, in vivo trials on animal models have shown some evident side effects such as damage to vessels and hemorrhage at the site of excitation (Maxwell et al.,2011). In order to overcome this problem another technique named as microtripsy was introduced and it showed better potential as compared to that of histotripsy.

Both histotripsy and HIFU are considered as noninvasive techniques of tissue destruction. Transcranial blood clots are typically subjected to HIFU by using a HIFU transducer system. Its latest design has the potential to be used for treating deep vein thrombosis. While histotripsy device contains a transducer that is transcutaneous and it can be utilized for deep vein thrombosis in the best possible way. These techniques of therapeutic ultrasound hold great potential for sonothrombolysis but it is needed to maintain a perfect balance of parameters of ultrasound.

2.5.2 Intravascular sonothrombolysis

This technique is site-specific and this feature makes it an attractive approach for sonothrombolysis because it greatly reduces the risk of damage to the vessel wall. Due to the evident benefits of catheter-based thrombolysis, its combination with US can further increase the outcomes of sonothrombolysis. EKOS EndoWaves system is currently used in clinical trials for the catheter-based system. It is targeting different types of thrombi such as deep vein thrombosis, pulmonary embolism etc. however it is yet undiscovered whether it is more effective than that of conventional approaches or not (Shi et al.,2018). further research is needed in order to explore the outcomes of the catheter-based system by designing new transducers and in combination with microbubbles.

2.6 Novel Contrast Agents for Sonothrombolysis

Wide range of studies have been conducted for lipid based microbubbles such as Definity and Sonovue both with and without thrombolytic agents (Auboire et al.,2018 and Petit et al.,2012) microbubbles have showngreat potential for replacing the conventional methods of treatment both alone and in the presence of thrombolytic drugs. However, there are certain issues that are needed to be addressed asuch as duration of contrast, required concentration of microbubbles and dosage of thrombolytic drug. Limitations of MB

mediated sonothrombolysis are being tried to be addressed by certain advance techniques such as by using magnetic microbubbles, nano droplets, targeted microbubbles. These approaches may help to address the concerns of conventional approaches. Due to improvements in targeted drug delivery and systemic travelling of contrast agents, they have been applied to treat deep vein thrombosis, ischemic stroke, and pulmonary

Nonspecific t-PA binding can be reduced by using drug loaded microbubbles. Because this drug is released only by exposure to US and US is only focused on the region of interest. This method can help to reduce the risk of hemorrhage and can also improve the outcomes of sonothrombolysis (Shaw et al.,2009, Laing et al.,2011 and Ren et al.,2011). Drug-loaded microbubbles have shown better outcomes as compared to that of using microbubbles or t-PA alone (Shaw et al.,2009, Laing et al.,2011 and Ren et al.,2011). It has been reported that required dosage of t-PA can be reduced by using drug loaded microbubbles which can ultimately reduce the side effects of sonothrombolysis. Recent studies have also used targeted and loaded microbubbles in combination and this has further improved the outcomes (Hua et al.,2010 and Hua et al.,2014). Nano droplets are also among recent advances, these droplets consist of very small size as compared to that of microbubbles (Pajek et al.,2014,Brubler et al.,2018 and Guo et al.,2019). This property may help to reduce the risk of vessel damage and can improve the rate of diffusion of drugs to the target site by creating pores in the thrombus. Another approach for making microbubbles more target specific, magnetic microbubbles are introduced. These microbubbles are targeted to clot site by using magnet (Victor et al.,2017). This also helps in reducing the risk of hemorrhage. Following figure shows the schematic of all of these mechanisms:

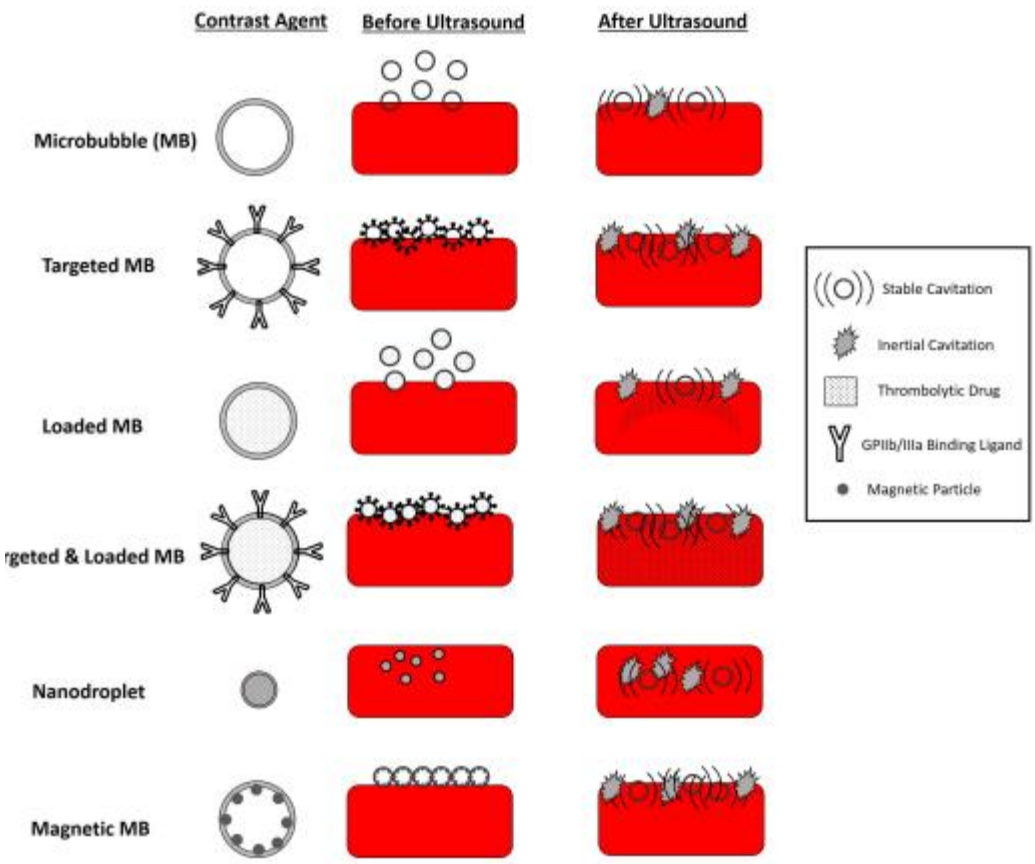

Figure 3: Schematic diagram of novel contrast agents for sonothrombolysis

2.7 Clinical challenges

In order to make sure patient safety and successful clinical outcomes of sonothrombolysis it is necessary to address certain challenges such as attenuation of US waves in living tissues, delivery of therapeutic agents

2.7.1 Attenuation of US in tissues and bone

The major benefit of sonothrombolysis is that it is a noninvasive technique. In order address issues like that of stroke it is necessary to address the problem of attenuation of US caused by tissue and bond bones. Upon propagation of US through the body, different tissues of body absorbs it and then emit it in the form of mechanical or thermal energy. Due to this absorption the desired depth of penetration of US is not achieved which then requires the use of higher acoustic pressure in order to target thrombus but this may serve

risky for the patient. This problem is very concerning particulary in case of treatment of stroke because cranium cause higher attenuation of US. For diagnostic US the temporal bone window that is very thin part of skull is used usually along with US contrast agents. However in some patients imaging through this acoustic window is not sufficient. It is difficult to predict exact number of people that incur this issue because of lack of generalization of data, however, it is estimated that about 15-20% of the population suffer from this issue. (Viguier et al.,2005 and Brown et al.,2011). At the frequency of 1MHz, about 80% of peak negative pressure is dropped even in the presence of a temporal bone window because of insonation (Potter et al.,2001). Even after attenuation significant amount of lysis can still be obtained at suitable acoustic pressure without even injecting thrombolytic drugs. This effect is reported by Porter et al in an Invitro experiment by using 1Mhz and 40 Khz (Potter et al.,2001) and in an invivo study by using a phantom that mimics tissue at 1.5Mhz by Xie et al (Xie et al.,2005) depth of penetration can be increased by using the low-frequency US with increasing attenuation because of tissue and bones but this should be done by keeping in view the risk of intracranial hemorrhage. Another side effect associated with attenuation of US is that skull may get subjected to thermal damage. A study has shown that a 1°C increase in temperature is observed upon exposure of US having intensity 0,4W/cm2 at 1 MHz (Nahirnyak et al.,2007).

2.7.2 Delivery of therapeutic agent to thrombus

For effective treatment it is necessary to create a balance between dosage and method of administration otherwise it may raise safety concerns. By increasing the concentration of microbubbles an increase in lytic process has been observed but this happens up to a certain point (Borrelli et al.,2012). However, by increasing beyond a certain limit there is risk of embolism due to the presence gaseous core or shielding of further microbubbles or target site from exposure of US (Owen et al.,2012). The method of delivery used for administration of microbubbles also limit the duration of treatment. Usually it is given in the form of bolus and a good enhancement of contrast is observed for shorter period of time (Rubiera et al.,2008 and Sarraco et al.,2009) however this duration can be enhanced by continuous infusion and was found to exhibit good enhancement, this approach has been applied for sonothrombolysis in clinical trails (Perren et al.,2008)). Lytic mechanism

and acoustic response also vary by patterns of bubble replenishment at the site of thrombus.

Another issue associated with efficient sonothrombolysis is difficulty in the delivery of microbubbles and drugs to the thrombus site where blood flow is already reduced. Culp et al highlighted the problem of transportation of microbubbles in his study in which he observed better recanalization by intragraft injection as compared to that of intravenous injection while working on atriovenous grafts (Cul et al.,2001). Another study was conducted in which continuous imaging of thrombus is carried out and higher intensities of US are only administered upon detection of microbubbles in the region of ischemia (Xie et al.,2009). This strategy was proven successful in recanalization without using any fibrinolytic drugs. By using this approach microbubble's efficiency can be enhanced because it allows the replenishing of microbubbles at the thrombus site. While continuous exposure of US can cause bursting of microbubbles even before reaching the target site. This image guided therapy allows real time monitoring of treatment and reduces the unnecessary exposure of US (Xie et al.,2009).

2.8 Effects of an underlying pathology

Most of the research conducted so far employed healthy animals. However multiple pathologies are involved in clinical studies and they may create hindrance in successful enhancement of sonothrombolysis. However, an in vivo study conducted on atherosclerosis that is main reason behind incident of stroke and myocardial infection has shown opposition to this argument because both healthy and diseased animals with myocardial infarction upon treating with microbubbles and drug at the frequency of 1.6MHz US has shown decline in size of perfusion n defect (Xie et al.,2011). However, these outcomes can't be implemented on humans because of complex physiologies and variabilities involved in it

2.8.1 Safety

Microbubbles are considered as safe and are being routinely used in clinical settings however rare side effects are seen such as nausea, chest pain and headache (European medical agency.,2006). These side effects vanish by themselves and are not much

concerning. Here we will discuss safety issues that are mainly concerning with microbubble enhanced sonothrombolysis.

2.8.2 Microembolisation

Fragmentation of thrombus may occur due to exposure of microbubbles and US (Potter et al.,2001) and there is an associated risk of embolization because these fragments may have delocalized to other capillaries and block them. conditions of exposure of ultrasound determines the size of fragments of clots (Wright et al.,2012 and Maxwell et al.,2009). A study was conducted in the absence of fibrinolytic drug and microbubbles and it was observed that 99% of fragments formed by lysis at 20KHZ through Invitro sonication were less than 10 µm in size (Ariani et al.,1991). According to author no case of systemic or pulmonary embolization has been observed in clinical studies. However, in order further enhance safety a study has excluded the patients having history of right to left cardiac shunt (Molina et al.,2009) in which there is high risk of re embolization.

2.8.3 Intracranial hemorrhage

The biggest safety issue associated with treatment of ischemic stroke is the bleeding risk. Clinical symptoms of intracranial hemorrhage are easily detectable and are usually very serious. Neurological manifestation is not observed in most of the cases usually there is asymptomatic transformation. Studies are still going on to determine that ether reperfusion is naturally accompanied with bleeding or it is linked with adverse outcomes (Kent et al.,2004). Research is being carried out in order to introduce such kinds of treatments for stroke where the risk of bleeding is very less particularly efforts are being made to avoid symptomatic incidents that are more damaging

An increase in risk of symptomatic and asymptomatic intracranial hemorrhage has been observed by using t-PA for the treatment of ischemic stroke (Wang et al.,2004) while using the US along with fibrinolytic drug at lower frequency has shown increased bleeding (Daffertshofer et al.,2005). Microbubble administration for the treatment can reduce the risk of bleeding by decreasing the threshold of cavitation and will ultimately reduce the damage caused to surrounding tissues. As microbubbles can enhance the rate of revascularization therefore their incorporation along with the US can reduce the risk of symptomatic bleeding which occurs due to delay in the process of revascularization (Clark

et al.,2000). However, endothelial damage may take place due to the implementation of inappropriate conditions. Still, much research is needed to understand the causes of hemorrhage because a study has reported that almost a similar trend of bleeding was observed with microbubble administration and with t-PA and US alone (Molina et al.,2006 and ,Perrent et al.,2008) reasons for hemorrhage upon exposure of microbubbles and US are yet needed to be fully explained. It is very important to understand hemorrhagic transformations for widespread usage of microbubbles in clinical applications for enhanced sonothrombolysis.

2.9 Optimization of treatment

The effect of microbubbles on the lytic process and acoustic response generated by them is dependent upon multiple factors such as the method of administration, time and frequency of US, etc.

2.9.1 Effect of parameters of US

There are multiple parameters that are needed to be balanced perfectly in order to maintain the safety and efficiency of sonothrombolysis enhanced by microbubbles. These parameters include frequency of US, duration of US exposure, and method of administration.

2.9.1.1 Pressure

In order to overcome the cavitation and attenuation for US-based enhancement of thrombolysis it is necessary to use pressure above the threshold level. This threshold can be reduced by using microbubbles. In older studies, it was very difficult to determine the optimum pressure required for efficient lysis because they involved a complex set of parameters such as exposure regime, clot models, and varying frequencies. These parameters such as artificial cavitation nuclei, material characteristics, and US frequency are responsible for the threshold of cavitation. While reporting these parameters certain issues are faced such as in clinical practices it is difficult to monitor cavitation in conventional settings and no frequent reports are available about the output of pressure from diagnostic probes.

2.9.1.2 Frequency

There are three factors that are responsible for choosing the desired frequency these include microbubbles size, which influences the frequency of resonance (Stride et al.,2009), by increasing frequency attenuation also increases that reduces the depth required for treatment at higher frequencies, at lower frequencies, there are increased chances for the formation of standing waves at the reduced frequency in the cerebral cavity (Ammi et al.,2008) with increasing frequency it has been observed that threshold of cavitation of microbubbles also increases (Apfel et al.,1991 and Sponer et al.,1990). According to previous reports generally either very high-frequency range (Mhz) are employed or at very low range of frequency (20-500Khz) are used (Datta et al.,2006). The most commonly employed range of frequency for in vivo studies is usually very high that is 1-2MHz. Because of larger volume insonation, better skull positioning and penetration has been observed by using lower frequency. However safety concerns must be taken into account while using US parameters because intracranial hemorrhage was reported in a clinical trial that was being carried out by using the frequency of 300kHz and due to this reason these trials were terminated(Daffertshofer at al.,2005). In the later studies, it was suggested that the Breakdown of blood-brain barrier may be the reason for these complications. (Reinhard et al.,2006) as there is the possibility of standing wave formation, and multiple reflections due to interference of pulses that leads to an increase in pressure of cavitation beyond the threshold (Barron et al.,2009), these problems can be addressed by varying the parameters of exposure (Meairs et al.,2012).

2.9.1.3 Pulse Length

It has been observed that when pressure is increased beyond the threshold level of inertial cavitation, within few cycles of exposure of US microbubbles get destroyed (Chen et al.,2003). Invitro studies have shown improvement in lytic efficiency by using longer pulses (Meunier et al.,2007). This may occur due to the fragmentation of microbubbles into smaller bubbles however other studies have shown opposite outcomes in which it has been observed that microbubbles can be preserved for longer periods of time by using shorter pulses (Borelli et al.,2012). It is important to optimize the pulse length in order to avoid adverse events such as that hemorrhage. Frequency and pressure are the only parameters that are considered in conventional US machines used for diagnostic

purposes based on the mechanical index and it has been observed that the threshold of cavitation for tissues and microbubbles is reduced with pulse length and holds improved potential for effective lysis in safe manner (Church.,2005).

2.9.1.4 Pulse repetition frequency

The duty cycle is defined as the ratio of the length of the pulse to the repetition of the pulse (Brien.,2007). A study was conducted on water and it was observed that cavitation threshold can be reduced by either by enhancement of pulse repetition frequency or by enhancement of duty cycle (Fowlkes and Crum 1988). very low duty cycles (~ 0.1%) are used for diagnostic imaging as compared to that of 10% used in pulsed Doppler (Apfel et al.,1991). Different frequencies of pulse sequence and pulse repetition have been used for studying about sonothrombolysis by utilizing higher duty cycles of about 20%,80% or by employing Doppler modes(Alexandrov et al.,2008 and Culp et al.,2011), or by using pulses from second upto minutes(Tiukinhoy et al.,2007). By a combination of high PRFs and longer pulses a sustainable cavitational response may takes palce (Chen et al.,2003). Clot lysis has been found to improved by using variable PRF, increment in duty cycle at a particular pulse length this may occur due to improvement in survival of microbubbles or better replenishment rate. Safety concerns should be addressed while carrying out invivo studies.

Chapter 3

3. MATERIALS & METHODS

3.0 Materials and Methodology

3.1 Materials

Sebacic acid, glycerol (99.9% purity), span 60(99%purity), and Tween 80, were obtained from Sigma-Aldrich. Perfluropentane was purchased from Shanghai Tianfu, PBS tablets were purchased from Oxoid UK and New light corporation supplied us with a nitrogen gas cylinder. Streptokinase was obtained from

3.2 Synthesis of Pre-PGS as shell material

Pre PGS was synthesized by using the melt condensation method. For this purpose equimolar ratio(1:1) glycerol (20.14ml) and sebacic acid (50g) were taken in a 3 neck flask . in order to make sure that the composition is homogeneous both of these material were heated for 15 minutes. Then the reaction was carried out for 3 hours in the presence of a continuous supply of nitrogen gas in order to maintain inert environment. In meanwhile continuous stirring was carried out and the temperature was maintained upto 80°c. by the end of reaction a thick yellow viscous sticky material was obtained(PGS). Upon cooling it turned to white waxy material. It was stored at -20°C.

3.3 Preparation of Perfluropentane emulsion

Separate preparation of PFP was carried out. For this purpose, 500 µl of PFP was added dropwise into 10 Ml of PBS in the presence of 20 µl of tween 80. In meanwhile continuios sonication was carried out by using Probe sonicator at 120 W or 60% amplitude. This sonication was carried out for 10 minutes and during every cycle of soniction 1 minute ofc rest was include in the ice bath in order to avoid heating of the emulsion. Then in order to obtain homogeneous size, this emulsion was extruded from 0.22 micron filter paper by using extrusion assembly.

3.4 Synthesis of Pre-PGS microbubbles

The solvent displacement method was used for the formation of microbubbles. Firstly 2 g of Pre-PGS was dissolved in 2Ml of ethanol (Organic phase) in order to obtain a 100% w/v solution. Then 180 µl of span was dissolved into it at room temperature. Nextly dropwise addition of PFP emulsion into organic was carried out under continuous stirring.

Then this stirring was continued for 3 hours at room temperature in order to evaporate the solvent. Then the pallet obtained was washed thrice by centrifugation at 1000rpm.

3.5 Preparation of streptokinase-loaded microbubbles

0.5 g of streptokinase was dissolved in PFP emulsion under continuous stirring. This emulsion was then extruded through extrusion assembly by using 0.22-micron filter paper. Nextly, this emulsion was added dropwise to organic phase(pre-PGS). The mixture was stirred continuously for 3 hours in order to evaporate the solvent. Then the pallet obtained was washed by centrifugation at 1000 rpm in order to remove any unloaded streptokinase.

3.6 Reference standard preparation

In order to prepare a reference standard solution, 4 reference concentrations of streptokinase were dissolved in dextrose water. The concentrations used for this purpose were 0.5,0.4, 0.3, 0.2,0.1 g/ml of streptokinase in dextrose water.

3.7 Standard calibration curve for drug loading efficiency

In order to determine the concentration of encapsulated drug in PGS microbubbles, the calibration curve was plotted. For this purpose, all reference standard solutions were subjected to UV visible spectrophotometric analysis at the wavelength of 278nm. The standard curve was plotted which was then used for calculating drug loading efficiency.

3.8 Drug encapsulation efficiency

In order to determine the amount of drug encapsulated in the microbubbles, firstly disruption of microbubbles is carried out in order to release the encapsulated drug. For this purpose methanol-water is added into the pellet and emulsion is subjected to the vortexing. In order to release the drug, chilled methanol-water containing pellet is centrifuged for 20 min at 5000 rpm. The supernatant obtained is used for UV visible spectrophotometric analysis at the wavelength of 278nm. Following formula is the applied for the calculation of amount of encapsulated drug:

$$\text{Encapsulation efficiency \%} = \frac{\text{Concentration of streptokinase in medium}}{\text{Concentration of streptokinase added initially}} \times 100$$

3.9 Fourier-transformed Infrared Spectrometry

For the characterization of pre-PGS microbubbles and the functional groups present in them, FTIR analysis was performed. For this purpose, potassium bromide discs were used, these pellets of KBr were synthesized by applying high pressure of 1000 and 5000 psi. Then the sample was placed on to that disc and FTIR was performed at the wavenumber of 450-4000 and resolution of 4cm-1. Spectrum was recorded by using spectrum-100 obtained from Perkin Elmer, USA.

3.10 Zeta sizing and Zeta potential

In order to obtain the average size distribution, zeta potential, and polydispersity index of microbubbles zeta size analyzer was used (Malvern Zeta Sizer ver 7.12, UK, Serial no MAL1168467). Samples were first diluted by adding PBS and then pipetted into the plastic cuvette and subjected to zeta analysis at room temperature and a specific pH.

3.11 Scanning Electron Microscopy (SEM)

For SEM analysis, samples were diluted by the addition of PBS at room temperature. Then this diluted sample was placed in the form of a drop onto the glass slide and it was completely dried. After this gold sputtering was carried out and SEM was performed by using SEM-JEOL model no. JSM-6490LA. Images were captured at different magnifications.

3.12 Contact angle measurement

For contact angle measurement pre-PGS microbubbles and drug-loaded microbubbles were spread on the glass slide. Sessile drop method was used for measuring the contact angle by using fibro DAT 1100 (Sweden).

3.13 Blood sample

Whole blood samples were obtained from volunteers by using the sterile venipuncture method. Blood samples were drawn from the cubital vein of volunteers and then they were stored in sodium citrate buffered tubes in the refrigerator until their use. All volunteers were in good health and were not taking any sort of contraceptives or hormones. Furthermore, they were not facing any bleeding, coagulative or hematological disorders.

3.14 Preparation of Invitro blood clot model

A method reported in the literature was used for the synthesis of a blood clot (Petit et al.,2017). For this purpose, 1ml of anticoagulated blood was mixed with 2.5ml of 100 mM of calcium chloride. This mixture was injected into silicone tubes that were selected for formation of model after 5 minutes. Air bubbles should not be allowed to be injected into the tubes so that proper retraction of blood clots takes place. Then these blood containing tubes were incubated for 3 hours at 37°C, afterwards it is stored in refrigerator for 3 days for better retraction of the clot.

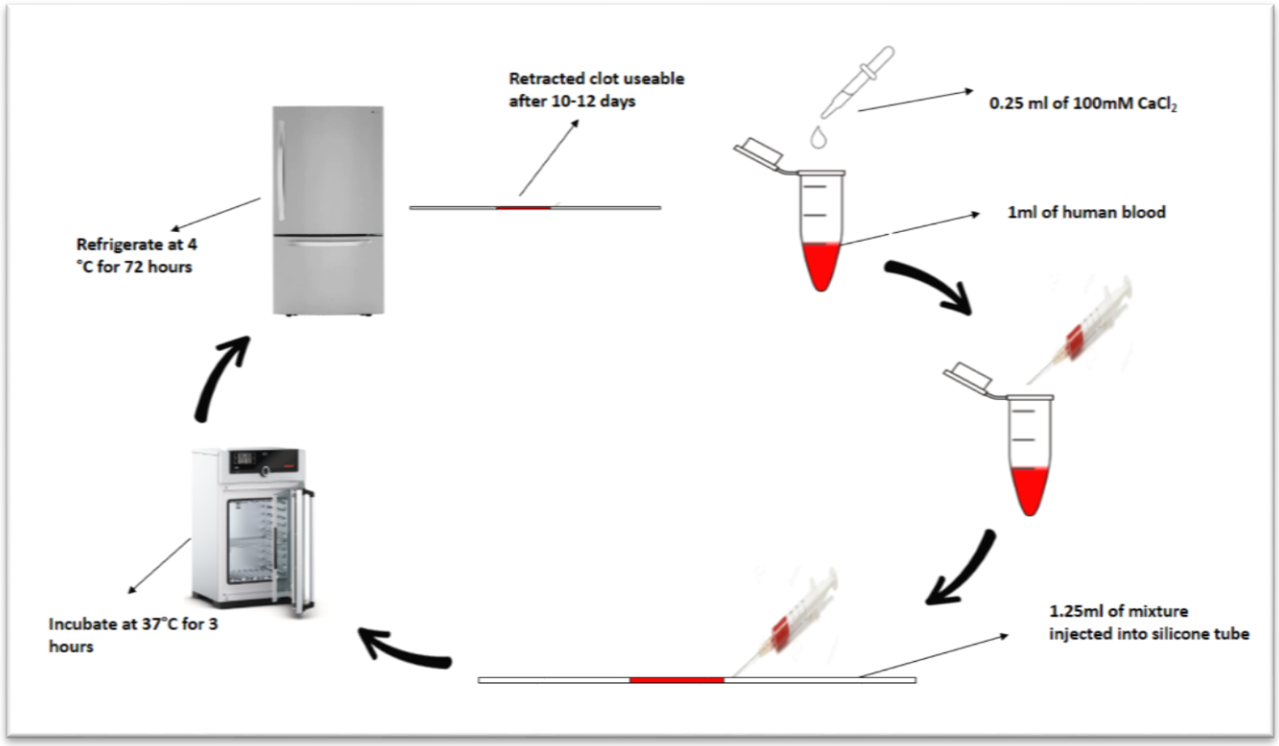

Figure 4: schemtic diagram of invitro blood clot formation

3.15 In-Vitro sonothrombolysis

The prepared blood clot model was subjected to Invitro sonothrombolysis by using clinical ultrasound machine. For this purpose, following parameters were used

Transducer probe

Mechanical index (MI) = 0.1,0.3,0.6.0.9

Frequency = 2.8 MHz and 3.2MHZ

Depth = 5 cm

The following study groups were subjected to sonothrombolysis:

Us alone

US+MB

US+Drug (Streptokinase)

US+Drug loaded MB

This process of sonothrombolysis was carried out for 30 minutes and the outcomes of sonothrombolysis were analyzed by measuring the mean gray intensity of ultrasound images, UV absorption, weight loss analysis and histological analysis.

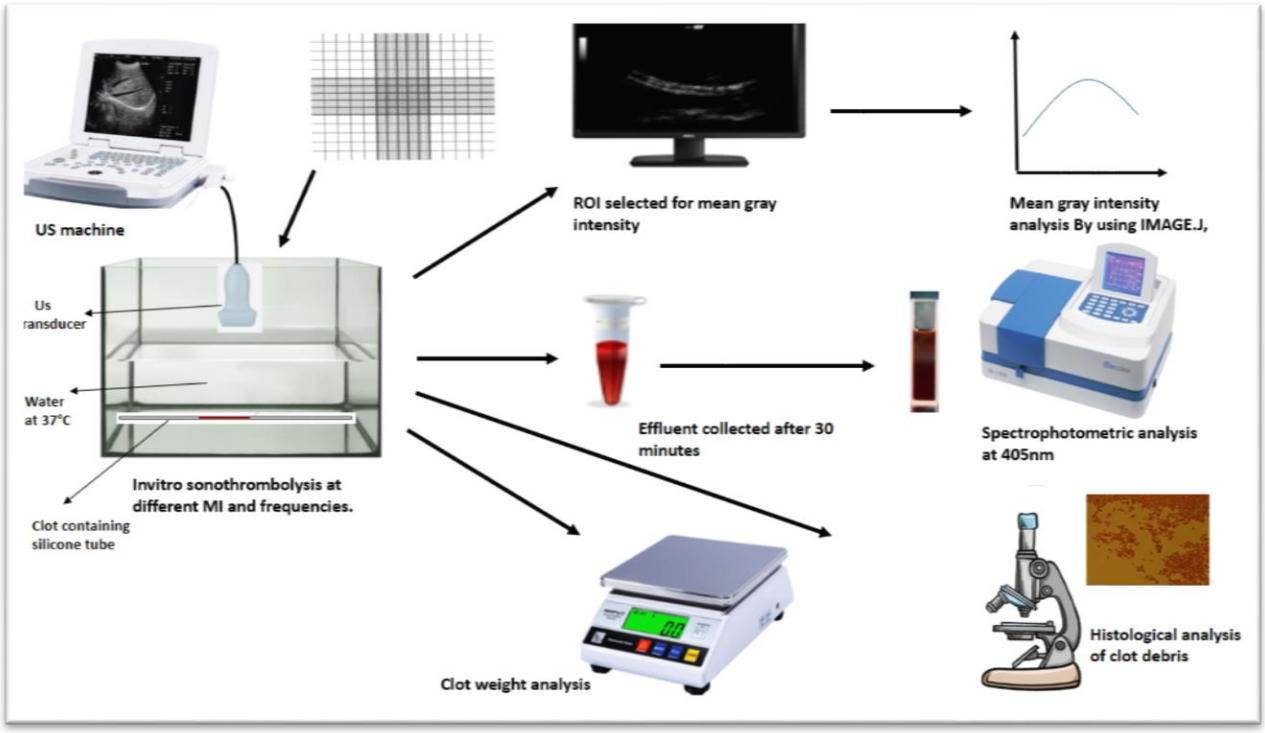

Figure 5: Schematic diagram of sonothrombolysis and monitoring of sonothrombolysis

3.16 Ultrasound Image analysis of Invitro clot model

During the process of sonothrombolysis, ultrasound images of Invitro model were captured and stored after every 5 minutes. These images were then analysed by using a software known as IMAGE.J. Mean gray intensity of clots was measured and certain parameters such as region of interest and X and Y coordinates of all images were kept constant.

3.17 Weight analysis of Invitro clot model

After 30 minutes of exposure to ultrasound weight analysis was performed. Initially, all clots were formed at the same weight of 4.56 g. then after the sonosthrombolytic treatment of 30 minutes' weight of all clots was measured again by using a scientific weighing balance.

3.18 Histology of blood clots

After sonothrombolytic treatments, all clots were fixed for a duration of 24 hours in formaldehyde, and then they were fixed in paraffin wax. Nextly H&E staining was carried out and then the slides were observed under an optical microscope. For properly understanding the effect of different groups histology of clots was observed after every 10 minutes of treatment with US alone, US+MB, US+ Drug, and US+Drug loaded microbubbles.

3.19 Spectrophotometric analysis of Hemoglobin of red blood cells

Among all study groups, red blood cells were released during lysis and they were stored for measuring the UV absorption by hemoglobin present in them. This analysis was carried out at the wavelength of 405nm for the effluents of all study groups and readings were recorded.

Chapter 4

4.RESULTS

4.0 Results

4.1 Chemical Characterization of Pre-PGS-Microbubbles and Streptokinase Loaded Microbubbles

4.1.1 FTIR

Pre-PGS microbubbles showed white color. FTIR analysis was carried out for characterization of the simple microbubbles, streptokinase-loaded microbubbles and streptokinase only for reference. Simple PGS microbubbles showed the characteristic vibrations of PFP due to C-F bond extending from 1100 cm^{-1} to 1460 cm-1..The peak observed at 1734 cm^{-1} denotes the stretching of the carbonyl (C=O) group in simple microbubbles. The other peaks appeared at 2926 cm^{-1},2955 cm^{-1} and 2688 cm^{-1} was due to stretching of C-H backbone. The broader peak observed at 3455.5 is due to OH stretching because of presence of alcohol groups found in pre polymer. While the charactestic peak of streptokinase- loaded microbubbles was observed at 1637 cm^{-1} which indicates the presence of amide group (Melo etal.2019) that is found in streptokinase. This indicates that drug has been loaded into the microbubbles. Other peaks seen in drug loaded microbubbles were almost similar to that of simple microbubbles, that includes broader peak of OH group at 3473 cm^{-1}, peaks of C-H backbone observed at 2920 cm^{-1}, 2845 cm^{-1}. Only the peak of amide group was not present in unloaded microbubbles. Following figure shows all the peaks observed in simple microbubbles, drug loaded microbubbles and Drug alone:

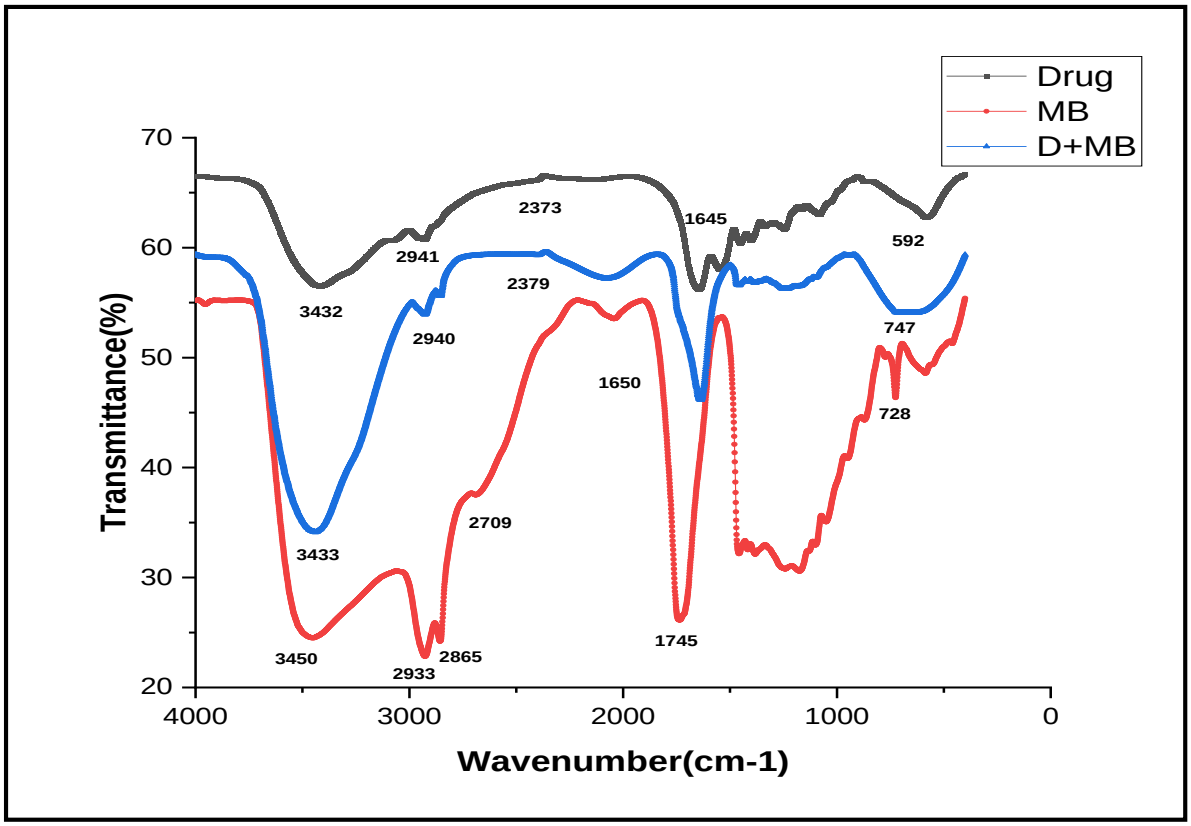

Figure 6: FTIR spectra of streptokinase, simple pre PGS microbubbles and streptokinase loaded microbubbles

Table 1: Interpretation of wavenumbers

Samples	Absorption peaks (cm^{-1})	Inference
Drug	3414	OH
	1645	CO-NH
MB	3455	OH
	2926	C-H
	2955	C-H
	1734	C=O
	1460-1100	C-F
Drug+Mb	3473	OH
	2920	C-H
	2845	C-H
	1650	CO-NH
	1460-1100	C-F

4.1.2 SEM analysis of Pre-PGS Microbubbles alone and Streptokinase-Loaded Microbubbles

Figure shows the scanning electron micrographs of Pre-PGS microbubbles(a) and Streptokinase loaded microbubbles at 50,000X performed at SCME, NUST. Pre-PGS microbubbles exhibited spherical shape with smooth texture and showed an average size of 28nm while Streptokinase loaded microbubbles showed a slightly larger size of 33 nm on average.

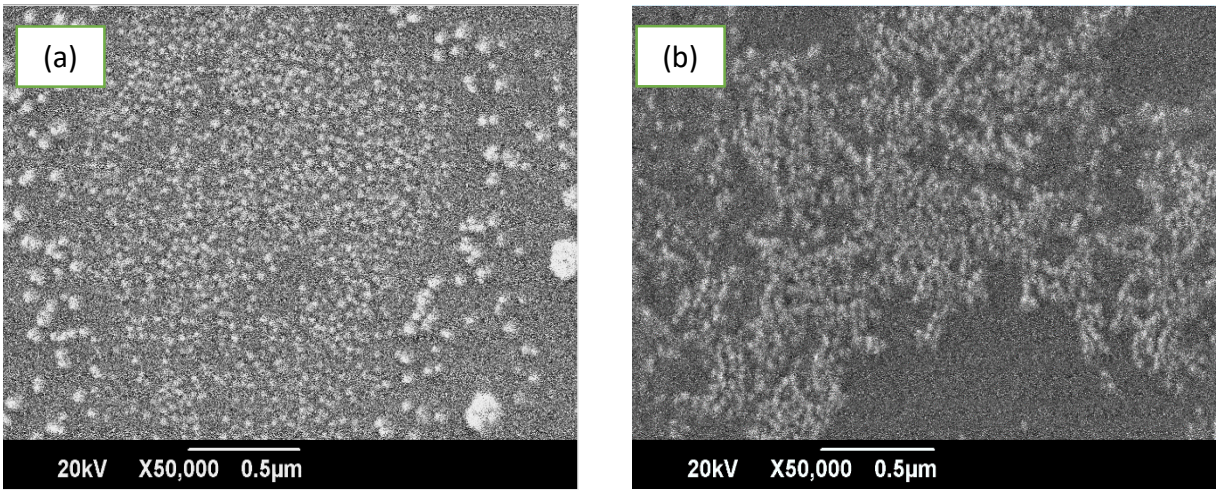

Figure 7:SEM images of (a)Pre- PGS microbubbles alone and (b) Drug loaded Microbubbles

4.1.3 Zeta size and Zeta potential

Polydispersity is observed in zeta analysis of PFCs as they reach closer to their boiling points because they consist of both liquid (non-phase-converted) and gaseous states (Phase Converted). This happens because some microbubbles get phase-converted near boiling points while others remain non-converted and exhibit smaller sizes our microbubbles also contain perfluropentane which is also liquid perfluorocarbon and the temperature for zeta analysis was 25°C. Microbubbles alone exhibited 2 peaks one Prominent peak that was observed at 240 nm that is 0.24 microns while Streptokinase loaded microbubbles exhibited a larger size of 492 nm that was 0.49 microns but it also showed 2 other peaks at 120 nm and another smaller peak at 5539 nm was observed similar to that of unloaded microbubbles. This happened because the boiling point of Perfluropentane is 27°C and zeta analysis was performed at 25°C. It is reported that

particle size increases upon phase conversion about 3-10 times. A similar trend was observed in this study. It was observed that the zeta size was larger than that was found during SEM images. This is because for SEM analysis dehydration occurs in vacuum conditions furthermore zeta sizer provides us with hydrodynamic diameter rather than that of particle size (9). Zeta potential of pre-PGS microbubbles was found out to be 14.6 mv while for streptokinase loaded microbubbles it was reduced to 7.08mv.

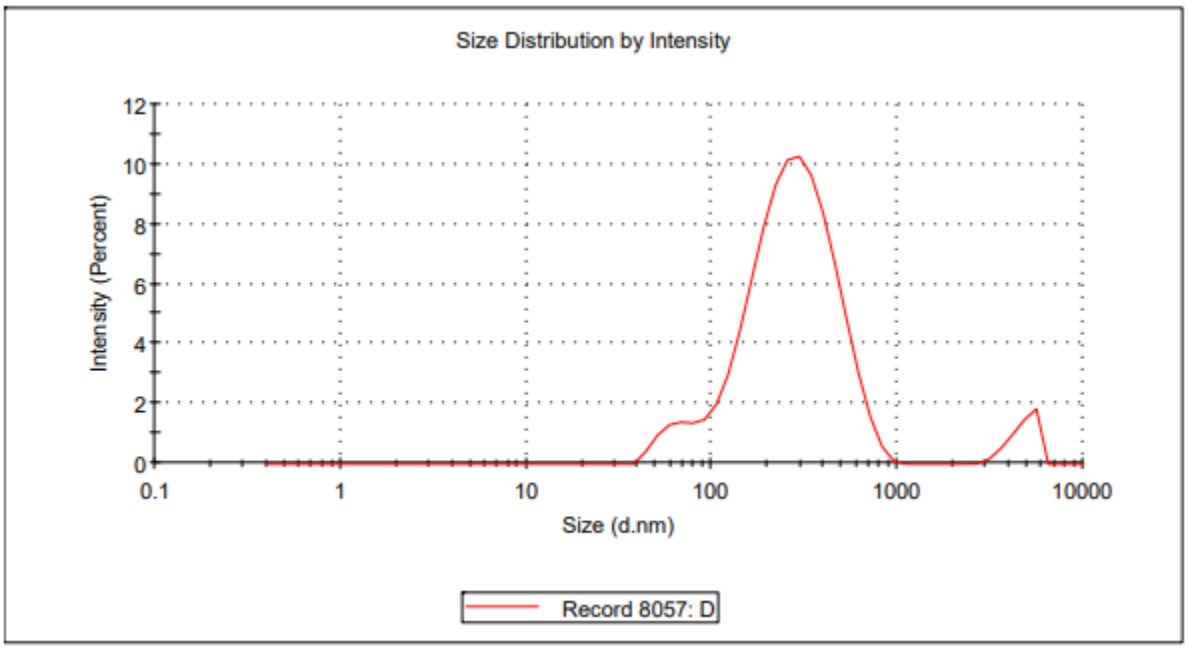

Figure 8:Zeta size analysis of Pre-PGS Microbubbles alone

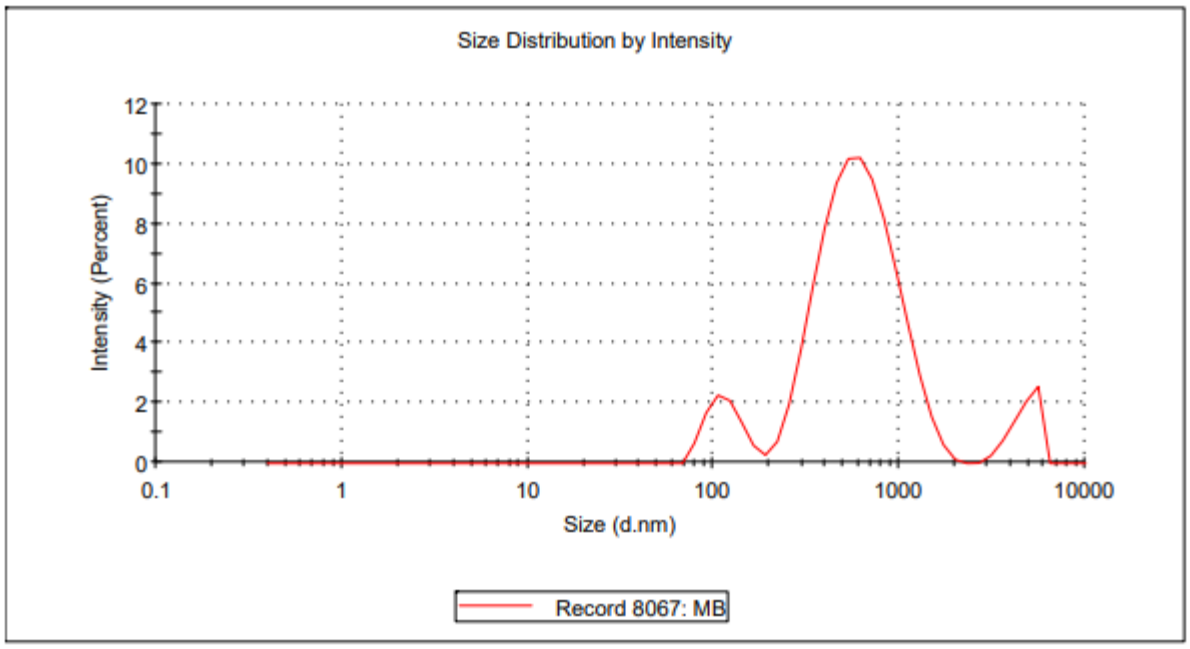

Figure 9:Zeta size analysis of streptokinase loaded Micro bubbles

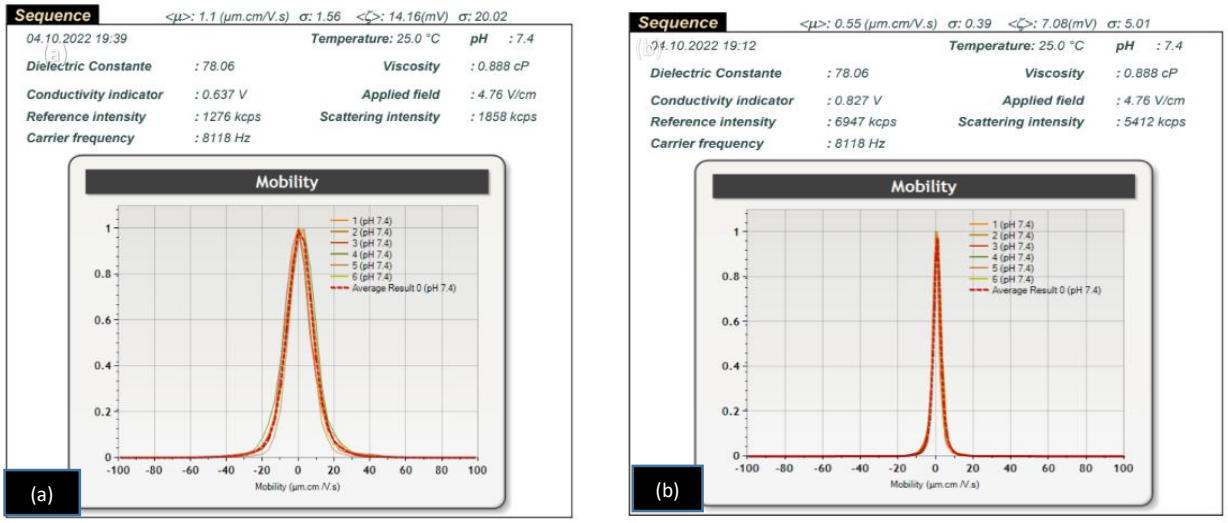

Figure 10:Zeta potential of (a)Pre PGS microbubbles and (b) streptokinase loaded microbubbles

4.1.4 Encapsulation efficiency

In order to calculate encapsulation efficiency, first of all, absorbance was measured at the wavelength of 278nm. Then standard curve was plotted fo absorbance of the UV spectrophotometer and the concentration of streptokinase. Following equation was used for the calculation of free drug from from the curve:

$$Y=mx+b$$

Hence:

$$x= (y - b/m)$$

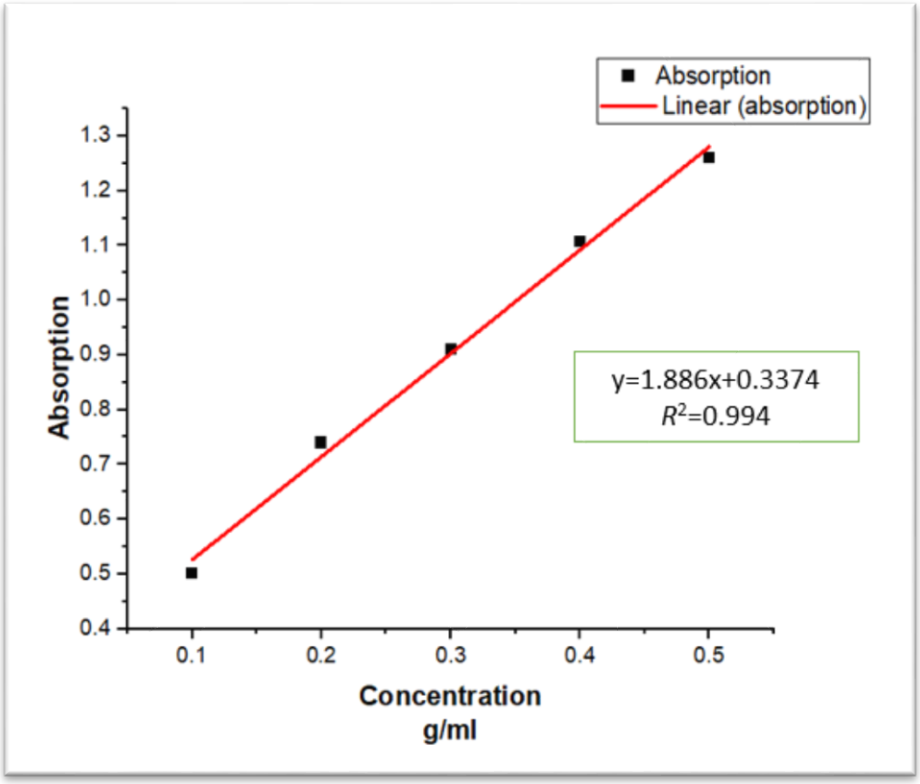

Figure 11: calibration curve for measuring drug loading effeciency

By using this equation, the encapsulation efficiency of streptokinase in microbubbles was easily determined. The following table depicts the detailed results of encapsulation efficiency

Table 2:absorbance at the 278 nm at the concentrations of 0.1,0.2,0.3,0.4 and 0.5g/ml of streptokinase

SK(g/ml)	Replicate 1	Replicate 2	Replicate 3	Mean	SD
0.1	0.51	0.49	0.52	0.5	0.33
0.2	0.78	0.73	0.71	0.74	0.13
0.3	0.89	0.94	0.92	0.91	0.66
0.4	1.12	1.18	1.02	1.106	0.66
0.5	1.27	1.26	1.25	1.26	0.33

Table 3:Streptokinase encapsulation efficiency in Pre PGS microbubbles, absorbance calculated at 278 nm for drug released in the medium

OD1	OD2	OD3	MEAN	SD	SK in Medium
1.082	1.054	1.082	1.072	0.0033	0.407

y= mx + b

y= 1.886x+0.3374

y= line intercept (Absorbance)

x= any point on the line (Drug Concentration)

y=mx + b

$$x = \frac{y - 0.3374}{1.886}$$

x=0.407 g/ml

$$\text{Efficiency}(\%) = \frac{\text{Drug loaded in medium}}{\text{Drug concentration used initially used}} \times 100$$

= 0.407/0.5×100

=81%

At start the total drug concentration loaded in Pre-PGS microbubble was 0.5g/ml and loading efficiency of microbubbles was found out to be 81% as illustrated in above calculations.

4.1.5 Contact angle measurement

Contact angle measurements were performed for 2 study groups and it was observed that for Pre-PGS microbubbles the contact angle was found out to be 60° which indicates that microbubbles are hydrophilic in nature but upon loading of streptokinase into the microbubbles it was observed that contact angle was slightly increased beyond 90° and it was found out to be 93° and was exhibiting slightly hydrophobic properties.

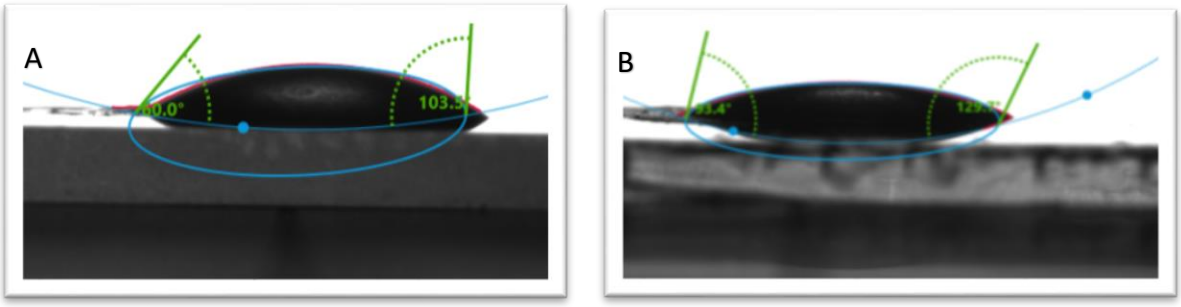

Figure 12:Contact angle measurement of simple Pre-PGS Microbubbles(A) and Streptokinase loaded Microbubbles (b)

4.2 Monitoring of Invitro sonothrombolysis

4.2.1 Mean gray intensity analysis of images of Clots captured during sonothrombolysis

In order to study the history of thrombus ultrasound is used. By interpreting the mean gray intensity during the process of sonothrombolysis we can measure the mass loss that occurred. Because the decrease in mean gray intensity is directly proportional to mass loss.

4.2.1.1 Invitro sonothrombolysis at 2.8 MHz by using US alone and US+MB

The figure shows the values of mean gray intensity recorded during 30 minutes of sonothrombolysis with a clinical ultrasound probe at the frequency of 2.8 MHz. These values were obtained by analyzing the mean Gray intensity from the images that were

captured after every 5 minutes of the procedure. Mean gray intensity values were obtained by using Image.J. Particular region of interest was selected and values were obtained. 2 study groups are presented in this graph first is (US) alone and 2nd is (US+MB). Upon analyzing the images, it was found that mean gray intensity values were decreasing with increasing mechanical index. The maximum reduction in mean gray intensity at the frequency of 2.8 MHz was recorded at MI 0.9 which was 23% for the study group of the US alone and 29% for the study group of US+MB.

Table is depicting the reduction of mean gray intensity of clot during Invitro sonothrombolysis at the mechanical index of 0.1,0.3,0.6 and 0.9 at the frequency of 2.8Mhz after duration of 30 minutes. In US only group mean gray intensity was reduced by 9%,20%,21% and 23% at MI 0.1,03,06 and 0.9 respectively. While in the US+MB group it was reduced by 13%,15%, 21%, and 29% at MI 0.1,0.3,0.6, and 0.9 respectively.

Table 4: is showing reduction of mean gray intensity of the clot during sonothrombolysis for US alone and US+MB at the frequency of 2.8 MHz at the MI of 0.1,0.3, 0.6 and 0.9

Mechanical Index (MI)	Mean gray intensity at the start of sonothrombolysis (US only)	Standard deviation (SD)	Mean gray intensity after 30 minutes of sonothrombolysis (US only)	Standard deviation (SD)	Mean gray intensity at the start of sonothrombolysis (US+MB)	Standard deviation (SD)	Mean gray intensity after 30 minutes of sonothrombolysis (US+MB)	Standard deviation (SD
0.1	37	0.66	34	0.66	40	0.33	35	0.33
0.3	39	0.33	31	0.66	41	0.33	35.	0.33
0.6	42	0.33	33	0.33	38	0.33	32	0.33
0.9	39	0.66	30	0.66	39	0.33	28	0.33

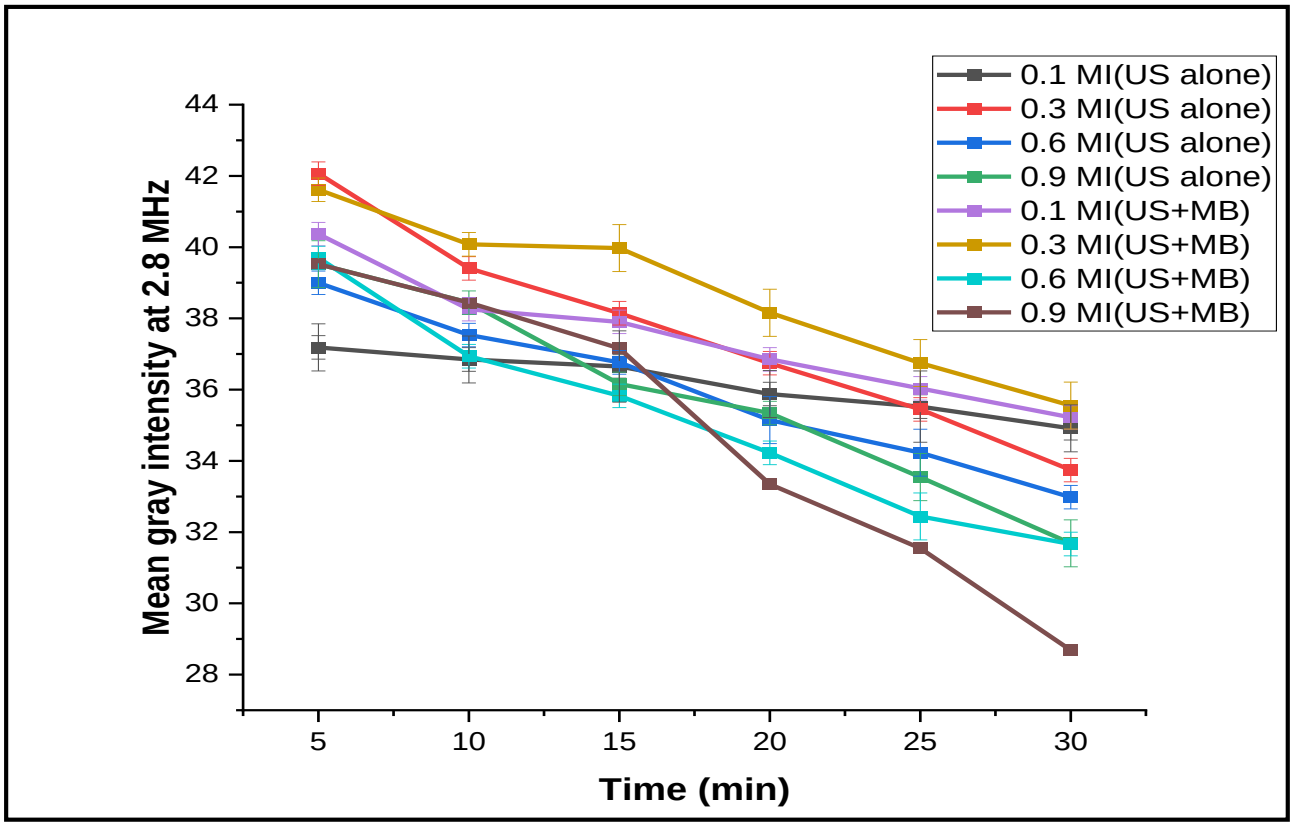

Figure 13: Mean gray intensity measured at 2.8 MHz for MI 0.1,0.3,0.6 and 0.9 for US only and US+MB

4.2.1.2 Invitro sonothrombolysis at 3.2 MHz by using US alone and US+MB

The figure denotes the mean gray intensity values that were recorded for sonothrombolysis of the Invitro model of the blood clot with an Ultrasound probe at the frequency of 3.2 MHz In this process, there were also 2 groups involved in it. The First was (US) only and 2nd one was US+MB. This analysis was also performed at multiple mechanical index (MI) ie. 0.1,0.3,0.6 and 0.9. values of mean gray intensity were obtained by image.J. it was observed that mean gray intensity values were decreasing with an increasing mechanical index. Furthermore, it was observed that with the incorporation of Pre-PGS microbubbles it was observed that Mean gray intensity was decreased more as compared to that of exposure to the US alone..40% of mean gray intensity was reduced in US only group while it was reduced to 49%.

The table shows the Reduction of the mean gray intensity of the clot during Invitro sonothrombolysis at the mechanical index of 0.1,0.3,0.6 and 0.9 at the frequency of 3.2 Mhz after duration of 30 minutes. In US only group mean gray intensity was reduced by 14%,22%,30% and 40% at MI 0.1,03,06 and 0.9 respectively. While in US+MB group it was reduced by 16%,32%, 36%, and 49% at MI 0.1,0.3,0.6, and 0.9 respectively

Table 5: Shows reduction of mean gray intensity of clot after 30 minutes of sonothrombolysis at the frequency of 3.2 MHz for MI 0.1, 0.3, 0.6 and 0.9 for US alone and US+MB

Mechanical Index (MI)	Mean gray intensity at the start of sonothrombolysis (US only)	Standard deviation (SD)	Mean gray intensity after 30 minutes of sonothrombolysis (US only)	Standard deviation (SD)	Mean gray intensity at the start of sonothrombolysis (US+MB)	Standard deviation (SD)	Mean gray intensity after 30 minutes of sonothrombolysis (US+MB)	Standard deviation (SD
0.1	44	0.66	38	0.66	43	0.66	36	0.33
0.3	40	0.33	31	0.33	41	0.33	28	0.33
0.6	39	0.33	27	0.33	41	0.33	26	0.33
0.9	42	0.33	25	0.33	41	0.33	21	0.66

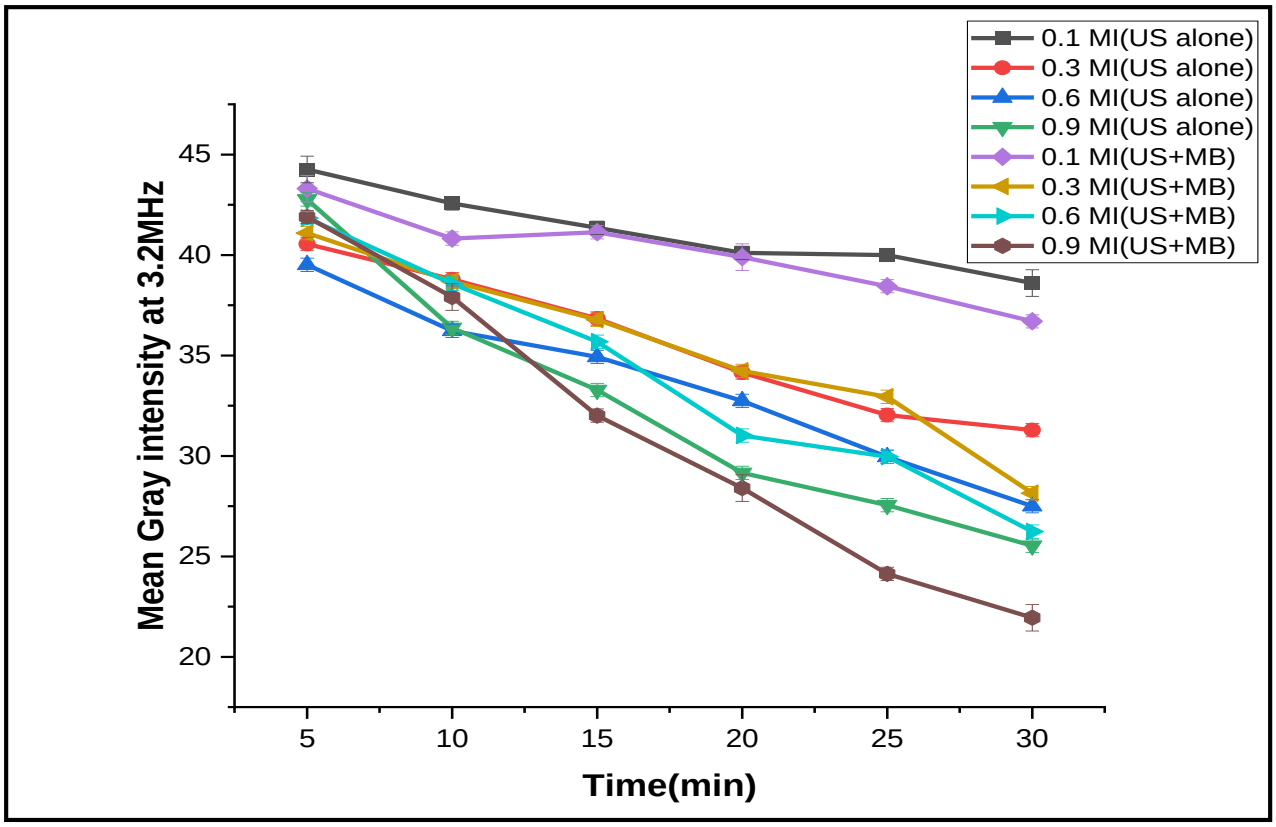

Figure 14: Mean gray intensity measured at 3.2 MHz for MI 0.1,0.3,0.6 and 0.9 for US only and US+MB

4.2.1.3 Invitro sonothrombolysis at 3.2 MHz by using US+ Drug and US+ drug loaded MB

For identifying the effect of the drug (streptokinase) and drug loaded microbubbles on in vitro sonothrombolysis, blood clots were subjected to a frequency of 3.2MHZ and the mechanical index of 0.9 for 30 minutes and it was observed that on the administration of streptokinase in the presence of US mean gray intensity was significantly reduced up to the 63% while the best outcomes were obtained by administration of streptokinase loaded microbubbles in which mean gray intensity was reduced by 80% as shown in the figure

The table shows the Reduction of the mean gray intensity of clot during Invitro sonothrombolysis at the mechanical index of 0.9 at the frequency of 3.2 Mhz after duration of 30 minutes. In US +Drug mediated sonothrombolysis mean gray intensity was reduced by 63%. in US+Drug loaded microbubbles mediated sonothrombolysis mean gray intensity was reduced by 80%.

Table 6:shows reduction in mean gray intensity at MI of 0.9 and frequency of 3.2 MHz for study group of US + Drug and US+ Drug loaded microbubbles

Mechanical index(MI)	Mean gray intensity at the start of sonothrombolysis (Drug only)	Standard deviation (SD)	Mean gray intensity after 30 minutes of sonothrombolysis (Drug Only)	Standard deviation (SD)	Mean gray intensity at the start of sonothrombolysis (US+Drug+MB)	Standard deviation (SD) Standard deviation (SD)	Mean gray intensity after 30 minutes of sonothrombolysis (US+ Drug+ MB)	Standard deviation (SD)
0.9	44	0.66	16	0.33	44	0.66	9	0.33

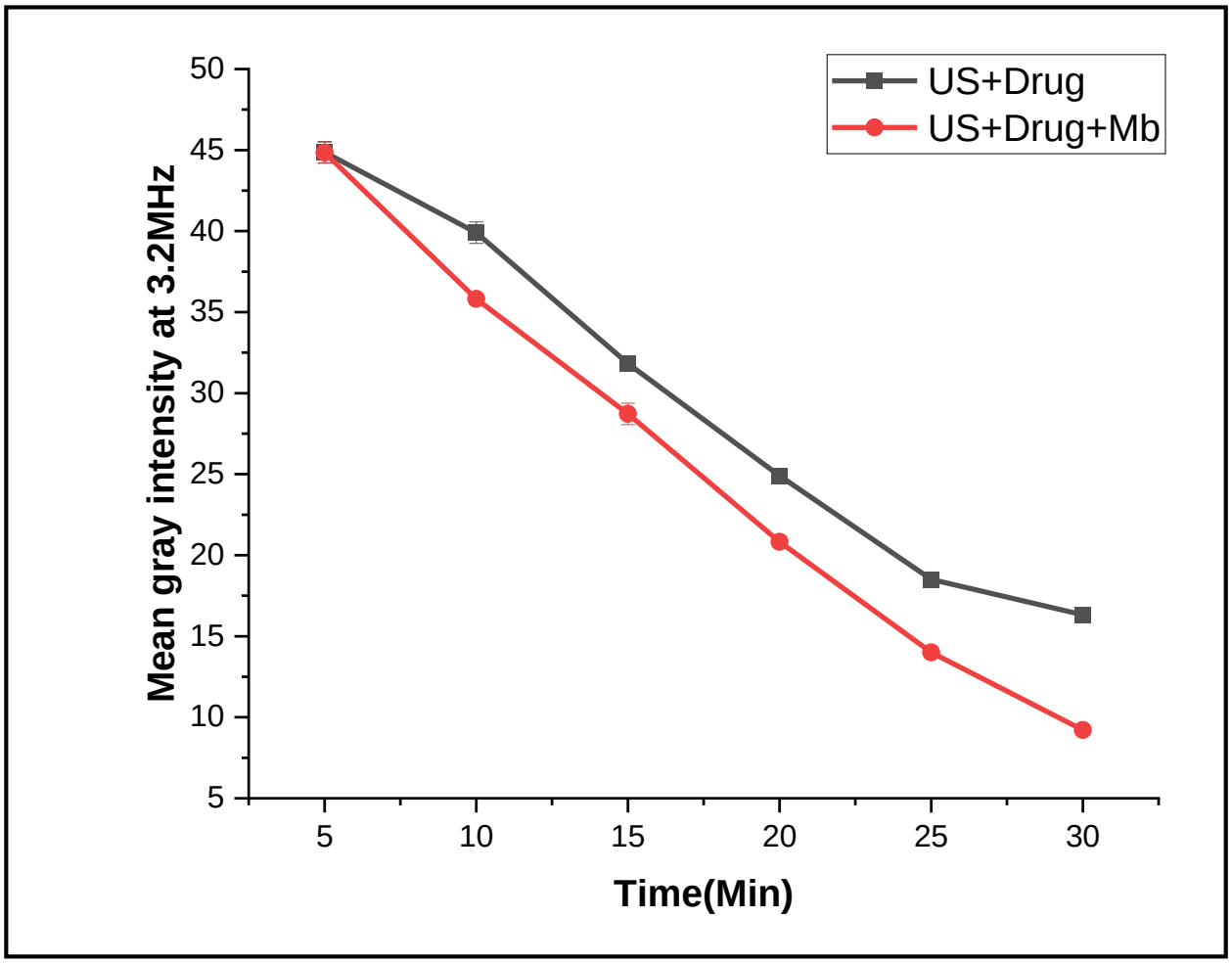

Figure 15: Mean gray intensity measured at 3.2 MHz at 0.9 MI for US+Drug and US+Drug loaded Microbubble

4.2.2 **Weight analysis at a frequency of 2.8 MHz with US only and US+MB**

The weight of clots before exposure to Invitro sonothrombolysis was measured and then after the procedure of 30 minutes, of sonothrombolysis weight was measured again at different MI of 0.1, 0.3, 0.6, and 0.9 for both study groups of US only and US+MB. It was observed that weight was reduced by 9%,14%,16%, and 18% at the MI of 0.1, 0.3, 0.6 and 0.9 respectively for US only group. While for the US+MB group it was reduced by 14%,16%,20% and 25% at the MI of 0.1,0.3,0.6 and 0.9 respectively as shown in the following Figure

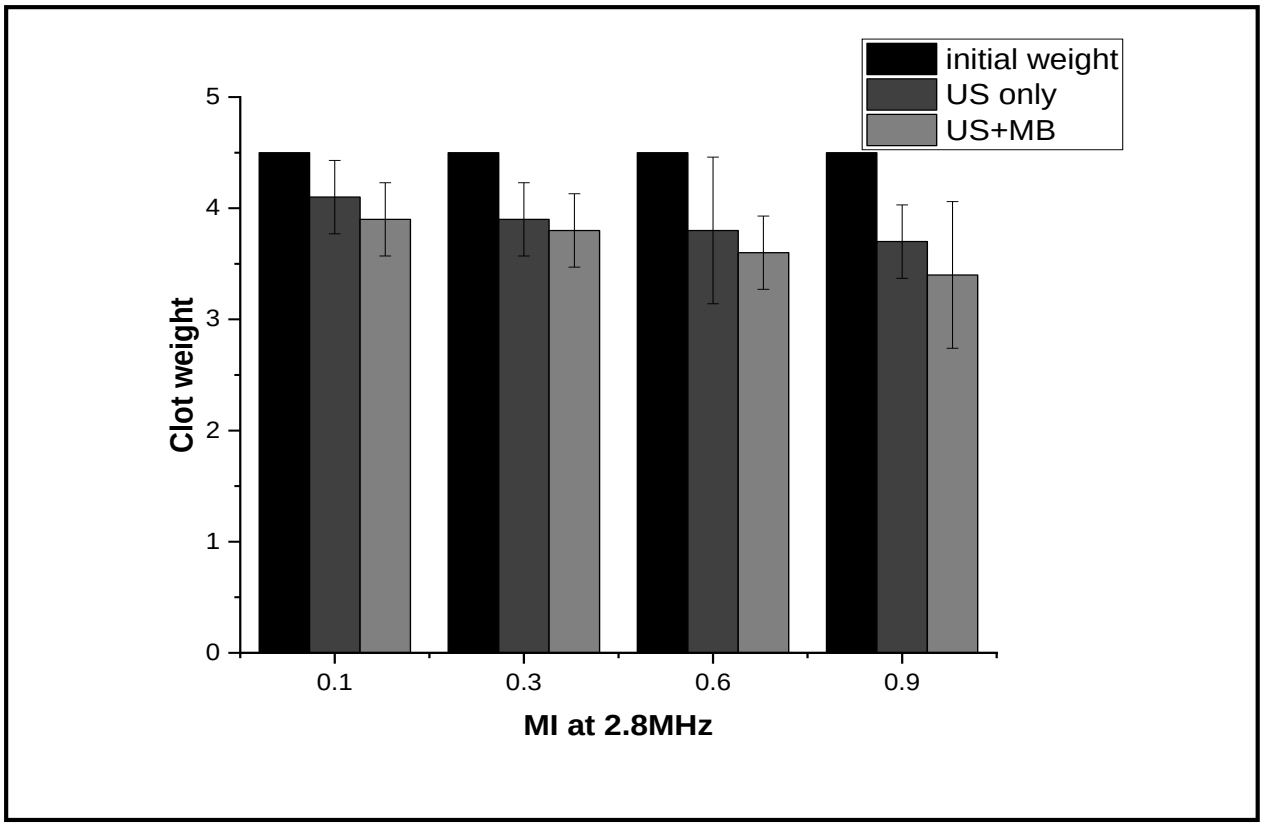

Figure 16: Clot weight analysis at 2.8 MHz for Study group US only and US+MB at 2.8 MHz

4.2.3 Weight analysis at a frequency of 3.2 MHz with US only and US+MB

Pre and post-weight of clot was measured for both groups of US only and US+MB at the frequency of 3.2 MHz and it was observed that the weight of clots after Invitro sonothrombolysis was reduced by 9%, 23%,25%, and 32% at the MI of 0.1,0.3,0.6 and 0.9 respectively for US only group and it was reduced by 18%,23%,32% and 38% at the MI of 0.1, 0.3, 0.6 and 0.9 respectively for the US+MB study group as illustrated in the following figure.

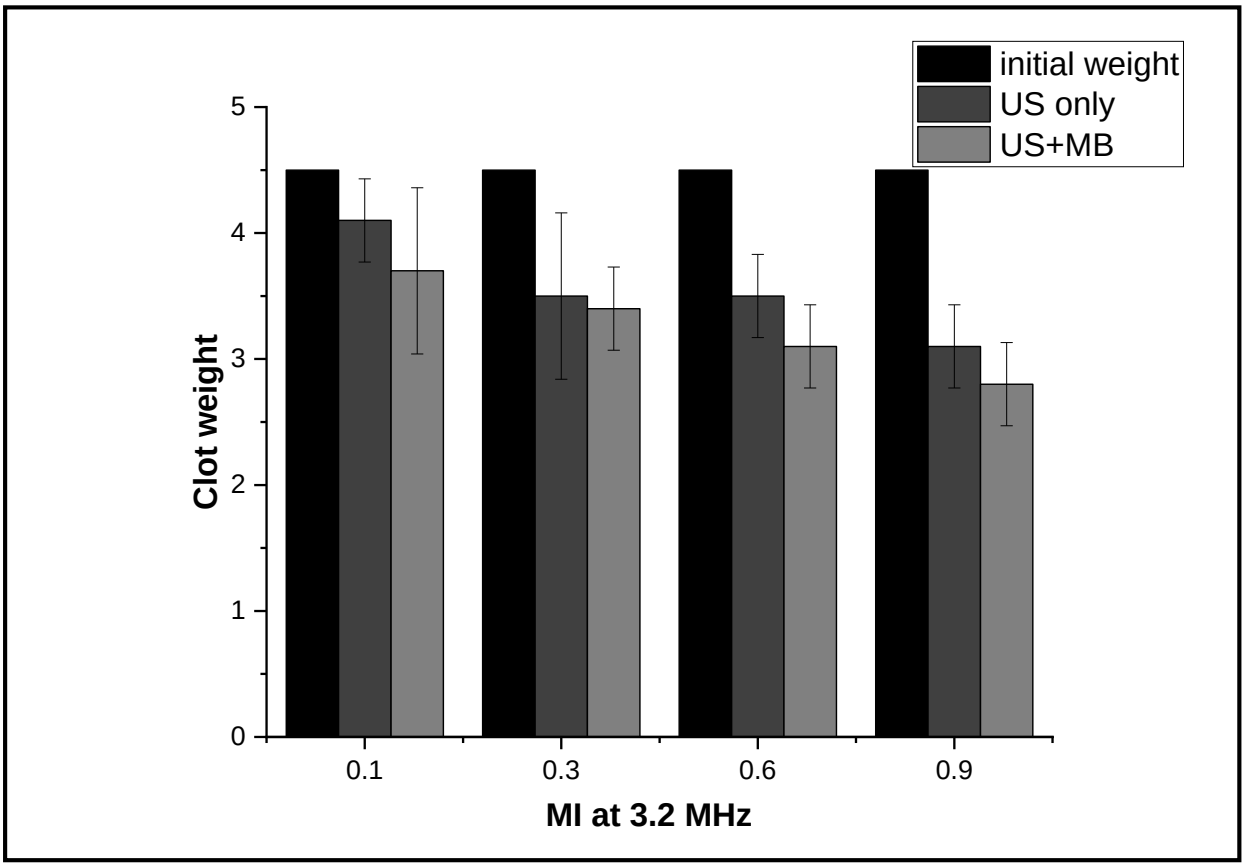

Figure 17: Clot weight analysis at 3.2 MHz for Study group US only and US+MB at 2.8 MHz atMI of 0.1,0.3, 0.6 and 0.9

4.2.4 Weight analysis at a frequency of 3.2 MHz with Drug only and Drug loaded MB

Upon administration of the drug(Streptokinase) at the frequency of 3.2 MHz weight analysis was performed at the mechanical index of 0.9 and it was observed that the weight of the clot was reduced by 45% for Drug mediated sonothrombolysis while it was reduced by 65% for drug-loaded microbubbles mediated Invitro sonothrombolysis as depicted in figure:

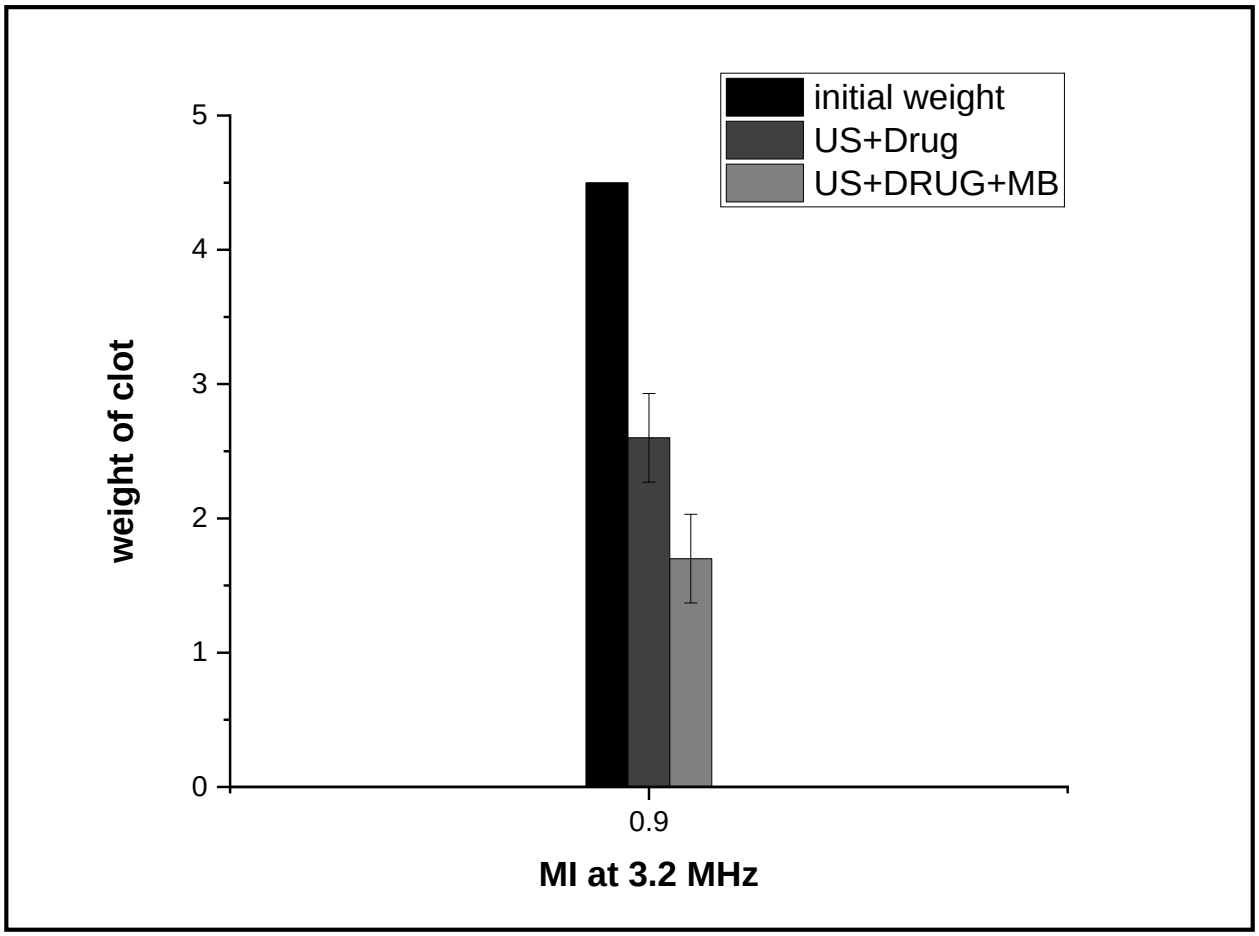

Figure 18: Clot weight analysis at 3.2 MHz for Study group US+ Drug and US+ drug loaded Microbubbles at 0.9 MI

4.3 Histological analysis

All slides were observed under a florescent microscope and it was observed that the clot remain intact with US alone treatment, then in the US +MB mediated sonothrombolysis it was observed that pores were created in blood clots as is evident in the figure below, Nextly, US+Drug treatment further showed improved lysis of fibrin network. Then upon treatment with drug-loaded microbubbles, it was observed that clot lysis was further enhanced and fibrin network was degraded significantly. As it can be seen in the following figure that Clot was significantly broken down. While after 30 minutes of treatment not enough residue was left that can be fixed in paraffin.

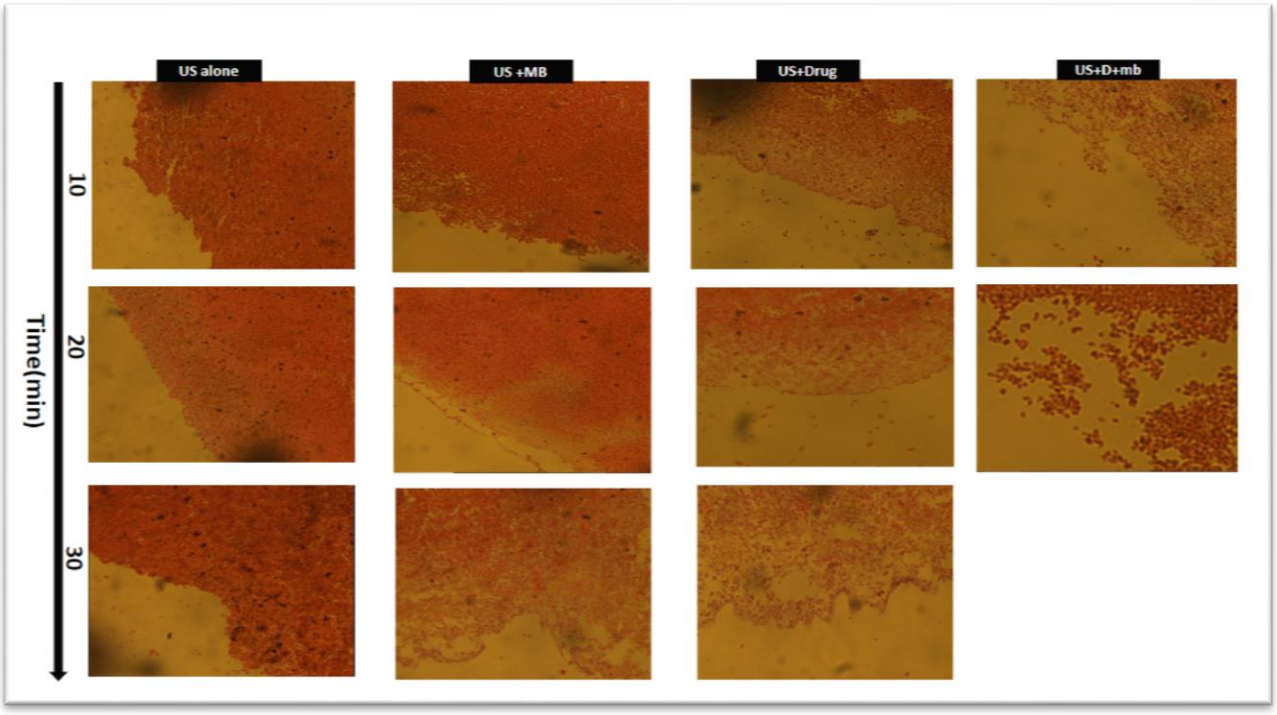

Figure 19: Histology of blood clots after every 10 minutes of sono thrombolysis for all study groups at different MI.

4.4 Spectrophotometric analysis of effluents of sonothrombolysis

Spectrophotometric absorbance was measured for the effluents collected after sono thrombolysis of all study groups that includes US alone. US+MB, US + Drug, and US+Drug loaded microbubble exposed to frequency of 2.8 MHz and 3.2 MHz at range of mechanical index (0.1, 0.3,0.6 and 0.9). this absorbance of hemoglobin found in red blood cells was measured at the wavelength of 405 nm. Following table shows the detailed readings of spectrophotometric absorbance**.**

Table shows the absorbance of hemoglobin at different frequencies and MI, maximum absorbance was found for the study group US+Drug loaded microbubbles at the frequency of 3.2 MHz that was 2.75±0.33.

Table 7: Shows absorbance of hemoglobin at 405 nm for effluents of clot from different study groups

	US (2.8 MHz)	Standard deviation (SD)	US+MB(2.8 MHz)	Standard deviation (SD)	US (3.2 MHz)	Standard deviation	US+MB (3.2 MHz)	Standard deviation	US+Drug (3.2MHz)	Standard deviation	US+drug loaded MB (3.2 MHz)	Standard deviation
0.1	2.14	±0.33	2.29	±0.21	2.40	±0.3	2.47	±0.34				
0.3	2.17	±0.28	2.33	±0.31	2.43	±0.33	2.53	±0.33				
0.6	2.23	±0.28	2.35	±0.26	2.49	±0.38	2.55	±0.31				
0.9	2.27	±0.33	2.38	±0.27	2.51	±0.33	2.59	±0.35	2.61	±0.33	2.75	±0.33

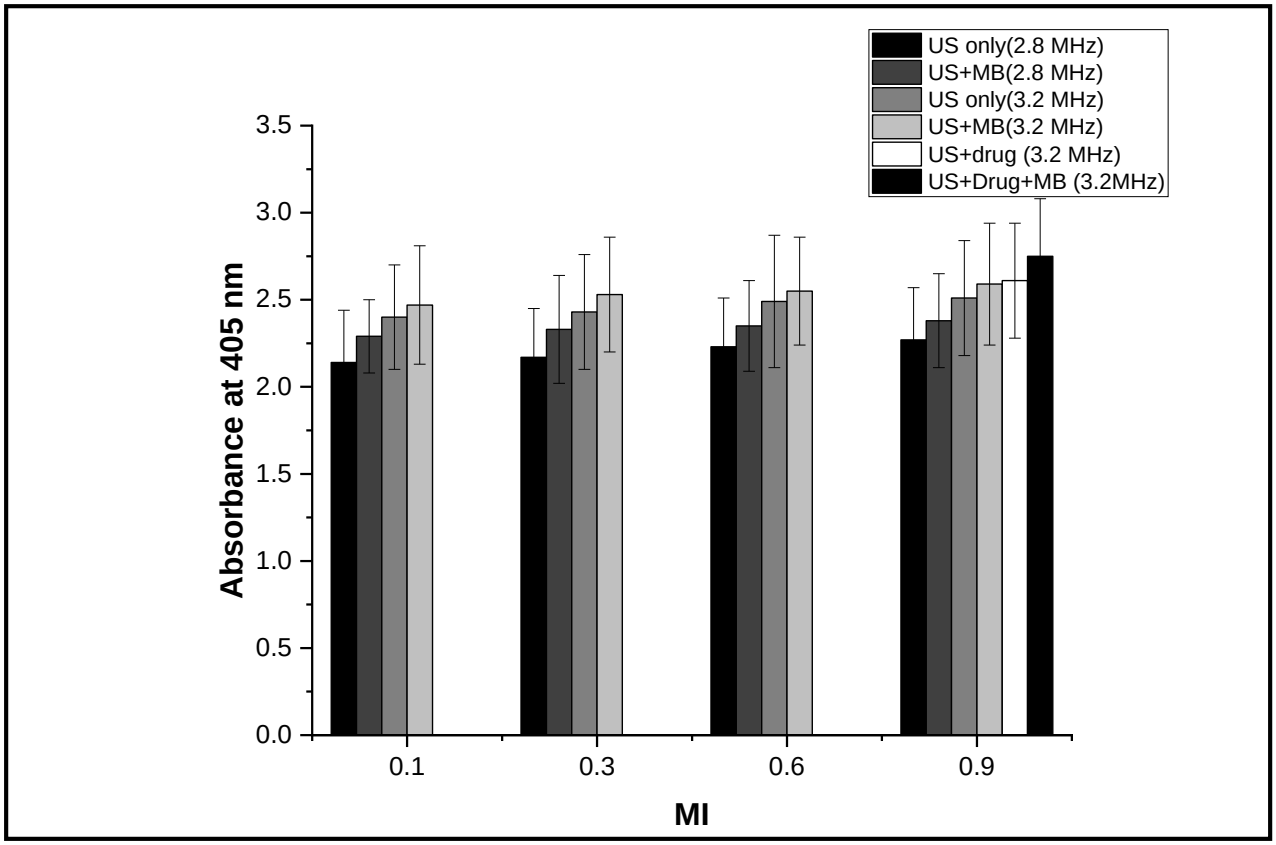

Figure 20: Absorbance of hemoglobin at 405 nm for different study groups at the MI of 0.1,0.3, 0.6 and 0.9

In the figure 20 it can be observed that by increasing the mechanical index, absorbance of hemoglobin increases because number of red blood cells increases in the effluent of clot. This effluent was collected after 30 minutes of the procedure of sonothrombolysis for all study groups that includes US only, US+MB at the frequency of 2.8 MHz and US only, US+MB, US+Drug and US+ Drug loaded microbubbles at the frequency of 3.2 Mhz at different MI of 0.1, 0.3, 0.6 and 0.9. Maximum absorbance was observed for the effluent of study group US+Drug loaded microbubbles that was found out to be 2.75 ±0.33

Chapter 5

5.Discussion

5.0 Discussion

Stability of microbubbles have increased the focus in ultrasound imaging and therapy. Many studies have been reported about synthesizing microbubbles with variety of shell materials and core gases in order to stabilize them. while application of microbubbles certain problems are encountered such as they are polydisperse in nature and how we synthesize them defines the resulting size of microbubbles (Harput et al.,2017). Now a day's main focus is on increasing the stability of microbubbles while keeping it cost effective approach. The material that is used for synthesizing the microbubble has great impact on defining its stability (Gharat et al.,2022). In our study we used PGS as shell material and PFP as core material by using solvent displacement method. In order to confirm the synthesis of pre-PGS microbubbles different analysis were performed that include zeta sizing, zeta potential, SEM analysis FTIR analysis. The average size of microbubbles as observed in SEM imaging was on average 28 nm for simple microbubbles. While zeta sizer indicated the size of microbubbles as 0.24 micron. This size is larger then that observed in SEM imaging because for SEM analysis dehydration occurs in vacuum conditions furthermore zeta sizer provides us with hydrodynamic diameter rather than that of particle size. Zeta potential of microbubbles were found out to be 14.6 mv. Zeta potential is an important parameter for determining the surface charge of Nano particles. If zeta potential is either strongly positive or strongly negative, it indicates that the Nano particles have good stability (Honary and Zahir 2013). The value of zeta potential from +30 mv to -30mv is considered as good and those particles which lie in between these values exhibit good colloidal stability. Our construct also showed zeta potential of 14.6 mv which indicates that they have good stability. If the value of zeta potential is too low, then it may result in aggregation of nanoparticles.

Pre-PGS microbubbles appeared as spherical in shape with smooth outer texture as indicated in SEM images while some particles were observed to form clusters. This happened because the slides were not completely dried before subjecting to SEM analysis and also because not enough sonication was performed while preparing sample for SEM imaging. Invitro blood clot model was prepared for performing sonothrombolysis. for this purpose, blood samples were taken from healthy individuals and were injected in silicone tubes. Once the clots were retracted then sonothrombolysis was performed in

different study groups. Firstly, it was performed at the frequency of 2.8 MHZ at the MI of 0.1,0.3, 0.6 and 0.9 for 2 study groups that are US only and US+MB. Then in the other study group it was performed at 3.2 MHZ for US only, US+MB, US+Drug and US+Drug loaded microbubbles. The US probe that was used for this purpose was of 3.5 MHZ.

For preparation of drug loaded microbubbles, streptokinase was loaded into the microbubbles, this drug has already been reported as an effective thrombolytic agent and then sonothrombolysis was performed. Its monitoring was carried out by analyzing mean gray intensity, pre and post weight analysis, Histological analysis and spectrophotometric analysis of effluent obtained from clot lysis.

Mean gray intensity can be used as a parameter for observing clot lysis as it is found to be reduced by decreasing the mass of clot. It has been observed that acoustic response of microbubbles is linear in nature when mechanical index is low from MI 0.1 to 0.5. but it has been observed that at the MI ≤0.5 microbubble's growth is reduced. It has been observed that oscillation and growth of microbubbles tend to increase by increasing the mechanical index beyond 0.5. at mechanical index ≥ 0.5 it has been observed that linear oscillations of microbubbles shift from linear to those linear oscillations in which rare fraction of microbubbles is higher then that of compression. This behavior tends to increase the lysis of clot and it is further increased by increasing the mechanical index. Due to this reason it has been observed that mean gray intensity was increased more in the study groups that were subjected to higher mechanical index in our study. This trend was followed among all study groups that include US alone, US+MB and US + drug loaded microbubbles.

As indicated in figure 15, that at mechanical index of 0.9 and frequency of 3.2 MHZ maximum reduction in mean gray intensity was observed. This was also confirmed by pre and post weight analysis as it was observed that maximum reduction in clot weight occurred upon exposure to mechanical index of 0.9.

In order to further ensure the results, histological analysis of clot residues was carried out and it showed very promising results in line with mean gray intensity reduction and weight loss. As shown in figure 19, it was observed that fibrin network was degraded with increasing mechanical index and frequency. Maximum degradation was observed in the

study group of US+Drug loaded microbubbles as indicated by the histolgical analysis after 20 minutes of sonothrombolysis. however histological analysis at MI 0.9 and frequency 3.2 MHZ was not performed after 30 minutes fro study group of US +drug loaded microbubbles because not enough clot residue was left that can be fixed in paraffin for histology.

Amount of clot lysis is proportional to decrease in mean gray intensity, weight loss, increased absorption of hemoglobin and degradation of fibrin network as observed by histological analysis of clot residues.

In previous research microbubble mediated sonothrombolysis was first time performed by using albunex microbubbles at the frequency of 170 KHz in order to enhance the rate of sonothrombolysis (Tachibana and Tachibana, 1995). It was observed that rate of sonothrombolysis was increased with incorporation of microbubbles. Several studies were conducted subsequently and it was observed in Invitro analysis that sonothrombolysis was enhanced at sub megahertz exposure of ultrasound and also by using frequencies that were used for diagnostic imaging. Another study was reported in which Sonovue microbubbles were used with rt-PA and it showed greater rate of sonothrombolysis as compared to that of using rt-PA alone (Goel et al.,2021). Invitro studies also confirmed that rate of sonothrombolysis was enhanced by using rt-PA in combination with microbubbles and rt-PA with US as compared to that of using them alone.

Our results were in line with previous reported studies in which rate of sono-thrombolysis was higher with US + drug loaded microbubbles was significantly higher than that of using US alone or US+MB only or drug only. Maximum reduction in mean gray intensity was observed in the study group of US+Drug loaded microbubbles at the MI of 0.9 and frequency of 3.2 MHz. similarly, maximum reduction clot weigh was also observed for the study group of US+Drug loaded microbubbles at 0.9 MI and 3.2 MHZ. furthermore maximum degradation of fibrin network was also observed in this study group. Similarly spectrophotometric analysis of clot effluents also confirmed that maximum clo breakdown occurred in US+Drug loaded microbubble group at the frequency of 3.2 MHz and MI 0.9.

This indicates that US + streptokinase loaded microbubbles resulted in effective Invitro sonothrombolysis at the frequency of 3.2 MHZ and 0.9 MI.

6.0 Conclusion

Pre-PGS microbubbles hold great potential for drug delivery and targeting. Synthesis of microbubbles was confirmed by FTIR, SEM and Zeta analysis then these microbubbles were used for Invitro sonothrombolysis. Various study groups were formed and were evaluated for sonothrombolysis. procedure was carried out for 30 minutes. Monitoring of sonothrombolysis was carried out by mean gray intensity analysis, weight analysis, histological analysis and spectrophotometric analysis. It was observed that mean gray intensity was significantly reduced, clot weight was also reduced and degradation of fibrin network was enhanced that was observed by histological analysis. Similarly, absorption values of hemoglobin were also increased for clot effluents that were subjected to spectrophotometric analysis. For effective Invitro sonothrombolysis we have successfully designed streptokinase loaded microbubbles that showed promising results at MI of 0.9 and frequency of 3.2 MHz. This approach is very convenient for conducting the thrombolysis in the presence of ultrasound.

7.0 References

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