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DEVELOPMENT AND CHARACTERIZATION OF ENCAPSULATED THROMBOLYTIC FORMULATIONS TO ENHANCE THROMBOLYSIS

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DEVELOPMENT AND CHARACTERIZATION OF ENCAPSULATED THROMBOLYTIC FORMULATIONS TO ENHANCE THROMBOLYSIS

A Dissertation APPROVED FOR THE
SCHOOL OF CHEMICAL ENGINEERING AND MATERIALS SCIENCE

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ABSTRACT

Therapeutic thrombolysis with plasminogen activators is an effective and potentially life-saving measure in the management of patients with acute myocardial infarction and cerebral ischemia. Liposomal encapsulation of plasminogen activators by multiple laboratories around the world has shown promise. However, liposomes suffer from physical instability that results in a short shelf-life. The use of polymer microcapsules as an encapsulation vehicle has now demonstrated comparable, if not superior, thrombolytic potential compared to liposomes with improved storage capability.

The capacity of liposomal (LESK) and microencapsulated streptokinase (MESK) to digest an occlusive arterial thrombus were compared to an identical dosage of free streptokinase (FREE SK) in a rabbit model of carotid thrombosis (Chapter 3). Compared to FREE SK (74.5 ± 16.9 min; mean ± SEM), LESK demonstrated significantly reduced reperfusion times (19.3 ± 4.6 min) while MESK exhibited even greater improvement (7.3 ± 1.6 min). LESK and MESK also resulted in reduced residual clot mass and greater return of arterial blood flow.

In vitro studies utilizing clots formed of whole blood or plasma in glass capillary tubes were completed to elucidate the mechanism of lysis with MESK (Chapter 4). In both clot types, lysis occurred more rapidly at higher pressures and MESK restored flow

faster than FREE SK. Light and confocal microscopies were used to capture images of clot digestion. FREE SK, like other free plasminogen activators, binds to the leading edge of the thrombus and initiates digestion through a series of localized reactions at the front. MESK, however, accelerates digestion by improving the distribution of streptokinase throughout the clot following greater penetration of the leading edge, a finding confirmed by a computer simulation (Chapter 5). Clot digestion occurs within the thrombus and at the front, thereby promoting faster clot digestion and restoration of flow.

The potential benefit of MESK was also explored in a canine model of coronary thrombosis, a clinically relevant model of myocardial infarction. Compared to FREE SK (68 ± 7 min), MESK significantly reduced the time to achieve sustained reperfusion of the occluded artery (29 ± 4 min). In addition, MESK-treatment resulted in substantial reductions in infarct size, residual clot mass, and bleeding complications.

MESK accelerates thrombolysis and the restoration of blood flow compared to identical dosages of FREE SK while also reducing systemic fibrinogenolysis and blood loss. Accelerated clot digestion is achieved by distributed intraclot thrombolysis following greater spatial distribution of the plasminogen activator. Microencapsulation may produce an improved dosage form for restoring arterial blood flow and reducing bleeding complications with thrombolytic therapy.

DEVELOPMENT AND CHARACTERIZATION OF ENCAPSULATED THROMBOLYTIC FORMULATIONS TO ENHANCE THROMBOLYSIS

CHAPTER 1

INTRODUCTION

Cardiovascular disease continues to be the primary cause of death in the Western world. Accounting for nearly 950,000 deaths (40% of total mortality) and $352 billion in medical expenditures annually, cardiovascular disease has a profound effect on all races and genders [1]. Although cardiovascular disease has a greater effect on the older generation, an increasing trend in younger victims of such crises is now being reported. CVD ends more lives each year than the next 5 leading causes of death combined; more than cancer, chronic lower respiratory diseases, accidents, diabetes mellitus, and influenza and pneumonia. Given the number of overweight Americans and the size of the aging population, CVD is likely to be a significant problem for years to come [2].

When considering the multiple causes of death within CVD, it is clear that coronary heart disease is a major player. More than one-half of all deaths attributed to CVD can be linked to coronary heart disease, with another 18% due to stroke. As a large percentage of events related to coronary heart disease and stroke are ischemic in nature, improved therapies to digest occlusive clots must be pursued in order to upgrade the prognosis of afflicted patients.

Effective reperfusion therapy is required to restore vessel patency quickly and reperfuse tissue once patients realize symptoms of occlusive crises. Reperfusion therapy is primarily composed of three methods: 1) thrombolytic therapy to digest the thrombus and restore blood flow; 2) percutaneous transluminal coronary angioplasty (PTCA) to push back atherosclerotic plaque that is limiting the cross-sectional area available for blood flow; and 3) stent placement to assure that the vessel remains open [3]. The use of each treatment modality requires various levels of clinical screening, trained personnel, and equipment.

Thrombolytic therapy is available as a means to dissolve the occlusive thrombus and achieve patency. Prior to infusion, the patient must be cleared of contraindications for plasminogen activators. For stroke victims, the type of stroke must be verified as ischemic in nature (and not hemorrhagic which is a contraindication for thrombolysis) by MRI or CT scan. Other contraindications include age over 75 years, recent surgery that might be complicated due to the potential for bleeding, and presentation for treatment after the recommended 3-6 or 6-12 hour windows for stroke or myocardial infarction, respectively. If the patient does not present any contraindications, the agent can be administered intravenously by personnel trained in initiating and monitoring intravenous

fluids. In contrast, angioplasty and stent placement require numerous specialized persons such as cardiologists, radiologists, nurses, and technicians to operate the equipment.

The benefits of thrombolytic therapy compared to angioplasty have been explored [4, 5]. Reviews of large trials have demonstrated an improvement in mortality, infarct size, and left ventricular function when patients receive angioplasty instead of thrombolytic therapy soon after symptoms arise. Other studies have examined the cost-to-benefit ratio of each therapy and found comparable results [6, 7]. Despite the reported benefit, only 20% of American clinics are currently outfitted with the necessary resources to provide angioplasty to a patient presenting with symptoms of an occlusive thrombus [7]. While it is thought that thrombolytic therapy and angioplasty can work effectively in concert, the consensus among physicians is that both therapies are effective and, barring contraindications, the therapy most readily available should be administered in order to restore blood flow quickly.

Thrombolytic therapy is characterized by the administration of medicinal agents, commonly referred to as "clot-busting" drugs, which initiate a cascade of events within the patient's own circulatory system to dissolve the thrombus and restore blood flow [8]. Two types of plasminogen activators are available: 1) indirect activators {streptokinase and staphylokinase} and 2) direct activators {tissue-type plasminogen activators (t-PA) and its numerous genetic variants, bat plasminogen activator, and urokinase}. While direct activators are potent, stand alone agents, indirect activators must first complex with plasminogen, a circulating plasma protein, and the conjugate actually serves as the plasminogen activator. The protein-plasminogen complex then combines with and

cleaves plasminogen, resulting in a protease known as plasmin. Plasmin can, in turn, digest the fibrin strands that provide structural integrity for the thrombus.

Successful thrombolytic therapy must overcome physiologic obstacles once infused for conversion of plasminogen to plasmin and subsequent clot digestion. First, plasminogen activator inhibitors such as PAI-1 act to neutralize the concentration of clot-busting drug which is present in the circulation. Additionally, circulating antibodies could potentially be present that could neutralize or deactivate a plasminogen activator of bacterial origin such as streptokinase or staphylokinase. Between 10 and 20% of the American population has reported substantial antibody titers to streptococcal or staphylococcal bacteria that could significantly affect the success of streptokinase or staphylokinase for thrombolytic therapy [9]. The three largest studies examining the effect of antibody titers on the success or thrombolytic therapy with streptokinase do not agree on the significance of high titers [10-12], with some reporting inhibition and others reporting no effect. Finally, once plasmin is formed from the cleavage of plasminogen, it is rapidly neutralized in the circulation by α_2-antiplasmin. The enzyme remains briefly with a reported half-life of 0.1 s in the circulation but 2 orders of magnitude greater when bound to fibrin [13].

Thrombolytic therapy has several limitations including its effect on numerous other circulating species. For example, substantial depletions of plasma proteins such as plasminogen (the driving force for thrombolytic therapy) and fibrinogen (which plays an integral role in the clotting process) have been routinely reported when utilizing plasminogen activators [14-17]. The reduction in plasminogen occurs primarily due to the conversion of plasminogen to plasmin. Additionally, clot-bound plasminogen

migrates into the free circulation due to the concentration gradient that is established during this process, a phenomenon described by Sobel et al. termed "plasminogen steal" [18]. Fibrinogenolysis (the breakdown of fibrinogen) has been reported by several investigators as well, which has resulted in increased bleeding tendencies for subjects receiving thrombolytic therapy [16, 17]. These issues must be considered to promote successful reperfusion therapy with plasminogen activators.

Despite the clear benefits that can be achieved, thrombolytic therapy is dramatically underutilized with less than 75% of eligible AMI patients and only 5-7% of eligible stroke patients receiving thrombolytic therapy [19, 20]. Possible reasons for underutilization include physician preference, patient contraindications, seeking treatment outside of the accepted treatment window, less than optimal results, the potential for bleeding complications, and cost. Factors such as physician preference and patient arrival at the emergency room can be addressed through education, public awareness, and instilling a sense of urgency to seek treatment upon symptom presentation. Other issues such as efficacy and bleeding complications are targeted for improvement by the administration of encapsulated plasminogen activators. For the 40-60% of patients who do not achieve vascular patency after thrombolytic therapy [21], additional reperfusion therapy must be administered such as angioplasty or, in extreme cases, coronary artery bypass graft surgery.

Since the first *in vivo* study reporting accelerated thrombolysis with encapsulated streptokinase in 1990 [22], other reports have demonstrated improvements in multiple endpoints when using encapsulated plasminogen activators compared to identical dosages of nonencapsulated agents. Laboratories in China [23], the Netherlands [24], and the

United States [25] have each reported enhanced clot lysis *in vitro* and *in vivo* with encapsulated plasminogen activators (Chapter 2). However, each of these studies has utilized the liposome, an aggregate of biological molecules formed of phospholipids. Although the results have been promising, liposomes possess limited stability and often require a lengthy preparation time [26]. For these reasons, this study has turned its focus towards the development of a more stable formulation of encapsulated plasminogen activator that provides similar or improved benefit to that observed with liposomal encapsulated agents.

The purpose of this research is to determine if the administration of a plasminogen activator encapsulated in polymer microcapsules could provide similar improvement in thrombolysis as seen with liposomal encapsulated plasminogen activators while possessing improved stability and hence, extended shelf-life. This study characterizes the ability of an encapsulated plasminogen activator to digest occlusive thrombi both *in vivo* and *in vitro* while exploring the mechanism of its success.

Previous studies examining the benefit of liposomal encapsulated plasminogen activators are reviewed in Chapter 2. Early work in this study utilizing liposomal encapsulated streptokinase (LESK) in a rabbit model of carotid thrombosis is described in Chapter 3. The first generation of microencapsulated streptokinase (MESK) was directly compared to identical dosages of LESK and free streptokinase (FREE SK) in a rabbit model to explore the efficacy of such a formulation.

In vitro characterization was initialized to clarify the mechanism of clot lysis with MESK. A simple in vitro system previously described by others [27, 28] was employed to examine encapsulated formulations' ability to digest thrombi formed of either whole

blood or human plasma while varying the applied pressure (Chapter 4). Microscopic techniques including light microscopy, real-time video of light microscopy, and laser scanning confocal microscopy were then used to explore the mechanism of clot lysis.

Lysis with both unencapsulated and encapsulated streptokinase has been approximated by a numerical model based on the convection-diffusion equation. A modified convection-diffusion equation has been utilized which includes numerous reactions as well as a Langmuirian-type isotherm to describe the adsorption phenomena. These results are described in Chapter 5.

Finally, the potential benefit of MESK was examined in a clinically relevant animal model. A canine model of coronary artery thrombosis was used to approximate the condition of acute myocardial infarction. Multiple endpoints were followed in the canine model including initial and sustained reperfusion times, residual clot mass, infarct mass, reocclusion episodes, and bleeding complications. These results are described in Chapter 6.

PREVIOUS ATTEMPTS TO ACHIEVE ENHANCED THROMBOLYSIS

CHAPTER 2

INTRODUCTION

As an alternative to invasive techniques such as angioplasty or bypass graft surgery, myocardial infarction and stroke can be treated medicinally with plasminogen activators. Commonly referred to as "clot-busting drugs", plasminogen activators initiate a cascade of events that result in the dissolution of the occlusive clot and the recanalization of the blood vessel. In order to be effective, the infused dosage must be significant enough to overwhelm various components within the circulatory system (i.e. plasminogen activator inhibitors, antibodies, α_2-antiplasmin), as well as compensate for the agent's short half-life. As with any agent, the large requisite dosage translates into potential side effects such as uncontrolled hemorrhaging. In order to fully understand the motivation for encapsulation, some background on plasminogen activators is provided.

BACKGROUND ON PLASMINOGEN ACTIVATORS

The most often-utilized plasminogen activators are tissue-type plasminogen activator and its genetic variants (e.g. alteplase, retavase, tenecteplase, lanoteplase), streptokinase, urokinase, and staphylokinase [29]. Tissue-type plasminogen activator (t-PA) and its variants are direct activators, converting plasminogen to plasmin without the assistance of another molecule. Streptokinase and staphylokinase, on the other hand, are classified as indirect activators. These two proteins must first combine with plasminogen, a plasma protein, to form a 1:1 molar ratio activator-plasminogen complex, which then becomes the activator.

The streptokinase-plasminogen complex, like the plasminogen activators listed above, is a serine protease that has the ability to cleave the Arg_{560}-Val_{561} peptide bond of plasminogen to form plasmin. Plasmin, in turn, degrades large proteins such as fibrinogen and fibrin. The degradation of fibrin results in the breakdown of thrombi (Figure 2.1) and the restoration of blood flow to oxygen-deprived tissues. Derived from streptococcal cultures, streptokinase is one of the smaller plasminogen activators with a molecular weight of 47 kDa and is cleared rather quickly from plasma (approximately 16 minutes by antibodies; 90 minutes by protein clearance). Owing to its lack of fibrin specificity, streptokinase activates circulating and clot-bound plasminogen indiscriminately. Fibrin specificity can be defined as the preference of a plasminogen activator to activate fibrin-bound plasminogen (such as with tPA) over circulating plasminogen. Reduced fibrin specificity has been implicated as a cause of potential bleeding complications. Plasmin formed in circulating blood is rapidly neutralized by α_2-antiplasmin (~0.1 s) and is therefore unavailable for clot lysis [8]. Plasmin that remains

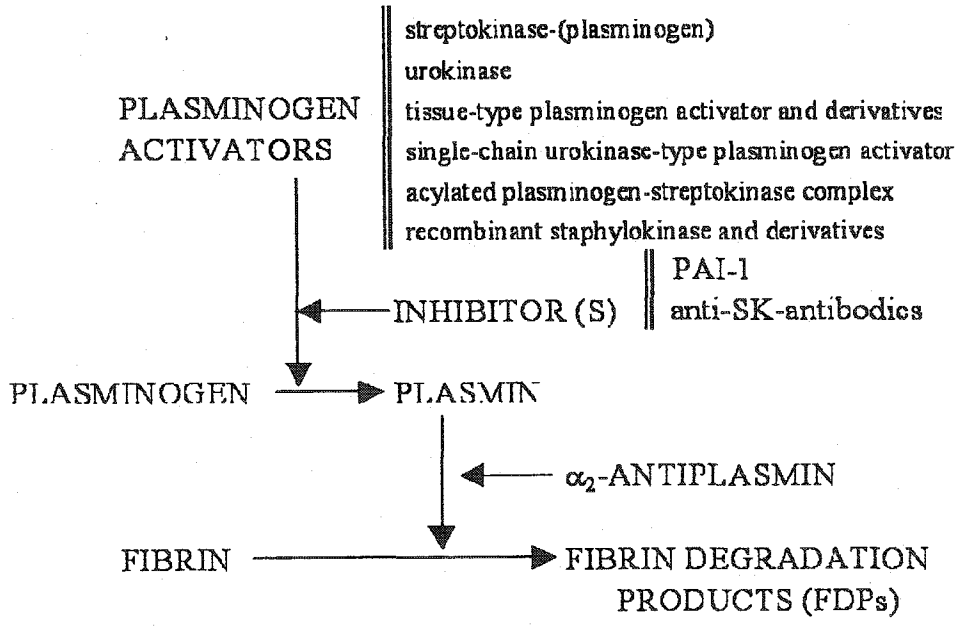

Figure 2.1: Mechanism of fibrin degradation with plasminogen activators [adapted from 29]

after the inhibitor is exhausted can degrade other plasma proteins such as fibrinogen, which can result in hemorrhaging. Plasminogen activators are known to deplete the concentration of multiple plasma proteins over the course of their existence [14, 15]. Use of streptokinase or staphylokinase also initiates the formation of antibodies due to their derivation from bacterial cultures. When infused, the anti-streptokinase titer rises to nearly 100 times the preinfusion level and has been reported to sustain such elevation for months or even years [30].

The transport of plasminogen activators into the thrombus has been shown both experimentally and analytically to play a significant role in clot digestion [31]. Magnetic resonance imaging studies by Blinc et al. [32, 33], along with light microscopy studies by Diamond et al. [28, 34], have clearly shown the effect of convection in addition to diffusion alone of these agents. While transport of the drugs by diffusion thoroughly and completely digests the thrombus, lysis is much slower than that observed in the physiological setting. In contrast, permeation of the thrombus by plasminogen activators digests the clot much more rapidly but incompletely compared to diffusion alone. Convection forces the plasminogen activator into the middle of the thrombus due to higher flow velocities in the center and is further encouraged by a reduced pressure drop over the length of the clot. Increased transport of the agent into the center results in residual thrombus on the vessel walls not observed with diffusion alone. Attempts have been made to analytically describe the phenomenon of clot lysis and explore the mechanisms of incomplete lysis [35, 36].

Efforts to improve upon plasminogen activators have focused primarily on two fronts. The development of new agents is one obvious method. By varying the protein

structure of current agents, new drugs can be developed that have improved characteristics such as longer half-life, increased fibrin-specificity, or more convenient infusion capabilities. Recombinant technology has allowed for the production of these proteins to become economically feasible. However, current cost estimates for drug development of new compounds are placed at more than $850 million over a 10-12 year period (Tufts Center for the Study of Drug Development, 2003). The risk and high cost of development limits the discovery and availability of new agents.

A second possibility for improvement of plasminogen activators is to consider the delivery of the agent to the clot. Intrathrombic delivery of plasminogen activators has been recently reviewed [37]. It was anticipated that delivery of the fibrinolytic agent directly to the clot would limit its interaction with plasma proteins, thereby promoting improved thrombolysis. However, the experimental benefit achieved did not merit the specialized personnel and equipment that would be required compared to an intravenous infusion of the same agent. Additionally, direct infusion of plasmin has been explored as a means to limit the difficulties observed due to the short half-lives of plasminogen activators. Intraaortic infusion of plasmin demonstrated benefit in a rabbit ear puncture re-bleed model by providing comparable thrombus digestion without hemorrhaging versus t-PA which resulted in bleeding in 9 of 10 animals [38]. Coincident use of ultrasound [39], concomitant administration of plasminogen [40, 41] and pulse-spray thrombolysis [42] have each shown a potential for enhancing thrombolysis. Despite promising preliminary results, each method has drawbacks such as the requirement of specialized procedures or an increased potential to form an embolus from the macerated thrombotic occlusion.

Chemical modification of plasminogen activators has been investigated as a means to improve thrombolysis without developing new pharmaceutical agents. "Pegylation" of proteins has proven an effective technique to extend their brief half-lives when administered intravenously. Dramatic improvements have been observed by covalently linking polyethylene glycol to insulin, interferons, interleukins, tumor necrosis factor, superoxide dismutase, and others [43]. In addition, t-PA [44], urokinase [45], and streptokinase [46] have each been pegylated. While extended circulation times were reported for each study, thrombolytic efficacy was not adequately explored.

Recently, targeted delivery of streptokinase has been attempted by means of linking an anti-fibrin antibody to inactivated thrombin, then coupling to a hirulog-streptokinase fusion protein possessing lytic activity [47]. Streptokinase is released upon the arrival of hirudin which separates the hirulog from the inactivated thrombin bound to the anti-fibrin antibody, and is now free to attack the components of the thrombus while sparing the circulating clotting factors. Similar studies have also been reported with urokinase [48]. This approach is designed to limit hemorrhaging but has yet to be reported as a successful thrombolytic complex in vivo.

Streptokinase is often underutilized because of its lack of fibrin specificity compared to agents such as t-PA. There is some evidence that the low affinity for fibrin contributes to the potential for bleeding. Recombinant modification has demonstrated the potential for improving the fibrin specificity of streptokinase [49]. Reed et al. removed the 59 amino-terminal residues on streptokinase to form a plasminogen activator that was fibrin specific and no longer capable of fibrin-independent plasminogen activation. The thrombolytic superiority of the mutant streptokinase was verified when comparing

recombinant streptokinase containing the NH_2 residues by the digestion of plasma clots. At the end of a six-hour exposure to the thrombi, amino-deleted streptokinase digested approximately 80% of the thrombus compared to only 50% with the full recombinant streptokinase. In addition, fibrinogen levels remained much higher with the deletion-type streptokinase versus unmodified streptokinase (10% degradation versus 97%, respectively), suggesting the reduced potential for bleeding complications with this technique.

While chemical modification of currently used agents has shown potential, physical association of the drugs for delivery in acute crises has not received much attention. Despite the many avenues pursued to improve fibrinolysis, few studies have examined the efficacy of encapsulating plasminogen activators in a vehicle. The vehicle should shield the protein for a period of time, thereby lessening the susceptibility of proteinaceous drugs to inhibition by circulating plasma proteins, premature activation, systemic removal by antibodies, or simple breakdown due to the body's harsh environment. While in the vehicle, active agents should remain unrecognizable to inhibitors, antibodies, and the environment. Encapsulation also could effectively increase the half-life of an agent by extending its circulation time in the body, as the short half-life plays a role in the reduction of total protein activity.

PREVIOUS USE OF ENCAPSULATION

Encapsulation of drugs is not a novel idea. In fact, many compounds have been encapsulated in a number of vehicles over the last three decades. Drugs are encapsulated

for a variety of reasons, most commonly to provide a prolonged, steady release rate and to limit the side effects that can result from these agents [50].

Drugs can be administered in a variety of modes other than the conventional oral route. The delivery of an active agent has a profound effect on its potential benefits, such as improved patient compliance and decreased dosage. For example, drugs utilized for chemotherapy have been trapped in polymer wafers and then implanted in the brain as a follow-up to surgical tumor removal [50]. By placing the wafers at the site of tumor removal, a reduction in total dosage of the chemotherapy agents is possible, thereby resulting in fewer side effects.

In contrast to controlled delivery's conventional goal of steady, sustained administration, this study is focused on the use of encapsulation to improve the properties of plasminogen activators. Tissue begins to suffer almost immediately following oxygen deprivation, and therefore, restoration of flow must be achieved quickly. New delivery methods of clot-busting drugs should be considered in order to better the survival rate of victims and improve upon the drawbacks of plasminogen activators.

As mentioned previously, encapsulation of thrombolytic agents has demonstrated dramatic improvements over free plasminogen activators by reducing the time required to digest a clot as well as increase the percentage of the clot that is digested (Table 2.1). Early studies at the University of Oklahoma were the first to report accelerated thrombolysis in vivo using an encapsulated plasminogen activator [22]. Multiple agents have shown benefits using two animal models [22-25]. Each study pertaining to liposomal encapsulated agents has proposed various hypotheses to explain how

	Reperfusion Time (min)	Reperfusion Time (min)	Percent lysis (%)	Percent lysis (%)
Animal model	canine	canine	rabbit	rabbit
Agent	streptokinase	urokinase	streptokinase	t-PA
Free agent	78 ± 43	69 ± 7	36.3 ± 3.4	26.3 ± 3.8
Encapsulated agent	32 ± 28	28 ± 7	47.4 ± 1.4	34.8 ± 3.6
Reference	22	23	24	25

Table 2.1: Previous in vivo studies utilizing liposomal encapsulated plasminogen activators (mean ± standard deviation).

encapsulation promotes thrombolysis. Nguyen et al. suggested that the liposomes rupture due to the shear stress on the vesicles that occurs when they arrive at and begin to pass the clot [22]. The liposomes then release their payload and lysis begins. Frézard states that "fluid" liposomes, such as those made from dioleoylphosphatidylcholine in the study of Nguyen et al., disintegrate and release their contents within a few minutes after their intravenous administration [51]. This is due in large part to the transference of the lipid molecule from the liposomal membrane to plasma high density lipoproteins. It is also likely that the liposomes protect the enzyme from components in the plasma that cause degradation or premature activation. Another study has shown that plasminogen binds to the surface of the liposome, leading the investigators to suggest that plasminogen acts as a "homing device" for the clot [52]. The plasminogen would then reduce the free circulation of the liposome and attract the encapsulated thrombolytic agent to the clot.

Encapsulation of streptokinase in liposomes has been shown to significantly prolong the systemic delivery of this agent [53]. Liposomes composed of

distearoylphosphatidylcholine and polyethylene glycol (PEG) increased the half-life and average systemic concentration by 16.3- and 6.1-fold, respectively, compared to streptokinase alone. However, it is not clear if increases in circulation times are due to the liposomes themselves or the addition of PEG to the liposomal membranes. As will be discussed in the next chapter, PEG resists plasma protein adsorption that would likely limit opsonization and removal from the circulation by the reticuloendothelial system. PEG has been successfully added to other liposomes to demonstrate extended circulation times [54].

Despite the clear enhancement of fibrinolysis achieved with liposomal encapsulation, liposomes are known to have limited stability in the aqueous state [22, 55], severely limiting the commercial potential of this system. While progress towards improved stability and encapsulation efficiency using liposomes has been reported [56, 57], other vehicles should be investigated. Therefore, efforts in our laboratory are underway to develop a second-generation vehicle which maintains the benefits observed with liposomal encapsulated thrombolytic formulations and yet avoids the limited stability observed with vesicles. A solid dosage form would be desirable in order to optimize and control shelf life. The development of a polymeric encapsulated thrombolytic formulation has been explored and is described in the forthcoming chapters.

ASSESSING THE POTENTIAL EFFICACY OF ENCAPSULATED STREPTOKINASE IN A RABBIT MODEL OF CAROTID ARTERY THROMBOSIS

CHAPTER 3

This chapter has been submitted in large part for publication.

INTRODUCTION

Despite the success achieved with intravenously administered thrombolytic agents, plasminogen activators suffer from several limitations that hamper systemic administration. As discussed in Chapter 2, encapsulation has been examined in various models as a means to enhance thrombolysis. Although the direct mechanism for improved lysis with liposomal encapsulated agents has only been theorized, the general consensus is that encapsulation would protect the drug from premature removal/ deactivation by components of the circulation such as antibodies and PAI-1. Protection from premature degradation would effectively extend the agent's half-life and allow for a greater drug dosage to initiate clot digestion. Canine coronary and rabbit jugular models of thrombosis have been successfully implemented to demonstrate the enhanced thrombolytic capability of liposomal encapsulated plasminogen activators compared to identical dosages of free activator. However, the potential of these formulations to treat

cerebral ischemia has not been examined. To that end, a rabbit model of carotid thrombosis has been implemented as an approximation of stroke to explore the potential efficacy of encapsulated streptokinase for treatment of cerebral ischemia.

Liposome preparation by most techniques is a lengthy process (approximately 4-7 days), and these vehicles lack stability in their aqueous form for periods greater than 48 hours [22, 55]. Depending on particle size, a large percentage of vesicles are susceptible to the first-pass phenomenon in which they are cleared from the circulation by components of the reticuloendothelial system (RES) such as the liver, spleen, and bone marrow following opsonization by plasma proteins [58, 59]. These difficulties have reduced commercial appeal for these agents despite the accumulating evidence for their efficacy. Therefore, we have sought to develop a formulation of encapsulated streptokinase that demonstrates comparable benefit to that observed with liposomal encapsulated streptokinase but with improved storage potential, which would likely translate into improved commercial viability.

Although there are several techniques to prepare liposomes including the freeze-thaw and extrusion methods, the detergent removal method is a popular and reproducible approach [51, 60, 61]. Liposomes are prepared by taking advantage of differences in hydrophobicity with various components. Vesicles are formed from phospholipids that are composed of a long chain hydrophobic fatty acid and a hydrophilic polar head group. When placed in an aqueous environment, a bilayer is formed as the hydrophobic tails arrange themselves to be in close proximity, while the hydrophilic heads point toward the aqueous environment (Figure 3.1). In this manner, dissolved proteins such as streptokinase can be entrapped within the confines of the phospholipid bilayer, oftentimes

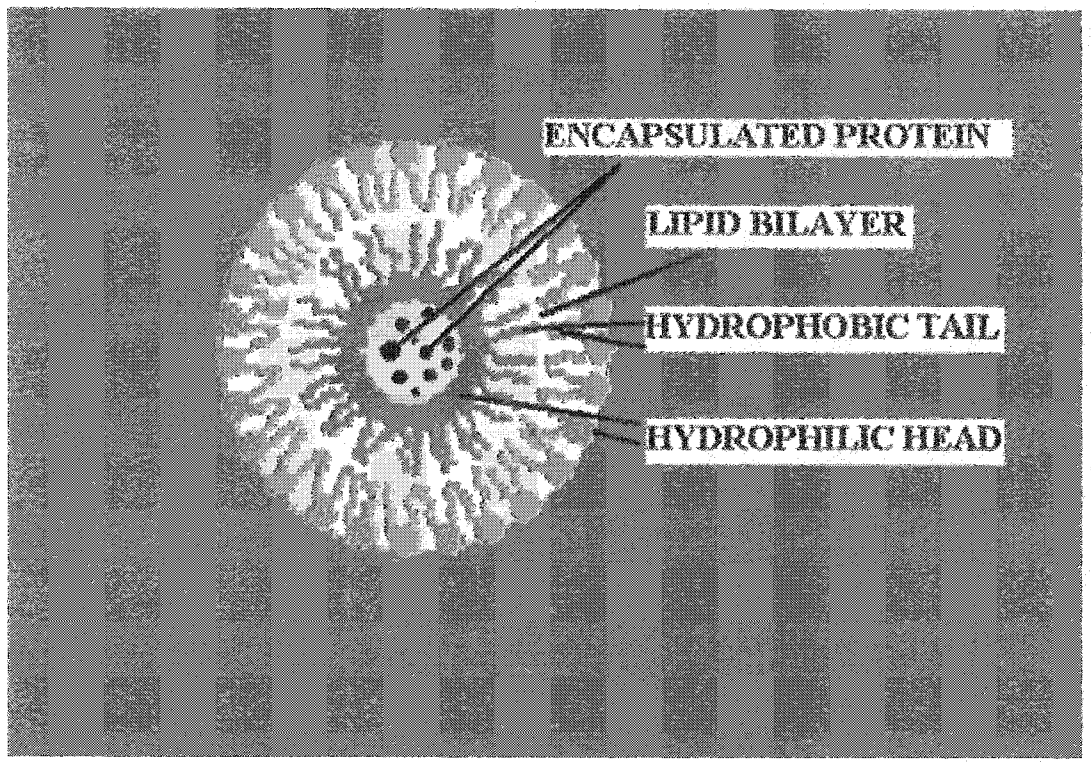

Figure 3.1: Schematic of liposome

resulting in spherical vesicles. For the detergent removal method, lipids are first solubilized with an aqueous solution of the detergent that also contains the protein(s) to be encapsulated. The detergent must have a high critical micelle concentration (CMC) so that it is easily removed, for instance by dialysis.

The fatty acids that make up the phospholipid bilayer are susceptible to oxidation after approximately 48 hours [26, 51], thereby compromising the bilayer's continuity and allowing the unpredictable release of the entrapped agent. This severely limits the ability to adequately assess the amount of encapsulated agent at times past 48 hours. For a liposomal encapsulated thrombolytic formulation to be usable in a widespread manner, clinicians would have to predict the need for such a formulation as anything that was prepared more than 48 hours previously would likely need to be discarded. The tremendous amount of wasted drug that would result from this scenario has certainly dampened the movement of such technology to the clinics.

Other vehicles or formulation processes must be considered to address this issue. While other laboratories are focusing on methods to improve encapsulation efficiency and storage characteristics of the liposomal formulations [56, 57], we have considered the use of other materials and processes to entrap streptokinase. Polymeric formulations would seem to be ideal for this application with their ability to be lyophilized into a dry powder, thereby providing extended storage possibilities compared to aqueous liposomal formulations.

Microencapsulation of proteins has been pursued for a number of years with significant success when entrapping various proteins such as insulin, superoxide dismutase, and even cellular material such as islet cells [62-64]. Traditional techniques

for microencapsulation seek to extend the release of the entrapped material for extended periods as in weeks to months. While there are a number of techniques to encapsulate proteins in microparticles, the double emulsion technique is a relatively simple method that has been successful for a number of proteins. Additionally, this formulation process affords many preparation steps that can be modified in order to affect particle diameter, size distribution, release rate, and porosity [65].

Release rate can be affected by a number of factors including the polymer molecular weight, the presence of cross-linking molecules, porosity, and particle size [65]. Increasing the molecular weight will prolong the degradation of the polymer, as will the presence of crosslinking moieties such as dialdehydes [66, 67]. Particles with greater porosity provide more channels for the invasion of fluid which promotes the degradation of water-soluble material. Lastly, smaller particles dissolve faster than larger particles as microparticulate degradation is dependent on the ratio of surface area to volume.

When selecting a vehicle for a specific application, one must first look at the requisite properties of the desired medium. For this particular application, it is desired to make use of a polymer that has a reasonable shelf life (> 3 months). Restrictions common to all biomaterials apply; it should be water-soluble, biodegradable, biocompatible, nontoxic, as well as dissolve rather quickly. After some preliminary investigation, microparticles were formed of polyethylene glycol with molecular weights of 10,000 (rabbit studies) and 20,000 (in vitro and canine studies).

Polyethylene glycol (PEG) has been shown to have a number of interesting properties that could result in potential benefits for this application [68]. Firstly, the

polymer has a high solubility in water that results in a short dissolving time. It is unusual in the group of water-soluble polymers in that it is soluble in a variety of organic solvents as well such as chloroform, methylene chloride and ethanol. The immobilization of PEG to other polymer surfaces greatly reduces protein adhesion. Lastly, covalent coupling of PEG to proteins decreases their immunogenicity and increases their half-life in plasma [69].

For the application of encapsulating streptokinase in a microparticulate system, the goal is not to delay release of the drug for long periods of time, but to delay the release for only enough time to allow the particles to reach the thrombus. As PEG offers a number of advantages for a polymeric material in contact with blood, the feasibility of microencapsulated streptokinase formed of PEG has been explored and directly compared to identical dosages of free and liposomal encapsulated streptokinase using a rabbit model of carotid thrombosis [70].

MATERIALS AND METHODS

Preparation of LESK

Unilamellar phospholipid vesicles were prepared through the detergent removal method [22, 26]. Vesicles were prepared in the Department of Medical Biochemistry and Genetics at Texas A&M University, College Station, TX. Briefly, POPC (160 mg; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphotidylcholine; Avanti Polar-Lipids) was dried under vacuum using a centrifugal evaporator, together with 0.1 μCi of 1,2-di[1-^{14}C]-oleoyl-L-3-phosphatidylcholine (Amersham Life Science) added for tracing purposes, and then resuspended in 2 mL of 1 mol/L OG (n-octyl-β-D-gluco-pyranoside; Sigma

Chemical) in HBS [HBS:50 mmol/L HEPES (pH 7.5), 100 mmol/L NaCl] at 37°C for 2 hr with occasional mixing.

SK (80,000 IU; Behringwerke AG) was dissolved in the resuspended POPC, and then OG was removed by dialysis against 4 L of HBS (6 changes, 12 hours/change) at 4°C. The resulting LESK was purified from unencapsulated SK by gel filtration in HBS at 4°C through a Sepharose CL-6B column (7 mm i.d. x 500 mm). LESK eluted in the void volume, as evidenced by the coincident detection of protein by absorbance at 280 nm and of phospholipid by radioactivity in the 1.2-mL fractions. Particle sizes averaged 339 ± 154 nm in diameter as determined by a NIDEC 270 Submicron Particle Sizer. The preparation process required 4 days, and the vesicles were shipped by overnight express mail for use the next day in the Animal Resources facility at the University of Oklahoma Health Sciences Center, Oklahoma City, OK.

Preparation of MESK

SK was entrapped in microparticles through a modified double emulsion method as illustrated in Figure 3.2 [62, 71]. PEG (1 gm of 10,000 MW, Sigma Chemical) was dissolved at room temperature in 1.3 mL chloroform (Fisher Scientific) and SK (40,000 IU; Behringwerke AG) was dissolved separately in 250 μL of distilled water, also at room temperature. The two solutions were mixed and sonicated for 45 s at 40-50 watts (Sonic Dismembrator 550, Fisher Scientific). SK activity was not altered by sonication at this intensity and duration. The protein/polymer solution was mixed with 1 mL of 2% (w/v) polyvinyl alcohol (PVA; average MW 30,000-70,000; Sigma Chemical) and vortexed for 20 s. A small volume (250 μl) of 25% (w/v) glutaraldehyde (Electron

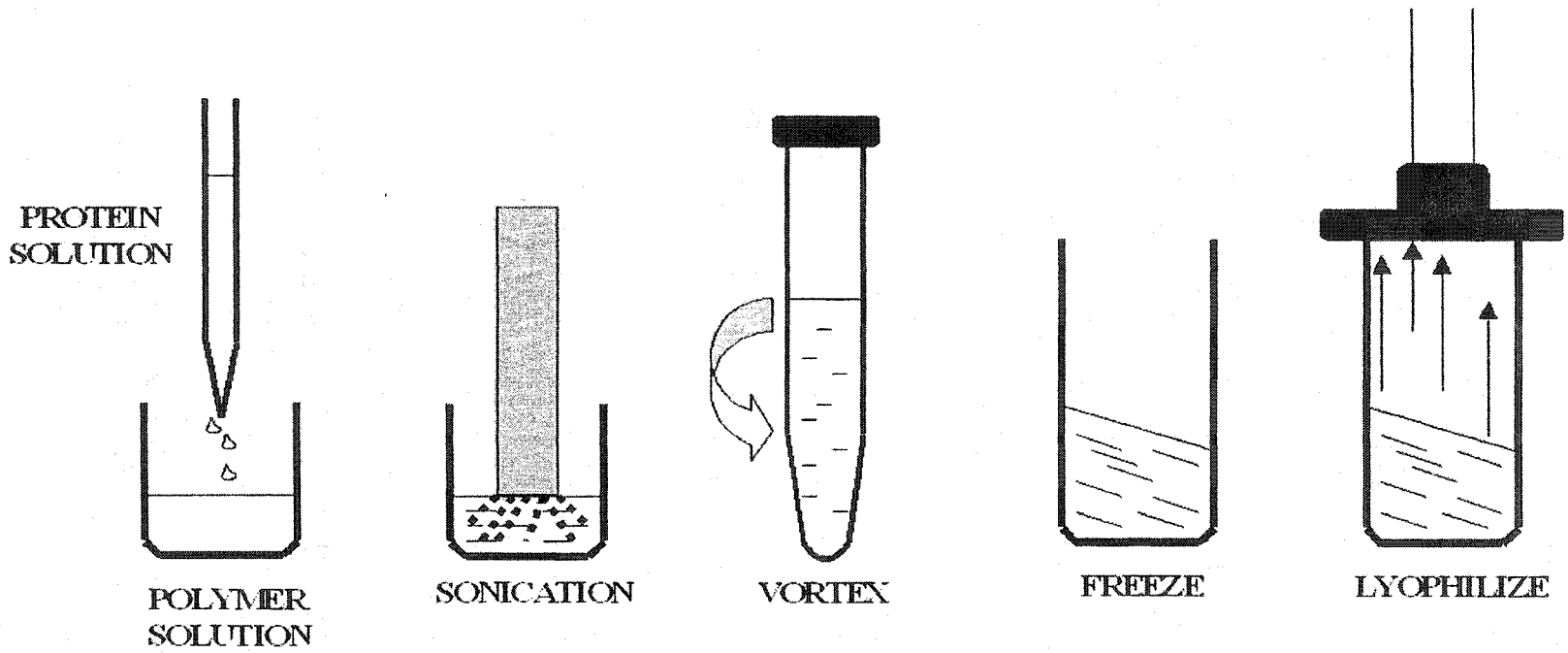

Figure 3.2: Schematic of modified double emulsion method.

Microscopy Sciences) was added to promote crosslinking and extend particle dissolution time, a technique used to slow release rates from both polymer [66, 67] and protein [72] microparticles. SK activity was not significantly influenced by this concentration of glutaraldehyde. The mixture was vortexed 20 s, and then placed in a –85°C freezer for 20 minutes. The mixture was lyophilized for at least 15 hours. After the water was extracted, the mixture was washed in hexane three times in a centrifuge at 1000 x *g* and 4°C for 5 min each and filtered under a vacuum in order to remove any externally bound protein. Final product composed of porous, mostly spherical particles with a number-averaged diameter of 810 ± 523 nm (mean ± SD; 90% < 1287 nm) was then stored at room temperature until use. An image of a representative microparticle is captured by scanning electron microscopy in Figure 3.3. Blank microparticles lacking entrapped SK were prepared by replacing the SK solution with an identical volume of distilled water and carrying out the preparation process as previously described.

Encapsulation Efficiency

SK content in the pooled LESK peak fractions was quantified with a commercially available chromogenic activity assay (Helena Laboratories) both in the presence and absence of OG (100 mmol/L final concentration) [22]. Octylglucoside was used to rupture the vesicles for quantitative determination of the encapsulated product. The activity of the released plasminogen activator from LESK after rupture was compared to standard curves of SK prepared in identical concentrations of octylglucoside.

SK content of the microparticles was quantified with the same activity assay. The

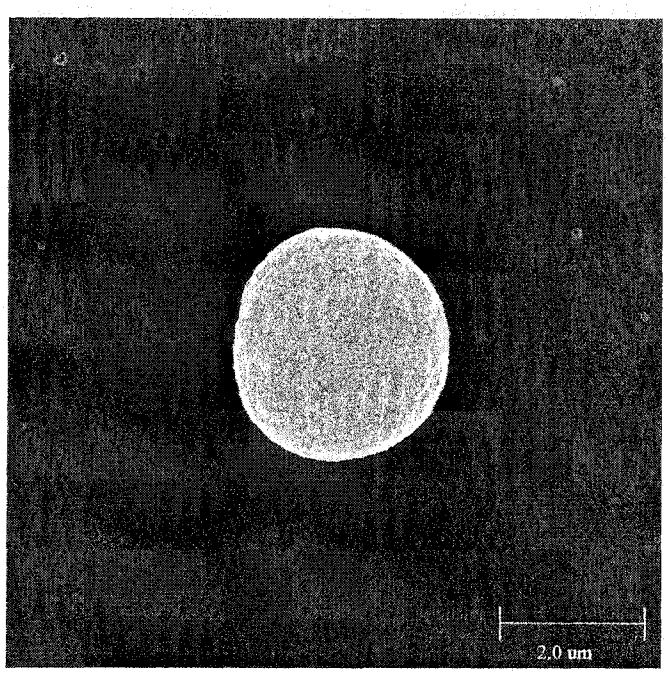

Figure 3.3: Scanning electron micrograph of a microparticle of encapsulated streptokinase as prepared with the modified double-emulsion technique.

microparticles were allowed to dissolve completely in distilled water for approximately 45 min. This process can be followed and confirmed by light scattering studies using a Coulter LS Particle Size Analyzer (Beckman Coulter). The activity of each sample was compared to that of a standard curve of SK in the presence of an identical concentration of completely dissolved blank microparticles.

Animal Model

Animals were treated under procedures that were examined and approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center, as well as the University of Oklahoma. The present study examined the

performance of four thrombolytic formulations (FREE SK, FREE SK + BLANK, LESK, and MESK) in vivo through the use of a rabbit model of carotid artery thrombosis [73] which was modified in our laboratory. New Zealand white rabbits of either sex weighing an average of 2.65 kg were anesthetized (5%) and maintained (2-3%) with inhaled isoflurane. Cardiac activity was monitored by ECG. The right carotid artery was isolated, as was the left jugular vein. The jugular vein was cannulated with PE-90 tubing, which was connected to a 20-mL syringe for administration of study formulations. Right carotid artery flow was followed with a 20-MHz pulsed Doppler probe cuff placed in direct contact with the vessel, a technique that measures the velocity of red blood cells passing through the artery. Autologous whole blood (0.1 ml) was withdrawn slowly over 10 s from the carotid artery into a glass syringe containing approximately 100 units thrombin (Sigma Chemical) before reinjection into a 5-10 mm long ensnared segment of proximal and distal right carotid artery. After 10 min, the proximal ligature was released, as was the distal ligature after an additional 5 min. Most vessels occluded with one or two thrombin injections. The thrombus was allowed to mature for 30 min prior to administration of any study formulation.

The relative dosages of the thrombolytic preparations were identical. A dosage of 6,000 IU/kg was determined as adequate to produce thrombolysis. The preparation was dispersed in normal saline to a total volume of 20 mL. An initial bolus of 20% preceded a constant intravenous infusion of the remaining formulation over a 30 min period. As a control, empty microparticles (1.5 gm) were mixed with 6,000 IU/kg of free streptokinase (FREE SK + BLANK; n=3) and administered via the jugular vein. Animals were observed for 2 h following the initiation of infusion. Reperfusion was documented by a

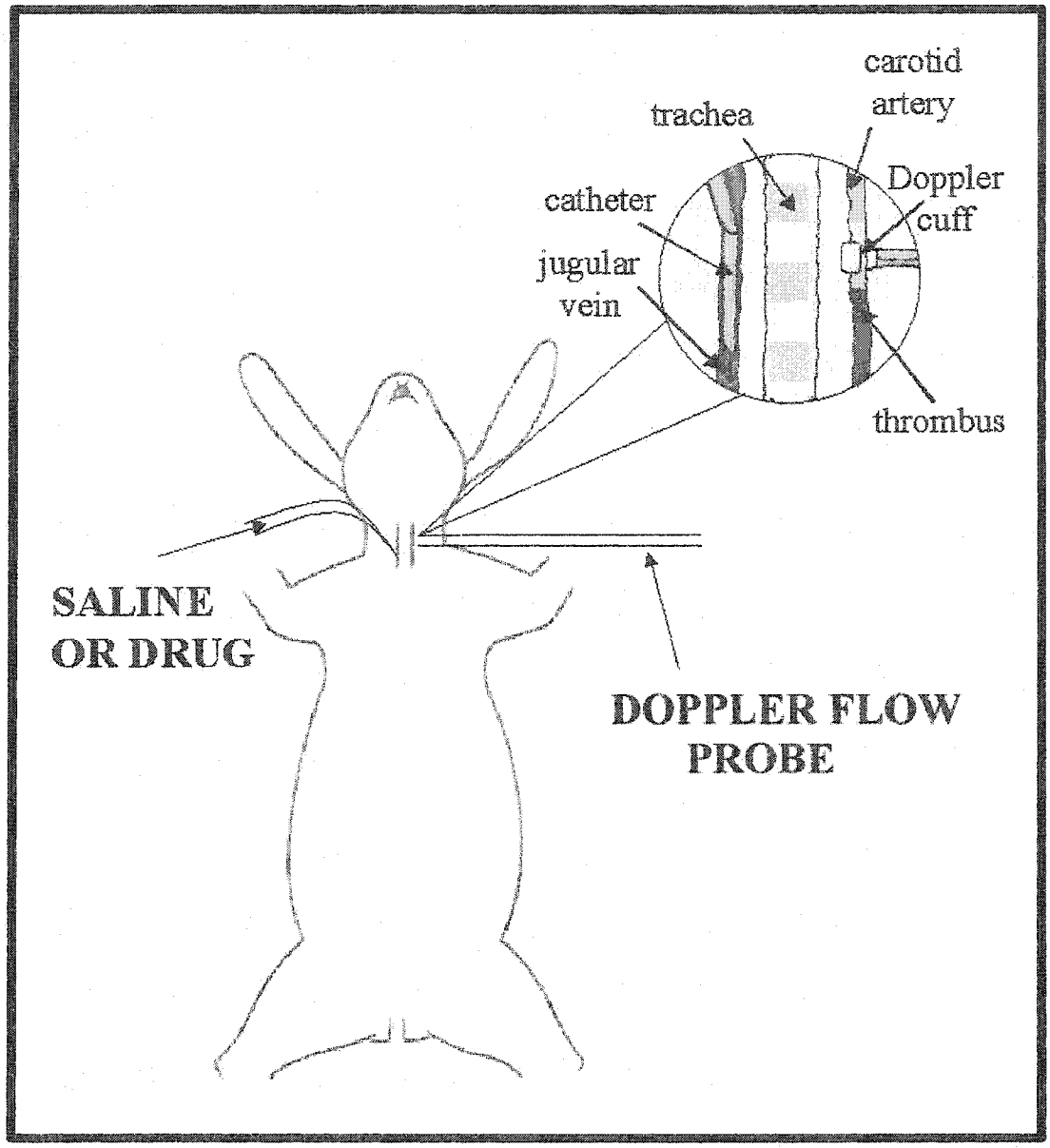

Figure 3.4: A rabbit model of carotid thrombosis was used to test thrombolytic efficacy of various free and encapsulated formulations of streptokinase.

recovery of right carotid arterial flow. Sustained reperfusion was defined as flow returning to at least 20% of the pre-thrombosis baseline flow for the remainder of the experiment without another occlusion event. At the conclusion of the experiment, the artery was dissected and residual thrombus mass determined gravimetrically.

STATISTICS

Using a nonparametric analysis of variance followed by an unpaired t-Test, p values < 0.05 were considered significant. Unless otherwise stated, all results are expressed as mean ± standard error of the mean.

RESULTS

Encapsulation Efficiency

The ability to encapsulate SK in a liposome varies greatly with the conditions of encapsulation and the method used. By employing the detergent removal method, an average of 30% of the initial SK was entrapped within the vesicles (150 IU SK/ mg of phospholipid). This incorporation rate compares well with other studies utilizing assorted techniques for liposomal entrapment of various plasminogen activators [22-26].

As with liposomal encapsulation, the efficiency of protein microencapsulation varies greatly with the reagents used, the conditions during preparation, and the techniques employed. At the conclusion of preparation, final product yield for the production of MESK (PEG + SK + PVA) was 82.0 ± 2.2% (n=7) by weight, while the encapsulation efficiency of viable SK was 29.0 ± 3.2% (11.1 ± 0.86 IU/ mg of PEG). On average, 1.47 ± 0.08 gm of MESK was infused for each rabbit.

Animal Model

The thrombolytic activity of LESK and of MESK was directly compared with FREE SK. The average time required for reperfusion with FREE SK (n=6) was 74.5 ± 16.9 min (Figure 3.4). Two animals treated with FREE SK did not achieve reperfusion and were given values of 121 min for the sake of statistics. In sharp contrast to FREE SK values, LESK (n=7) greatly reduced thrombolysis times to 19.3 ± 4.6 min ($p = 0.006$ vs. FREE SK), with one reperfusion observed as quickly as 8 minutes. MESK (n=6) reduced the time to restore blood flow even further to 7.3 ± 1.6 min ($p = 0.003$ vs. FREE SK). Admixing of FREE SK and blank microparticles (FREE SK + BLANK) resulted in comparable reperfusion times (54.7 ± 3.9 min; $p>0.05$; ns) to those FREE SK animals which reperfused (51.3 ± 10.6 min). As expected, an equal volume of saline alone (n=4) failed to result in reperfusion of the occluded carotid artery. A trend of decreasing residual clot mass can be observed with decreasing reperfusion time for each encapsulated formulation (Figure 3.4). Substantial differences in clot mass can be observed with both encapsulated formulations as compared to FREE SK alone. FREE SK + BLANK demonstrated similar clot masses to FREE SK alone.

In addition to the dramatic reduction in the time required to achieve reperfusion when utilizing both encapsulated streptokinase formulations, we also observed an increase in the percentage of baseline flow that was returned with LESK and MESK (Figure 3.6). Flow through the occluded carotid artery was monitored through the use of a 20-MHz pulsed Doppler flow probe. Baseline flows were determined prior to injection of thrombin, and readings were collected at various time points throughout the experiment. Determination of the percentage of returned blood flow was calculated by

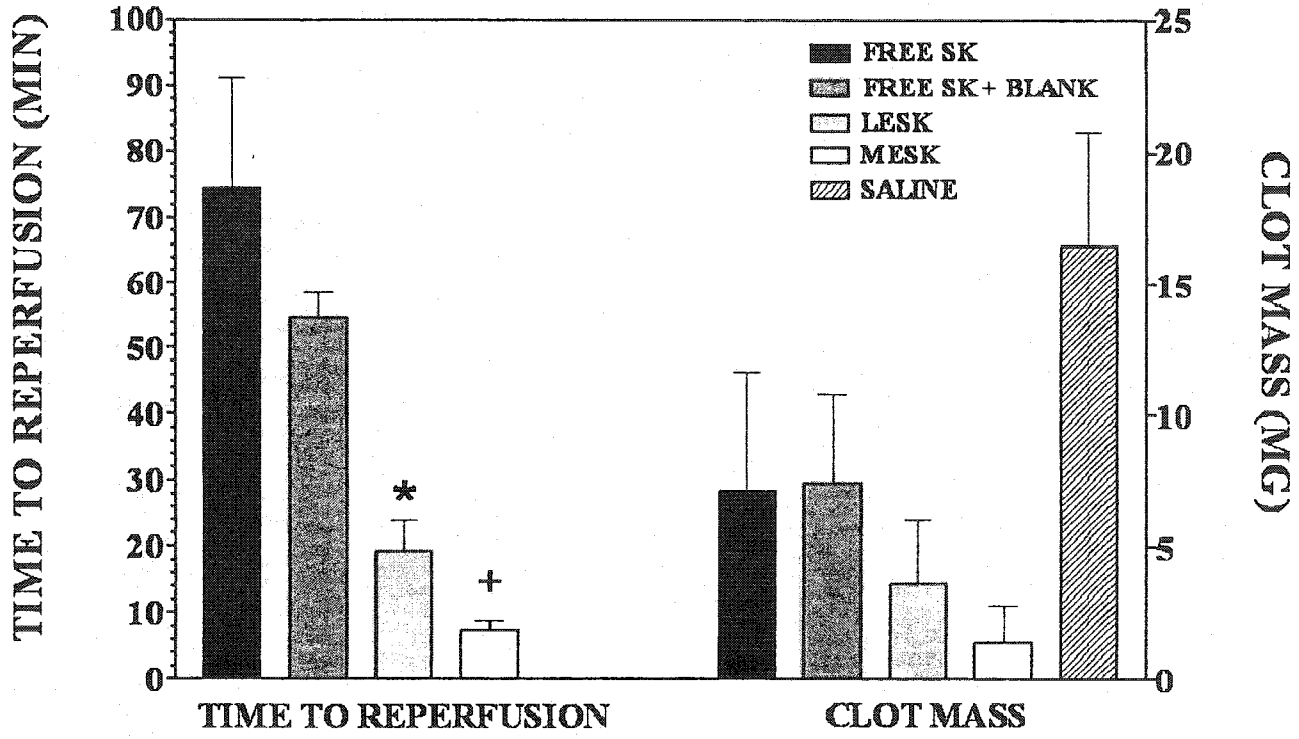

Figure 3.5. Examination of initial reperfusion time and residual clot mass in the rabbit. Reperfusion was not observed for saline after 120 min. Clot masses for all SK-treatment groups were significantly reduced versus saline but were not different from each other.

*** $p < 0.05$ vs. FREE SK; + $p < 0.05$ vs. LESK**

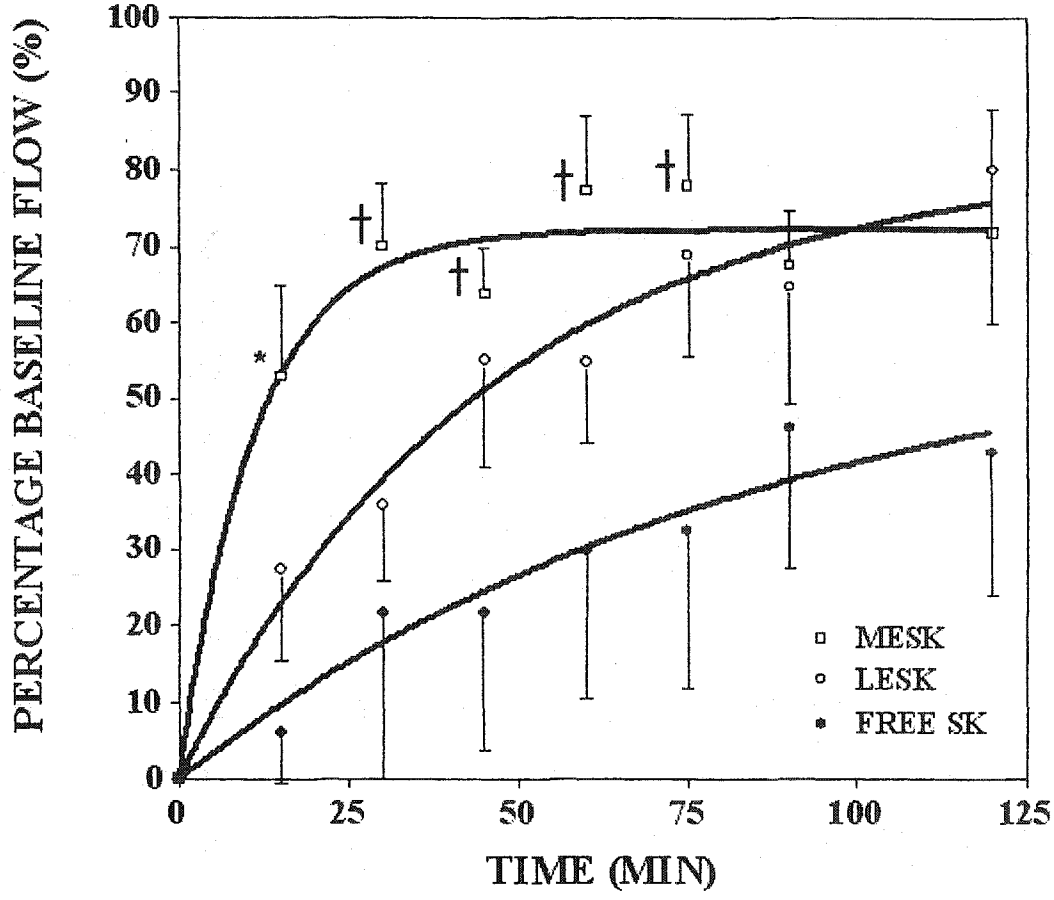

Figure 3.6. Percentage of returned arterial blood flow compared to baseline flow.

*** p < 0.05 vs. FREE SK: † p < 0.1 vs. FREE SK**

taking the ratio of the mean flow velocity at specified time points to the pre-thrombosis mean flow velocity. The best-fit lines through the data points are generated with a one-phase exponential model (FREE SK $R^2 = 0.952$; LESK $R^2 = 0.972$, MESK $R^2 = 0.969$).

As seen in Figure 3.5, a greater percentage of original baseline flow is returned earlier with both encapsulated formulations than with FREE SK. MESK seems to reach its optimum return of flow at approximately 30 minutes; the flow remains constant for MESK until the end of the experiment, while LESK and FREE SK exhibit a gradual increase in flow throughout this time period. At the conclusion of the experiment, both LESK and MESK resulted in a return of flow noticeably higher than that obtained with FREE SK. The substantial increase in end flow shows that encapsulation has a profound effect on the level of blood flow restoration, as well as on the time course of restoration.

For experiments in which encapsulated formulations were administered, an absence of reocclusion was documented. It is not uncommon to observe episodes of reocclusion following reperfusion with streptokinase. The mechanism of reocclusion is unknown but possible causes include thrombi traveling downstream, the release of various chemicals that cause restenosis due to spasm of the blood vessel, or the exposure of active surfaces that initiate platelet response. In rabbits treated with FREE SK, an average of 1.0 ± 0.69 reocclusion events occurred per rabbit within 2 h after initiation of treatment. Incidences of reocclusion were reduced with both encapsulated formulations, with LESK averaging 0.71 ± 0.29 events and MESK averaging 0.33 ± 0.21 episodes (p=ns vs. LESK or FREE SK) per rabbit. Occlusions treated with LESK and MESK tended to remain open after initial reperfusion, while the use of FREE SK was often

accompanied by multiple episodes of reocclusion and subsequent reperfusion. The infusion of FREE SK + BLANK resulted in 0.33 ± 0.24 reocclusions per animal.

Streptokinase administered intravenously is known to invoke a systemic lytic state. More specifically, the hemostatic plugs that seal preexisting sites of vascular injury are susceptible to clot lysis like the larger, occlusive blood clot. Streptokinase is also known to degrade the concentration of circulating fibrinogen, a plasma protein necessary for normal blood clotting processes [15, 16]. In animals receiving FREE SK, extensive bleeding from the injury sites was qualitatively observed compared to those animals receiving either LESK or MESK. Substantial bleeding was routinely observed from the thrombin injection site of the carotid artery, as well as the surgical incisions with rabbits receiving free streptokinase, while such bleeding was not evident in rabbits receiving the encapsulated formulations for treatment of thrombosis.

DISCUSSION

For victims of cardiovascular crises, it is imperative to receive treatment as soon as possible. Studies involving myocardium in dogs have shown that infarct mass increases with the duration of coronary artery occlusion preceding reperfusion [74]. Human clinical studies have shown that a treatment delay of only 43 minutes results in a larger infarct size and reduced left ventricular function [75-77]. Therefore, the prognosis for patients improves dramatically when achieving reperfusion soon after symptoms occur.

This study is directed towards reducing the time necessary to restore the flow of blood to the tissue once treatment is initiated. As illustrated in Figure 3.5, the infusion of

LESK and MESK resulted in significantly faster reperfusion times than were observed with FREE SK. In addition to the reduced time required to restore blood flow through the occluded vessel, a greater percentage of the flow was returned more rapidly with both encapsulated formulations than with FREE SK, with MESK reaching a maximum level of flow one hour faster than LESK (Figure 3.6). The faster reperfusion times and reduced residual clot sizes observed in this model likely result from a reduction in reocclusion episodes during treatment with LESK and MESK. It would be reasonable to assume that reocclusion is limited in the latter cases by the increased rate and extent of clot lysis observed with the encapsulated formulations when compared to free streptokinase.

One theory for the efficacy of encapsulation in thrombolysis is the blocking of channeling by the particles and a subsequent increase in pressure at the leading edge. Heeremans et al. compared the thrombolytic ability of free t-PA to free t-PA + empty liposomes in a rabbit jugular vein model [24]. Freely infused t-PA resulted in a similar degree of clot lysis as that obtained with an identical dosage of t-PA + empty liposomes (26.3% vs. 21%; $p>0.05$; ns). Perkins and coworkers also observed no statistical benefit with FREE SK + empty liposomes [25]. Due to this small difference in the percentage of lysed clot, it is unlikely that an increase in pressure due to the blocking of channeling plays a sizable role in the lysis of clots with encapsulated formulations. In this model, FREE SK + BLANK resulted in no significant improvement over FREE SK. Similar results have been obtained in our laboratory in vivo using FREE SK + empty liposomes or microparticles. Clearly, the true benefit is only observed when the plasminogen activator is closely associated with the vehicle.

The mechanism of action by MESK will be addressed in detail in Chapter 5 in which a number of in vitro experiments are discussed. In brief, streptokinase is protected from circulating plasma proteins by sequestration within the microparticles. Perhaps more importantly, encapsulation of streptokinase within the microparticles facilitates clot permeation whereas free streptokinase binds to the surface due to reactions with plasma proteins. Improved permeation of the thrombus leads to greater distribution of streptokinase within the thrombus as the particles dissolve.

In this study, SK was encapsulated in microparticles formed of PEG. Mammalian enzymes are unable to breakdown the polyether backbone of PEG, leaving the degraded material to be filtered from the blood compartment by the kidneys [69]. In vitro experiments in our laboratory have demonstrated the time-dependent microparticle dissolution and release of fluorescently-labeled SK from MESK in plasma (data not shown). Over the 120 min period of this experiment, the Tyndall effect can be observed during the solubilization of microparticles along with the release of a green dye.

Based on these preliminary results, microencapsulation of thrombolytic drugs offers a promising alternative for current products used to treat thrombosis with the potential to salvage more tissue and reduce bleeding complications. Further experiments were undertaken to explore the mechanism of encapsulated streptokinase's action as well as examine its potential in a model of greater clinical efficacy. This work will be explained in Chapters 4 and 5, respectively.

ASSESSING THE POTENTIAL EFFICACY OF ENCAPSULATED STREPTOKINASE IN VITRO IN ORDER TO EXPLORE ITS MECHANISM OF ACTION

CHAPTER 4

INTRODUCTION

Significant differences in thrombolytic ability have been observed between free and encapsulated streptokinase in a rabbit model of arterial thrombosis. In order to explore the mechanism utilized by microencapsulated streptokinase to digest thrombi more rapidly than free streptokinase, an in vitro system was used so as a number of variables could be easily controlled (i.e. clot age, clot structure, pressure drop, etc.). The system allows for changes in clot type to investigate the effect of clot retraction, as well as variances in applied pressure.

As previously shown by Diamond, Blinc, and others, thrombolysis is a pressure-mediated phenomenon [31-36]. With diffusion as the sole mode of plasminogen activator transport within the clot, drugs would move only millimeters over hundreds of minutes. Clearly, diffusion is not the dominant transport mechanism. Convection, or

forced movement, is the prevailing method of transport within the thrombus. Interstitial flow of plasma through a thrombus can be adequately described by Darcy's law of flow through porous media:

$$v = \frac{Q}{A} = \frac{k}{\mu}\frac{\Delta P}{L} \tag{4.1}$$

where v is the interstitial fluid velocity, Q is the flowrate, A is the cross-sectional area available for fluid passage, k is the permeability of the media, μ is the fluid viscosity, and $\Delta P/L$ is the pressure drop across the media. It is apparent that increases in pressure drop over the length of the clot should result in faster lysis due to faster inflow of plasminogen activator. Such increases arise when the length of the clot is reduced due to clot digestion. Previous in vitro studies used low pressure drops much like that observed in the case of venous thrombosis [28]. Higher pressure drops are required to better approximate the coronary thrombotic condition in which pressure drops can reach 60 mmHg/cm-clot.

To that end, the thrombolytic potential of MESK has been investigated using a simple in vitro system with two clot types (plasma and whole blood) and at three increasing initial pressure drops per clot type. Initial pressure drops utilized for plasma clots were 5, 10, and 15 mmHg/cm-clot, while whole blood clots were exposed to initial pressure drops of 10, 20, and 30 mmHg/cm-clot. Although these values are still significantly smaller than those that occur across arterial clots in vivo, these experiments are a better approximation of physiological conditions compared to previous studies.

MATERIALS AND METHODS

Preparation of microencapsulated streptokinase (MESK)

Streptokinase was entrapped in a water-soluble polymer through a modified double emulsion technique [70, 71]. Briefly, 2 g of polyethylene glycol (20,000 MW; Polysciences, Inc., Warrington, PA) was dissolved at room temperature in 3 ml chloroform (Fisher Scientific, Pittsburgh, PA) and streptokinase (Streptase®, 40,000 IU, Behringwerke AG, Marburg, Germany) was dissolved separately in 0.2 ml distilled water, also at room temperature. The aqueous protein solution was added to the polymer solution and immediately dispersed by sonication (Sonic Dismembrator 550, Fisher Scientific, Pittsburgh, PA) at 10 W for 1 min to form the first emulsion. Sonication for this length of time did not significantly alter the activity of streptokinase. The protein/polymer solution was mixed with 8 ml of a 2% (w/v) polyvinyl alcohol solution (average MW 30,000-70,000, Sigma Chemical, St. Louis, MO) and vortexed for 20 s. The mixture was placed in a freezer at -80°C for 2 h and lyophilized for 15-20 h. The lyophilized product was washed with hexane in a centrifuge at 600 *x g* for 10 min at 4°C, and then filtered under vacuum through 0.45 μm membranes to remove any protein bound to the external surface. The product was allowed to dry in a fume hood for 1 h. Final product (MESK) composed of porous, primarily spherical-shaped particles of 0.793 ± 0.026 μm average diameter (mean ± SEM) was then stored at room temperature until use. Blank polymer was prepared by replacing the streptokinase solution with an identical volume of distilled water. The remainder of the preparation was carried out following the same protocol as MESK. Microparticulate formulations with higher

concentrations of encapsulated streptokinase per particle were prepared by initially adding greater concentrations of streptokinase (250,000 IU in 0.4 ml) prior to sonication.

Fluorescent labeling of streptokinase

Streptokinase was labeled with a commercially available fluorescent marker (Alexa Fluor 488, Molecular Probes, Eugene, OR) using the accompanying protocol at room temperature. Briefly, 6 mg (60,000 IU) of streptokinase was dissolved in 1 ml PBS. A small volume of 1 M sodium bicarbonate solution (50 μl) was added to 0.5 ml of the streptokinase solution to raise the pH for optimum label attachment. The protein solution was added to a vial of the reactive dye and stirred for 1 h. Unattached dye molecules were separated from the protein-dye conjugate by filtration through the accompanying column. Labeled streptokinase was collected and either used immediately in its free form, encapsulated, or frozen in small aliquots at -80°C until use. Fluorescent labeling of streptokinase did not significantly affect the agent's activity.

Preparation of whole blood and plasma clots

Clots formed of whole blood or plasma were prepared as described [28]. Human fibrinogen (Grade L; American Diagnostica, Greenwich, CT) was dissolved in 0.05 M Tris-HCl, pH 7.4 and dialyzed against 0.05 M Tris-HCl with 0.1 M NaCl and 5 mM $CaCl_2$, pH 7.4 for 18 h with changes every 6 h and frozen in small aliquots at -80°C until use. Thrombin from human plasma (~ 3000 NIH units/vial; Sigma Chemical, St. Louis, MO) was dissolved in Nanopure distilled water to a concentration of 2 NIH units/ml and frozen in small aliquots at -80°C until ready for use.

Glass Natelson blood capillary tubes (Fisher Scientific) were prepared for clot formation by coating the interior surface with a thin layer of fibrin as described previously [78]. Briefly, the tubes were etched in a dilute solution of hydrofluoric acid (Fisher Chemical, Fair Lawn, NJ) for approximately 20 min, washed thoroughly in distilled water, and baked 1 h at 100°C. The tubes were allowed to cool to room temperature. The interior surface of the etched capillary tubes was coated with a fibrin solution to enhance adherence of the clots to the surface. A 1:1 mixture of fibrinogen and thrombin was rapidly mixed and injected into the etched tubes. The mixture was allowed to form a fibrin gel for at least 1 h at 37°C. After fibrin formation, the gel was gently removed with low air pressure, leaving a residual layer of fibrin deposited on the wall of the tube. The tubes were stored overnight at room temperature before use.

Whole blood was collected from healthy donors and anticoagulated with 0.13 M sodium citrate (1 volume part of sodium citrate per 9 volume parts of blood). Platelet-rich (PRP) and platelet-poor plasma (PPP) were obtained by centrifugation at 150 x *g* for 10 min and 4600 x *g* for 20 min, respectively. Platelet counts for PRP and PPP were significantly different (2.8e6 ± 1.4e5 platelets/ml and 1.5e5 ± 1.1e5 platelets/ml, respectively) as determined using a hemacytometer.

Clots of either whole blood or plasma were prepared in primed capillary tubes. Whole blood clots were formed by the addition of 0.25 M $CaCl_2$ at a ratio of 8 µl $CaCl_2$:100 µl whole blood. A small volume (~ 80 µl) of freshly calcified whole blood was injected into a capillary tube to form a clot of approximately 3 cm in length. Clots were allowed to solidify for 2 h at 37°C. Plasma clots were formed by injecting small volumes of a mixture of 50 µl PRP, 50 µl PPP, 100 µl thrombin, and 16 µl of 0.25 M

$CaCl_2$ and allowed to gel for 1 h at 37°C. Final thrombin concentration was 1 NIH unit/ml of plasma. Clots that formed with visible structural deformities such as interior void volumes or incomplete adherence to the tube wall were excluded from the study. Following clot formation, the capillary tubes were connected to Tygon tubing and a 5 ml glass pipette (as a flowmeter) filled with a solution of 5 ml normal saline, 2 ml PPP, and the fibrinolytic study formulation (Figure 4.1).

Fibrinolysis of whole blood and plasma clots by 2500 IU streptokinase was investigated in vitro at room temperature. Study formulations included free streptokinase (FREE SK), microencapsulated streptokinase (MESK; approximately 150 mg), free streptokinase + polymer (FREE SK + BLANK), and a high concentration of encapsulated streptokinase per particle (HI MESK; approximately 50 mg). Pressure was applied by the addition of air monitored by a pressure gauge. Lysis of plasma clots was studied at initial pressure drops of 5, 10, and 15 mmHg/cm-clot. The applied pressure was kept constant throughout the experiment. Whole blood clots were examined at initial pressure drops of 10, 20, and 30 mmHg/cm-clot, also keeping the applied pressure constant. The time required to dissolve the clot was monitored, along with the flow rate of perfusing formulation and the length of the clot at numerous time points.

Preparation of clots for confocal microscopy studies

Plasma clots were prepared for examination with confocal microscopy in a similar manner to that described above. Fibrinogen labeled with Alexa Fluor 594 marker (Molecular Probes, Eugene, OR) was dissolved in 1 M sodium bicarbonate solution at a

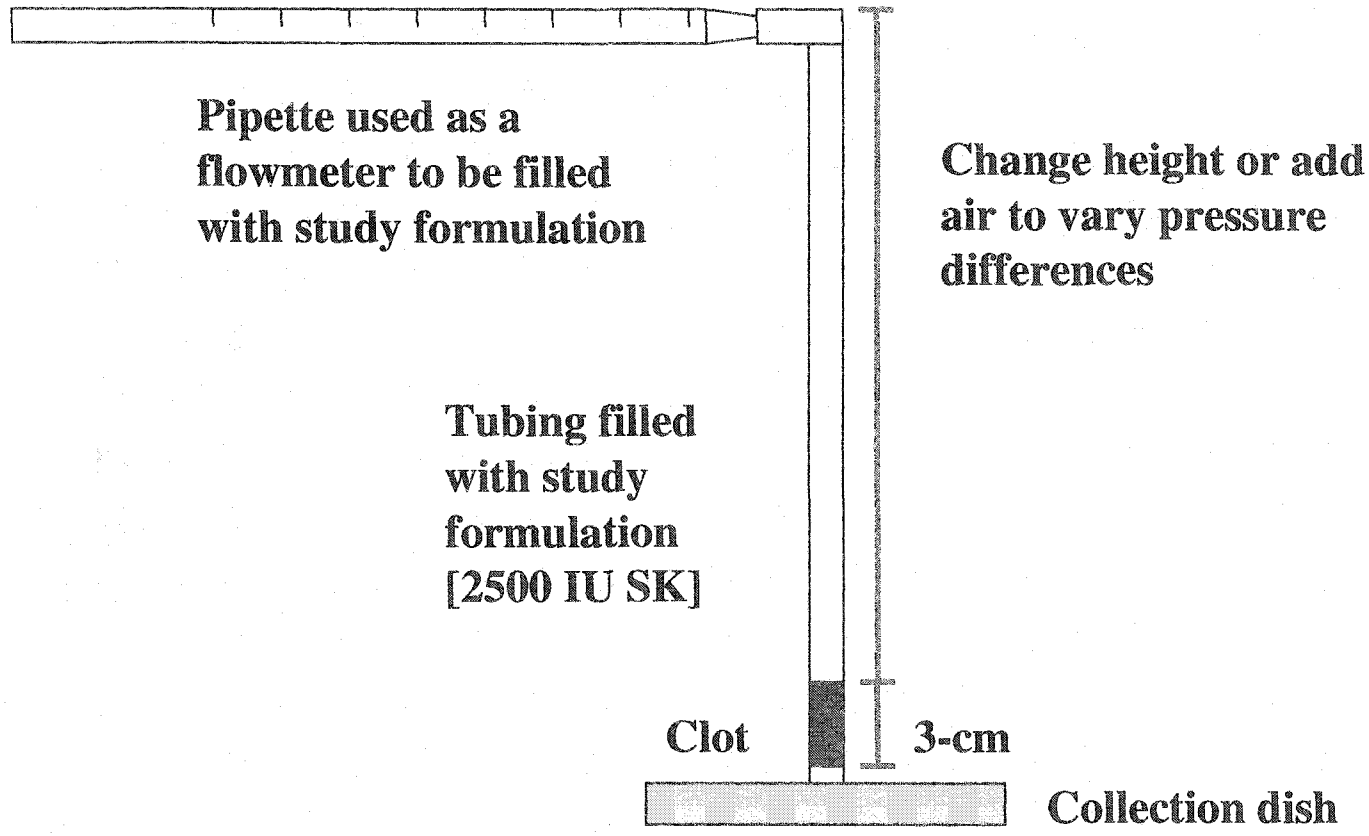

Figure 4.1: In vitro apparatus for examining clot digestion. Two clot types are utilized, and the initial pressure drop across the clot is varied by adding air pressure or changing the length of the tubing.

concentration of 1.5 mg/ml. Rectangular glass capillary tubes were prepared for clot formation by etching and coating in an identical manner as described above. To form plasma clots, 100 μl PRP was mixed with 12 μl of labeled FBG, 15 μl of 0.25 M $CaCl_2$, and 88 μl of thrombin. Approximately 100 μl of the mixture was injected into fibrin-coated tubes and allowed to incubate for 1 h at 37°C.

Following clot formation, the capillary tubes were connected to Tygon tubing and a 5 ml glass pipette filled with a solution of 5 ml PBS, 2 ml PPP, and the study formulation consisting of 10% labeled streptokinase and 90% unlabeled plasminogen activator.

Microscopy studies

Real-time video of plasma clot lysis with light microscopy was acquired using a 5x Plan-Neofluor objective on a Zeiss Axiovert 200 equipped with a Dage-MTI cooled CCD-300. Images were processed prior to being recorded using an Argus-20 Image Processor (Hamamatsu) and recorded on a Sony SVO-9500MD Videocassette Recorder using S-VHS video tapes for greater resolution. The images were captured digitally using a Flashbus MV Video Frame Grabber and analyzed with Metamorph Version 4.6r8 software (Universal Imaging Corporation).

Plasma clot lysis with confocal microscopy was observed with an Olympus XL Fluor 4X objective equipped with a 0.28 numerical aperture on an Olympus Fluoview FV-500 Confocal Laser Scanning Biological Microscope. The optical resolution (about 3.2 μm in the x- and y-directions) allowed for an adequate view of both the thrombus front and at least 2 μm of the thrombus interior. Samples were fluoresced using Argon

488 and Helium-Neon 543 lasers. Images were collected in 20 μm slices; collecting a single complete image took 40 s. Images were analyzed with Olympus MicroSuite B35V software (Soft Imaging System) to determine the intensity of the fluorescence signal as a function of position in the clot.

STATISTICS

Using the Student's t-Test, p values < 0.05 were considered significant. Unless otherwise stated, all data are presented as the mean ± standard error of the mean.

RESULTS

Streptokinase microencapsulation efficiency

The efficiency of the encapsulation process was measured in three ways: 1) total mass of product at the completion of preparation; 2) concentration of entrapped protein using a protein assay; and 3) the activity of the entrapped enzyme. At the conclusion of preparation, 91.2 ± 0.8% [n=11] of the total product was recovered.

The concentration of the encapsulated protein was determined with a commercial protein assay kit (Pierce, Rockford, IL) based on the Lowry method of protein determination. Microparticles (10 mg in 1 ml PBS) were allowed to dissolve completely at room temperature. The streptokinase concentration in each sample was compared to a standard curve of protein in an identical concentration of polymer. A majority of the initial protein, 90.0 ± 3.9%, was entrapped within the microparticles as observed with this assay.

Viable streptokinase content in the microparticles was quantified using a commercial chromogenic activity assay (Helena Laboratories, Beaumont, TX). The microparticles were allowed to dissolve completely in distilled water for at least 2 h, a period sufficient for complete dissolution. This process was followed and confirmed by light scattering studies using a Coulter LS Particle Size Analyzer (Beckman-Coulter, Fullerton, CA). The activity of each sample, characterized as the ability of streptokinase to cleave plasminogen and form plasmin, was compared to that of a standard curve of streptokinase in the presence of an identical blank microparticle concentration. Although most of the protein was encapsulated as evidenced by the protein concentration assay, a significant portion of the enzyme was deactivated during the process, with observed sample activity of 55.5% ± 5.3% of an identical concentration of non-encapsulated streptokinase.

Measurement of clot lysis

Fibrinolysis of whole blood and plasma clots was investigated by administering a perfusate of various streptokinase formulations (n=8 for all groups) with the results presented in Tables 4.1 and 4.2. Three general trends can be observed: 1) Clot lysis is accelerated by increases in pressure drop for either clot type and with all thrombolytic formulations; 2) clot lysis is accelerated with MESK versus either free streptokinase formulation; and 3) clot lysis is retarded by the infusion of FREE SK + BLANK. Additionally, infusion of the higher concentration per particle formulation of MESK (HI MESK) often resulted in further reduction in lysis times compared to MESK,

	10 mmHg/cm (min)	20 mmHg/cm (min)	30 mmHg/cm (min)	Average Change from FREE SK (%)
FREE SK	46.0 ± 5.3	26.4 ± 1.5	19.1 ± 1.8	--
FREE SK + BLANK	55.9 ± 3.6	34.1 ± 3.6	27.9 ± 1.8*	+ 32.3 ± 7.3
MESK	46.5 ± 4.7	19.4 ± 1.5*	14.1 ± 1.4*	- 17.2 ± 9.2
[HI] MESK	31.8 ± 2.7*†	18.5 ± 0.9*	14.4 ± 0.7*	- 28.5 ± 2.0

Table 4.1: Whole blood clot lysis times at various initial pressure drops with four formulations of streptokinase (mean ± SEM).

* = $p < 0.05$ vs. FREE SK; † = $p < 0.05$ vs. MESK

	5 mmHg/cm (min)	10 mmHg/cm (min)	15 mmHg/cm (min)	Average Change from FREE SK (%)
FREE SK	30.1 ± 3.4	29.1 ± 1.9	17.8 ± 1.6	--
FREE SK + BLANK	40.0 ± 8.1	36.4 ± 3.3*	20.4 ± 3.2	+ 24.2 ± 5.3
MESK	23.9 ± 3.3	23.8 ± 1.0*	12.0 ± 1.4*	- 23.8 ± 4.5
[HI] MESK	21.1 ± 1.3*	13.1 ± 0.8*†	10.8 ± 0.6*	- 41.4 ± 7.3

Table 4.2: Plasma clot lysis times at various initial pressure drops with four formulations of streptokinase (mean ± SEM).

* = $p < 0.05$ vs. FREE SK; † = $p < 0.05$ vs. MESK

although usually not a statistically significant reduction.

Interestingly, lysis of whole blood clots with FREE SK was preceded by a significant portion of erythrocytes passing through the clot, leaving a clot composed primarily of plasma and fibrin. Although some level of lysis had already occurred, clot dissolution proceeded more rapidly due to the reduction in resistance from erythrocytes. The phenomenon of erythrocyte washout has been reported previously [79]. However, erythrocyte washout was not observed with MESK- or FREE SK + BLANK-treatment of clots.

By visualization of clot lysis through an Olympus BH-2 light microscope equipped with a 4X objective, the method of fibrinolysis for FREE SK and MESK appeared quite different. FREE SK appeared to dissolve the clot from the leading edge alone, and movement of the front could be observed with the naked eye. Channeling, also known as dissolution fingering, regularly occurred through the clot, which was a function of the pressure drop over the length of the thrombus. The channeling phenomenon has been previously reported as a function of the applied convective forces [33, 34, 36]. Oftentimes there was a substantial portion of the thrombus remaining on the tube wall when flow was restored. Clots treated with FREE SK + BLANK exhibited similar behavior.

In contrast to FREE SK, thrombus exposure to MESK resulted in the formation of voids due to lysis in advance of the clot's leading edge (Figure 4.2). A sufficient concentration of streptokinase was allowed to penetrate the front and begin dissolution within the thrombus. In collecting real-time video of plasma clot lysis, two frames were isolated that demonstrate dissolution with FREE SK (Figure 4.3). The leading edge

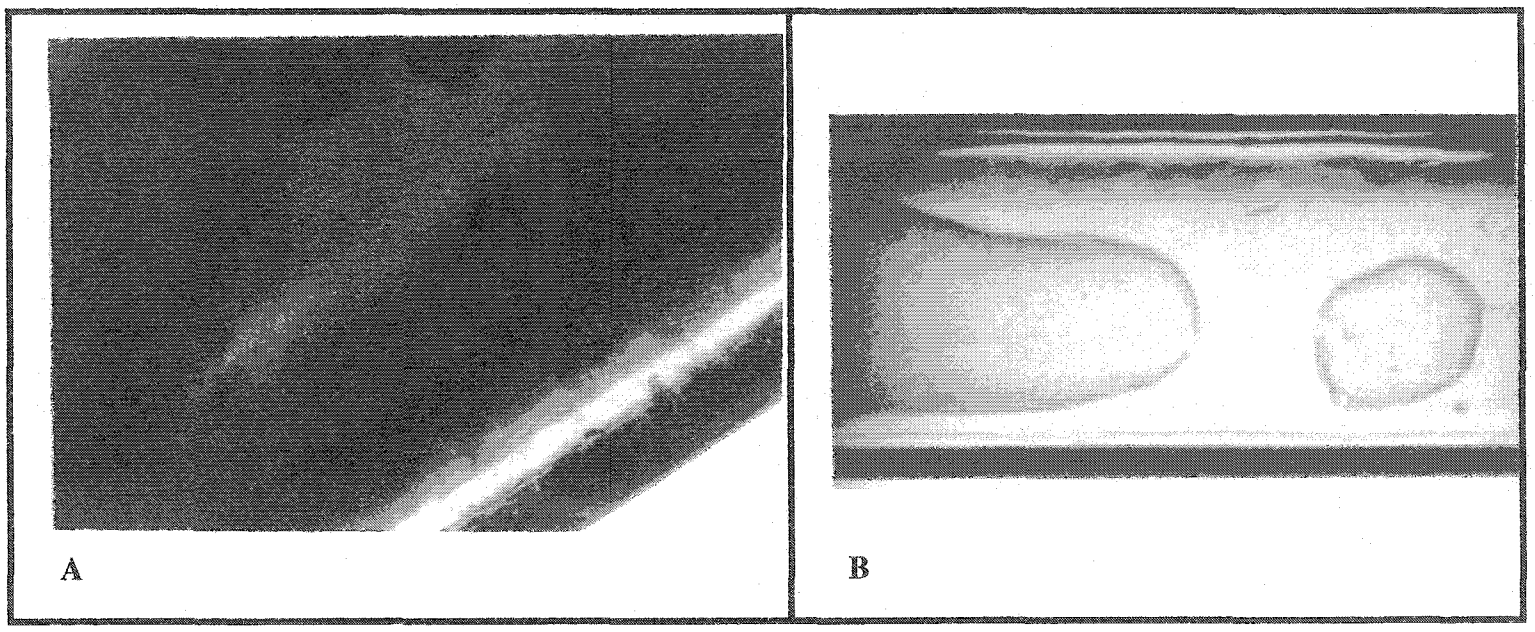

Figure 4.2: Microscopic view of whole blood (A) and plasma clot (B) undergoing distributed intraclot thrombolysis upon infusion of MESK.

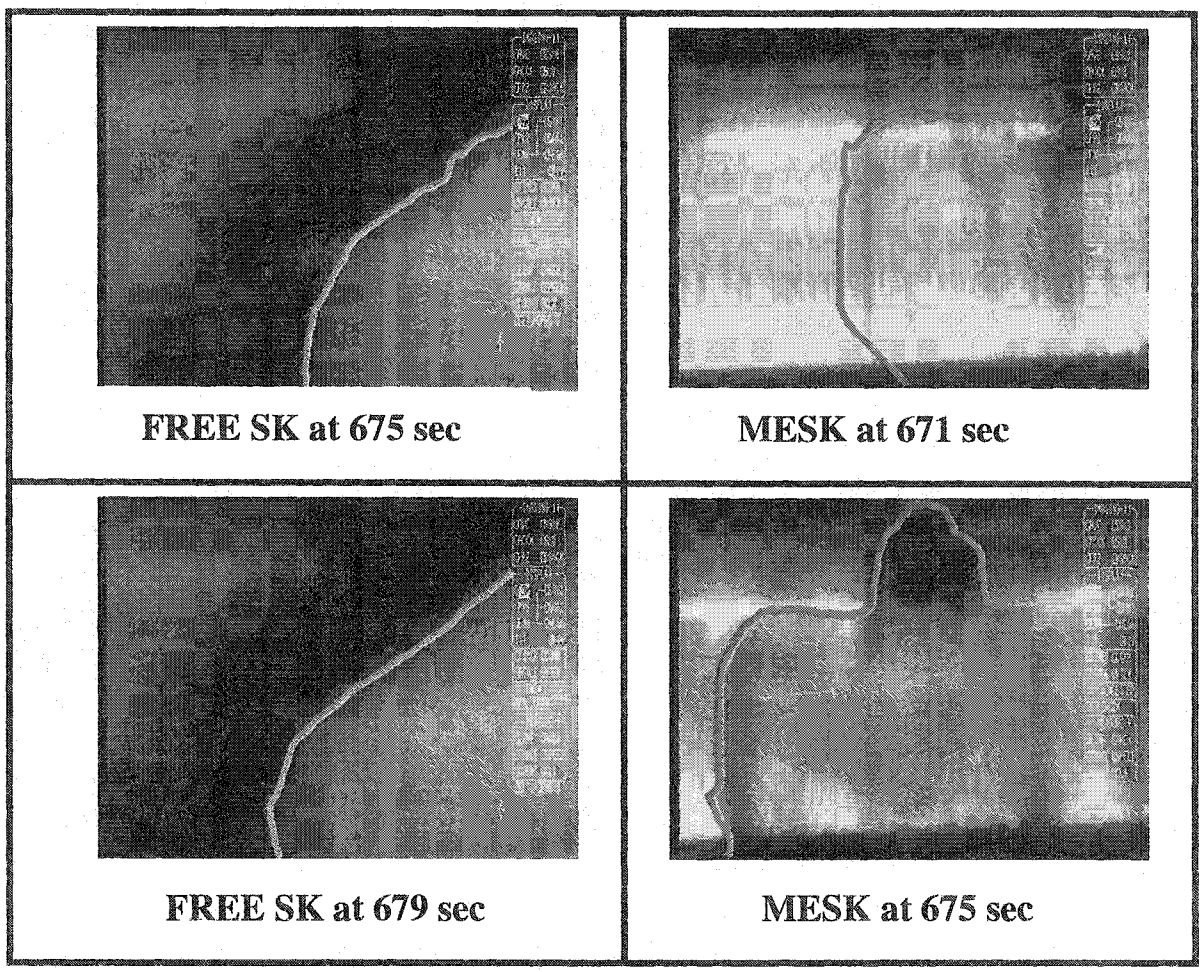

Figure 4.3: Frames of real-time video of plasma clot lysis using light microscopy. The leading edge of the thrombus is highlighted in blue.

of the clot moves slowly and in a predictable fashion. Frames isolated from an MESK-treated clot at approximately the same time period illustrate a very different method of lysis. The front moves faster with more of the thrombus dissolving in both the x- and y-planes instead of just one-dimension as seen with FREE SK.

Similar techniques were utilized to examine clot lysis with confocal laser scanning microscopy. As observed with light microscopy, FREE SK tended to bind to the thrombus front, as demonstrated by the intense region of fluorescence at the front (Figure 4.4A). When considering the map of fluorescence versus position extracted from the streptokinase concentration (Figure 4.5, bottom left), one significant peak of fluorescence intensity routinely occurred at the front, verifying the high concentration of streptokinase. MESK-treated clots resulted in improved permeation of streptokinase as evidenced by the formation of voids in the interior of the clot (Figure 4.4B). These voids were similar to those seen with light microscopy. Fluorescence mapping of a thrombus treated with MESK demonstrated multiple peaks of fluorescence as a function of position (Figure 4.5, bottom right), suggesting greater distribution of labeled streptokinase, an occurrence not seen with FREE SK. As seen in Figures 4.4 and 4.5, streptokinase is dispersed more effectively within the thrombus with MESK, while most of the FREE SK is bound to the leading edge. This is potentially due to decreased streptokinase adsorption to the front with MESK compared to FREE SK, thereby allowing greater penetration of the thrombus.

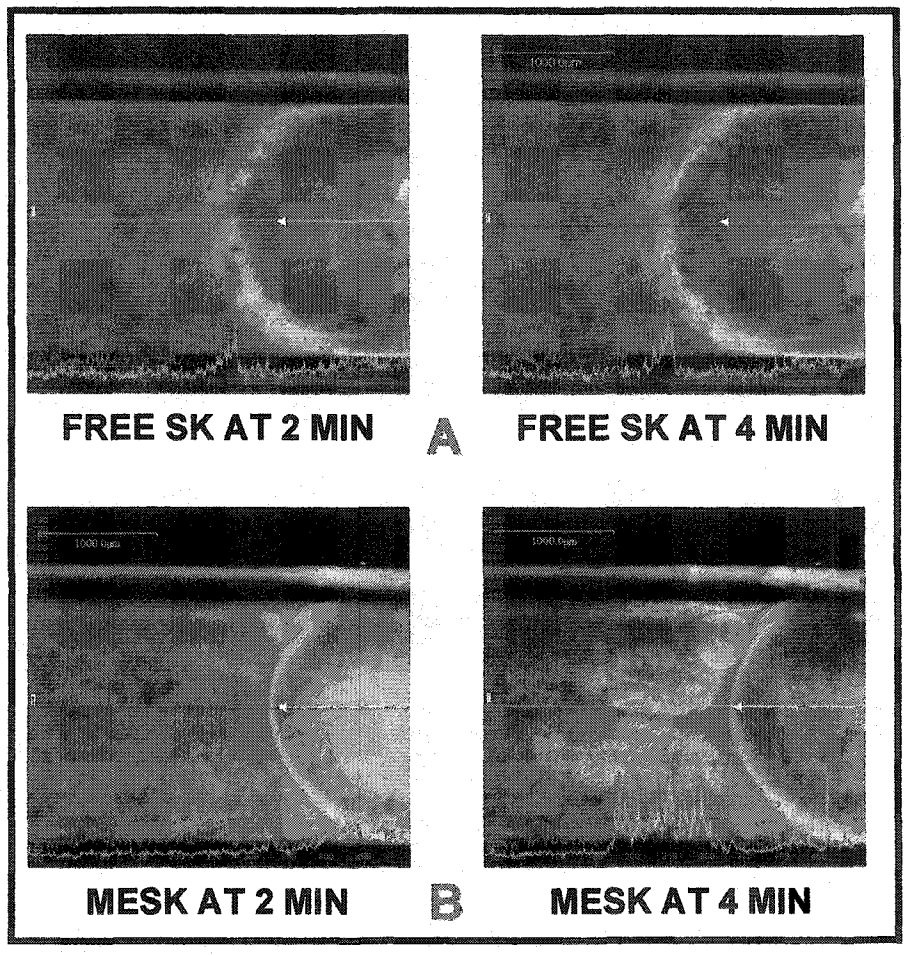

Figure 4.4: Confocal microscopy is used to monitor clot lysis with FREE SK (A) and MESK. (B).

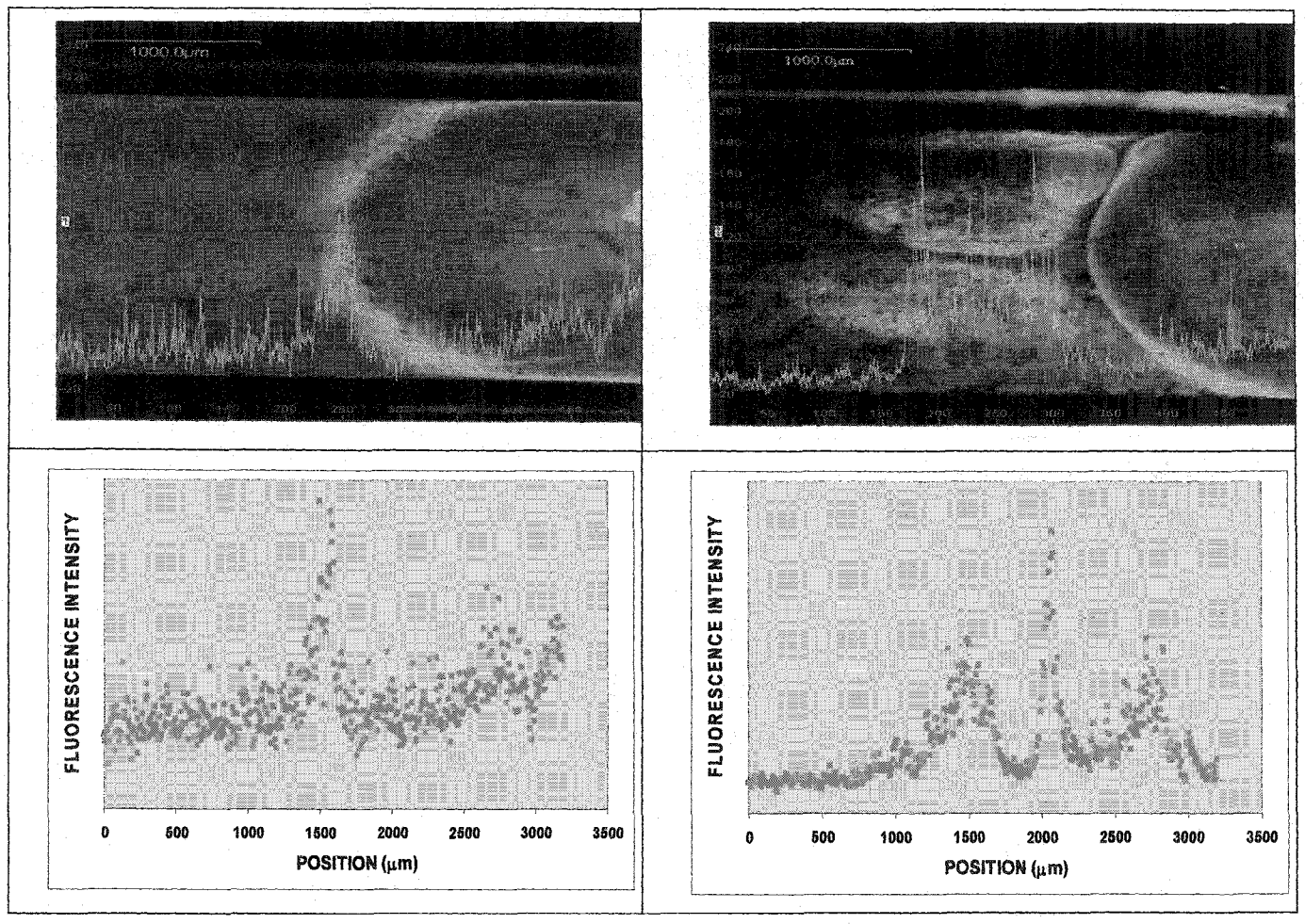

Figure 4.5: Greater distribution of streptokinase is observed with MESK (top right) compared to FREE SK (top left) by increased fluorescence of streptokinase within the clot (bottom).

DISCUSSION

Administration of liposomal encapsulated plasminogen activators has demonstrated enhanced clot lysis in numerous *in vitro* and *in vivo* models [22-26]. Multiple endpoints are dramatically improved such as a reduction in the time to obtain reperfusion and a greater percentage of dissolved thrombus. Encapsulation of streptokinase in polymer microparticles has shown comparable benefit to liposomal encapsulated streptokinase with improved shelf-life (Chapter 3). In these studies, accelerated reperfusion times have been demonstrated using microencapsulated streptokinase *in vitro*.

Multiple reports have demonstrated the occurrence of clot lysis from the leading edge alone with freely administered plasminogen activators [32, 34, 81-83]. Plasminogen activators normally bind to the clot front due to protein adsorption at the surface. The results of frontal-edge binding can be seen by the routine occurrence of steady frontal lysis or channeling, in which clots are dissolved from the front at the site of the lowest pressure drop across the length of the clot. Although the formation of a channel allows the restoration of much-needed blood flow, a large portion of the clot often remains on the vessel wall in the form of a mural thrombus. The presence of residual clot on the walls results in reduced available cross-sectional area for flow and increased probability for reocclusion.

In order to examine the ability of encapsulated streptokinase to digest thrombi of varying composition and in different conditions, a simple in vitro system has been utilized. As seen in Tables 4.1 and 4.2, reperfusion times were reduced with increasing

initial pressure drops for both whole blood and plasma clots when using MESK compared to identical dosages of FREE SK.

This simple in vitro model has the benefit of few variables to control, thereby allowing the investigator to look closely at the effect of one component (i.e. encapsulated vs. free streptokinase). However, the simplicity of the system results in several significant shortcomings. Many proteins, including plasminogen activators, perform quite differently at various temperatures. Performing the experiments at room temperature (25°C) compared to an ideal physiological temperature (37°C) undoubtedly results in slower clot lysis. Despite likely increases in clot lysis when performing tests at 37°C, the comparisons between study groups are still valid as all were examined at the same temperature. Also, this system does not afford the opportunity for removal of free or encapsulated streptokinase. This missing factor deviates from the in vivo setting in a manner that likely skews the differences that might be observed with MESK over FREE SK. Lastly, the applied pressure must be kept constant by the investigator, opening the door for experimental error to become prevalent.

The mechanism for encapsulated lytic agents' improvement has only been proposed in earlier studies. The most plausible theory is that encapsulation or entrapment protects the enzyme from premature inactivation or removal while in the circulation. Protection of the agent effectively lengthens the half-life and circulation time of the agent, thereby allowing a greater concentration of thrombolytic agent to convert plasminogen into plasmin. While the continued protection of these agents is likely to play a role in its efficacy, results from these experiments have elucidated a more precise mechanism for the success of encapsulated plasminogen activators.

In this chapter, evidence is presented in support of a hypothesis entitled Distributed Intraclot Thrombolysis (DIT) as a mechanism for thrombolysis with MESK. In contrast to free streptokinase which works through a series of localized reactions at the leading edge, MESK avoids significant adsorption at the frontal edge of the clot and is allowed to penetrate to the interior portion of the thrombus. Images collected with confocal microscopy verify greater concentrations of streptokinase within the thrombus with MESK compared to FREE SK at identical times (Figure 4.4B). As streptokinase is dispersed throughout the polymer vehicle, the protein is continuously released as the particle erodes, resulting in both free streptokinase and encapsulated streptokinase arriving at the site of occlusion. The streptokinase released by MESK on the interior of the thrombus results in the formation of voids within the clot.

Permeation of the thrombus by MESK might also provide a method to limit "plasminogen steal," the effect of plasminogen depletion that occurs in the presence of non-fibrin-specific plasminogen activators. This phenomenon relates to the movement of clot-bound plasminogen out of the clot and into the plasma compartment as a result of depletion of plasminogen in the outer plasma by plasminogen activators and the occurrence of a concentration gradient. Encapsulation limits the total concentration of free plasminogen activator at the surface of the clot, and although not specifically considered in this study, should limit the depletion of plasminogen in the circulation.

Decreased adsorption is likely a function of two factors: 1) the material and 2) sequestration of the drug within a vehicle. Polyethylene glycol is known to resist plasma protein adsorption, an appealing characteristic for this application. Secondly, only streptokinase that is released into the fluid phase is free to bind to the clot. Streptokinase

entrapped within the particles cannot react with plasma proteins and is permitted to continue progressing through the clot until its release from the vehicle.

In order to better understand some of the factors associated with clot lysis using FREE SK and MESK, a numerical simulation has been developed and described in Chapter 5.

NUMERICAL ANALYSIS OF CLOT LYSIS WITH FREE and MICROENCAPSULATED STREPTOKINASE

CHAPTER 5

INTRODUCTION

The limitations of molecular transport of plasminogen activators during thrombolytic therapy have not been well appreciated. Understanding and addressing these limitations could dramatically enhance the performance and improve the promise of these agents for treatment of cardiovascular crises. The same affinity of the plasminogen activators for fibrin necessary to achieve the clot digestion ultimately undermines the species' effectiveness in restoration of blood flow. More specifically, this strong affinity coupled with the high surface area of the fibrous fibrin network and the extremely slow flow into the clot results in rapid removal of the plasminogen activator shortly after blood enters a thrombus. Plasminogen activation therefore occurs primarily at the leading edge of the clot. The localization of the reaction near the liquid-solid interface has been demonstrated using in vitro experiments and computational modeling of lysis of fibrin gels in capillary tubes [32, 34, 80-82].

Results from in vivo (Chapters 3 and 6) and in vitro (Chapter 4) studies confirm that enhanced thrombolysis is achieved with microencapsulated streptokinase compared to free streptokinase. As described in Chapter 4, evidence is growing to support the hypothesis that enhanced thrombolysis with encapsulated plasminogen activators occurs as a result of distributed intraclot thrombolysis. In short, this theory puts forth that the encapsulation vehicle facilitates penetration of the plasminogen activator into the clot by inhibiting adsorption. As a result, fibrinolysis occurs throughout the clot in 3 dimensions (x, y, and z) instead of being restricted to 2 dimensions (x and y) as observed with free agents. Figures 4.2-4.5 show that encapsulated streptokinase digests thrombi in a very different manner than free streptokinase, with greatly improved spatial lysis occurring with encapsulated streptokinase. In order to better understand the mechanism of clot lysis with microencapsulated streptokinase, a first-generation computational analysis has been developed.

METHODS

Clot lysis by streptokinase is described using a convection-diffusion-reaction equation which takes into account the adsorption of free species to the thrombus. The thrombus has been modeled as a homogeneous, isotropic structure with a horizontal orientation. The problem has been reduced to changes in only one-dimension (the x-direction). To facilitate the modeling of thrombus digestion, the appropriate equation is the species mass transport equation:

$$\nabla \bullet N_{activator} + \frac{\partial C_{activator}}{\partial t} - R_{activator} - A_{activator} = 0 \qquad (5.1)$$

where $N_{activator}$ is the molar flux through a region, $\partial C_{activator}/\partial t$ is the rate of accumulation, $R_{activator}$ is the loss of plasminogen activator through reactions, and $A_{activator}$ is the loss of plasminogen activator through reversible adsorption.

Enzymes from blood may be introduced by diffusion and convection into the thrombus through a single plane, which is referred to as the blood-thrombus boundary. As the thrombus dissolves, the blood-thrombus boundary moves into the positive direction of the clot (x-direction). To simplify the problem, velocity of interstitial fluid is calculated from in vitro studies described in Chapter 4 and held constant, an assumption previously implemented by Blinc in earlier models of thrombolysis [13]. Therefore, changes in pressure drop over the length of the clot are not considered. Additionally, physical characteristics of the clot such as permeability and porosity are held constant; properties that would otherwise be changing during clot digestion. The thrombus initially contains no functional streptokinase, and the concentrations of bound plasminogen and antiplasmin within the thrombus are taken as constant.

The conversion of plasminogen to plasmin by streptokinase is modeled by a series of reversible and irreversible reactions which occur in the fluid phase of a thrombus:

1. $SK + PLG \leftrightarrow SK - PLG$ $(\mathbf{k_1/k_{-1}})$ (5.2)
2. $SK - PLG + PLG \rightarrow SK - PLG + PLM$ $(\mathbf{k_2})$ (5.3)
3. $PLM \rightarrow PLM - AP$ $(\mathbf{k_3})$ (5.4)
4. $PLM + FBN \rightarrow PLM + FDP$ $(\mathbf{k_4})$ (5.5)

where SK is free streptokinase, PLG is plasminogen, SK-PLG is the streptokinase-plasminogen complex, PLM is plasmin, PLM-AP is plasmin inactivated by α_2-antiplasmin, FBN is fibrin, and FDP is fibrin degradation products. The reaction rate constants, k_i, are identified in parentheses after each reaction.

The adsorption of various species to the thrombus from the fluid phase drives the dissolution of the clot. Adsorption is modeled using a Langmuirian-type isotherm in which the amount of adsorbed species to vacant sites on the thrombus is directly proportional to the concentration of the species. For example, the change in concentration with respect to time of plasmin is described by the following equation:

$$\frac{\partial}{\partial t} C_{PLM}^{\,b} = k_{ADS}^{\,PLM} \cdot C_{PLM}^{\,f} \cdot \left(\theta_{PLM}^{\,b} - C_{PLM}^{\,b} \right) - k_{DES}^{\,PLM} \cdot C_{PLM}^{\,b} \tag{5.6}$$

where the superscript "b" represents bound species, "f" represents fluid species, θ is the concentration of available vacant sites, and k_{ADS} and k_{DES} are the kinetic constants of adsorption to and desorption from the thrombus, respectively.

When all components are integrated into the equation, the general form of the convection-diffusion-reaction equation utilized in this model is represented as follows:

$$\frac{\partial}{\partial t} C_i^{\,f} = D_i^{\,f} \cdot \frac{\partial^2}{\partial x^2} C_i^{\,f} - \left[v \cdot \left(\frac{\partial}{\partial x} C_i^{\,f} \right) \right] - \left[\left(\frac{1-\varepsilon}{\varepsilon} \right) \cdot \frac{\partial}{\partial t} C_i^{\,b} \right] + \Sigma R \tag{5.7}$$

where D is the diffusion coefficient of species "i" through the fluid phase, v is the velocity of the interstitial fluid, ε is the porosity of the thrombus, and ΣR is the summation of ongoing homogeneous and heterogeneous reactions. Similar equations are written for each species with accompanying boundary conditions listed in Appendix B.

Release of streptokinase from the microparticles is modeled using the Hixon-Crowell equation [83]. This model is based on the requirement of the particle surface area being proportional to the cubic root of its volume. Therefore, the following equation was generated:

$$W_0^{\frac{1}{3}} - W_t^{\frac{1}{3}} = K \cdot t \tag{5.8}$$

where W_0 is the amount of drug within the particles initially, W_t is the amount of drug in the particles at time t, and K is the release rate. Dividing the left side of equation 5.8 by $W_0^{1/3}$ and simplifying gives:

$$\left(1 - f_t\right)^{\frac{1}{3}} = K \cdot t \tag{5.9}$$

where f_t is the fraction of drug released at time t. A plot of the left-hand side of the equation versus t will generate the release rate constant, K, as the slope.

When the Hixon-Crowell model is used, it is assumed that the release rate is limited by the drug particles' dissolution rate and not by the diffusion that might occur through the polymeric matrix [83]. The model also assumes that the particles diminish proportionally over time.

The addition of MESK to the system requires a fifth reaction with an appropriate reaction rate.

$$5.\ \mathit{MESK} \rightarrow \mathit{SK} + \mathit{PEG} \qquad (\mathbf{k_5}) \tag{5.10}$$

where k_5 is the dissolution rate/release rate from the polymer as determined using the Hixon-Crowell model.

In order to calculate changes in species concentration with respect to both position and time, a finite-difference technique using a central difference method to approximate a derivative and the Crank-Nicolson method to approximate second derivatives has been implemented to arrange equation 5.7 into a mathematically tractable set of equations [84-88]. A full description of the model's derivation including all assumptions is available in Appendix B. The FORTRAN codes are located in Appendix C.

Clot lysis was determined by changes in fibrin concentration. The clot was considered digested at a specific position when the fibrin concentration dropped to 2/3 of its baseline level. When the fibrin concentration dropped below 3.882 μM, a zero was installed in the matrix. Minimum levels of various concentrations were defined within the simulation to facilitate stability of the calculations. Streptokinase dosage was 2500 IU in 7 ml of fluid (0.76 μM) to be consistent with in vitro studies discussed in Chapter 4.

The model was executed using a Pentium III 900 MHz processor with a FORTRAN compiler. In order to maintain stability of the model during calculations, time steps of 0.01 seconds (1.67E-3 min) and position steps of 0.015 cm were used. Clot lysis was modeled for a maximum of 30 minutes. Simulations of 30 minutes of clot lysis required approximately 15 minutes of computation time. Other parameters are listed in Table 5.1. A flowchart describing the calculation process is shown in Figure 5.1.

MODEL PARAMETER	VALUE		REFERENCE
Diffusivity of proteins (D)	3.00E-06	cm^2/min	Diamond and Anand (1993)
Diffusivity of MESK (D_{MESK})	5.00E-08	cm^2/min	--
k_{ADS} for SK-PLG to fibrin	5.75E-03	μmol/(L*min)	Fears et al. (1985)
k_{DES} for SK-PLG to fibrin	3.99E-03	min^{-1}	Fears et al. (1985)
θ (available sites) for SK-PLG to fibrin	0.39	μmol	Anand et al. (1997)
k_{ADS} for plasmin to fibrin	3.00E-02	μmol/(L*min)	Diamond and Anand (1993)
k_{DES} for plasmin to fibrin	3.26E-03	min^{-1}	Diamond and Anand (1993)
θ (available sites) for plasmin to fibrin	1.0	μmol	Diamond and Anand (1993)
k_{ADS} for MESK to fibrin	5.75E-05	μmol/(L*min)	--
k_{DES} for MESK to fibrin	3.99E-05	min^{-1}	--
θ (available sites) for MESK to fibrin	0.39	μmol	--
k_1	60	min^{-1}	Tuliani and O'Rear (1988)
k_{M1}	1.80E-03	min^{-1}	Tuliani and O'Rear (1988)
k_2	22.02	min^{-1}	Tuliani and O'Rear (1988)
k_3	0.24	min^{-1}	Tuliani and O'Rear (1988)
k_4	1500	min^{-1}	Tuliani and O'Rear (1988)

Table 5.1: Model parameters for simulations.

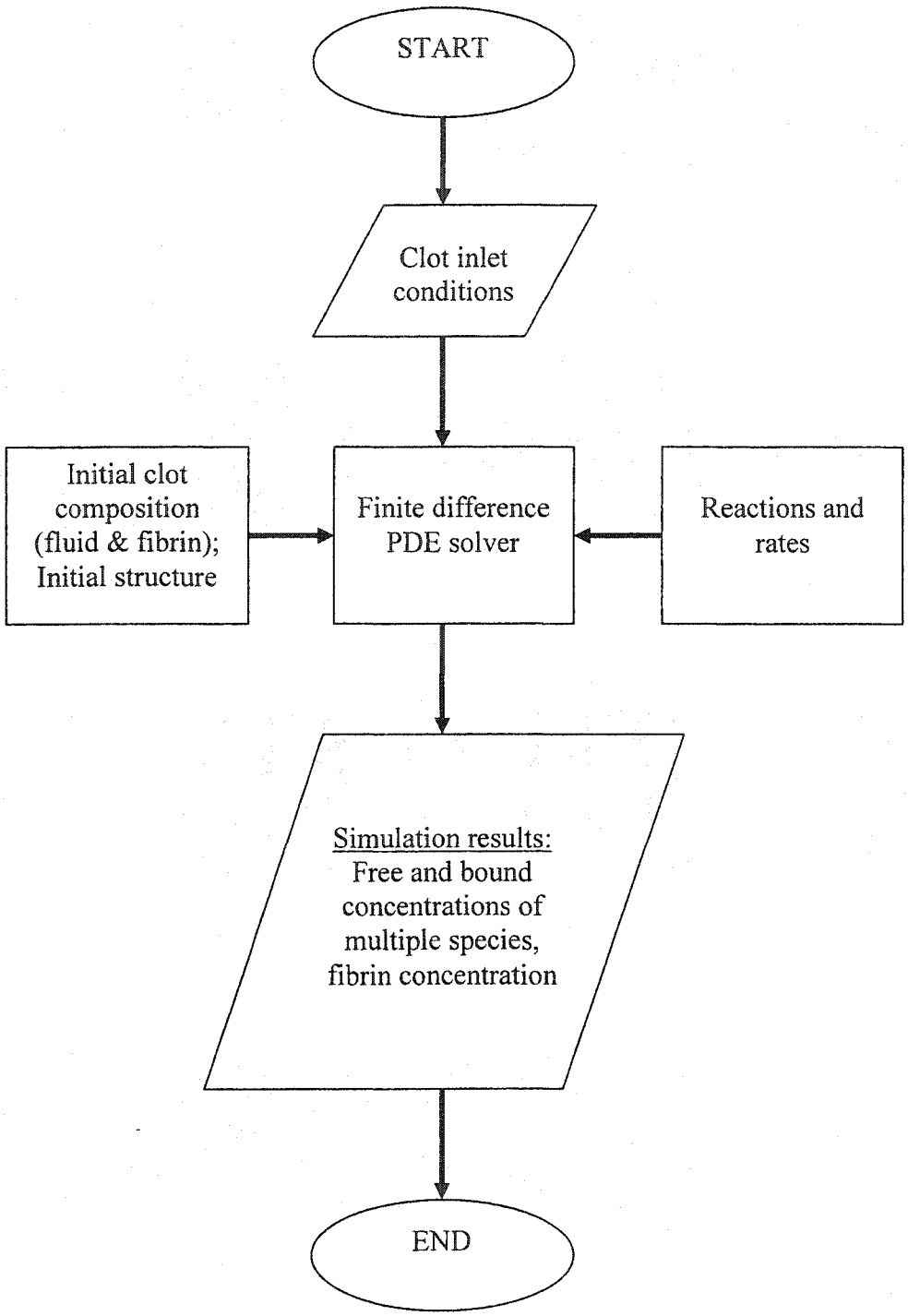

Figure 5.1: Flowchart for simulations of clot lysis with FREE SK and MESK.

RESULTS

Free Streptokinase

The ability of the model to accurately describe clot dissolution was examined first for free streptokinase. As predicted by Darcy's law, one would expect clot lysis to be more rapid at higher fluid velocities. Therefore, the constant velocity was varied from its initial value of 0.095 cm/min to both 0.95 cm/min and 0.0095 cm/min, and clot lysis was simulated with all other parameters remaining constant.

As seen in the top panel of Figure 5.2, fluid velocity has a significant effect on clot lysis times. More than 70% of the clot was digested in 30 minutes at 0.095 cm/min. When the velocity is increased by an order of magnitude, more than 70% of the thrombus was digested in less than 3 minutes. In this case full clot lysis is not evident, most likely because a majority of the free streptokinase moves through the clot too quickly. When velocity is reduced to 0.0095 cm/min, less than 10% of the clot is degraded in the 30 minute simulation. This velocity approximates the condition of diffusion-mediated transport alone, a condition described previously [28, 33].

It is also logical to expect that clot lysis would proceed faster with a greater initial concentration of plasminogen activator. To examine the effect of streptokinase concentration, initial streptokinase levels were examined at 0.076, 0.76, and 7.6 μmol (Figure 5.2, bottom). Streptokinase concentrations of 0.76 μM correspond to concentrations used in humans (~300 IU/ml or 1.5e6 IU). More than 70% of the clot was lysed in 30 min using the baseline value of 0.76 μmol, while only 50% dissolved at 0.076 μmol. The entire clot was digested at the highest initial concentration of streptokinase.

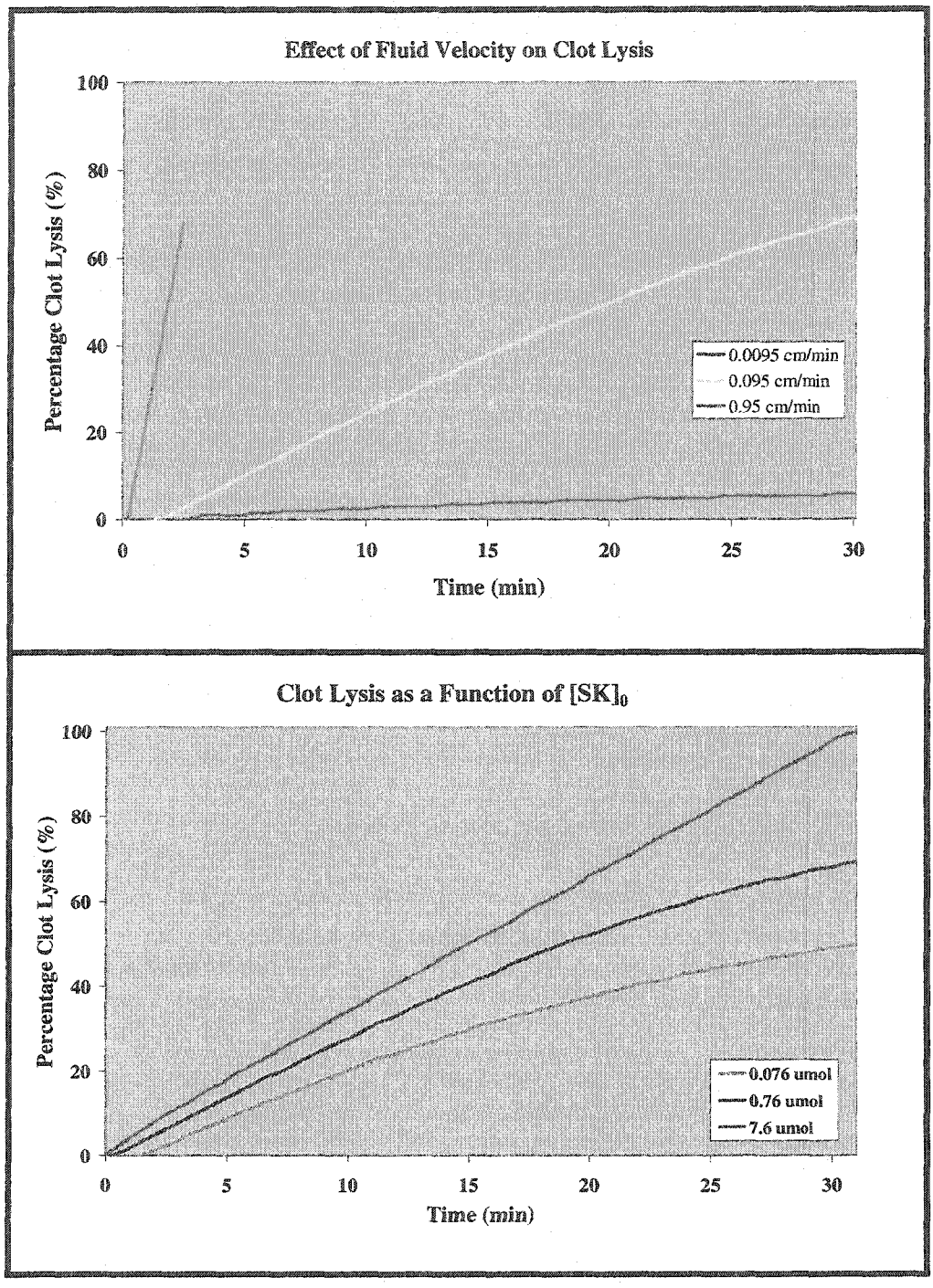

Figure 5.2: Clot lysis with FREE SK is greatly affected by fluid velocity (top) and initial concentration of free streptokinase (bottom).

This high concentration, however, is not a viable clinical option due to the greatly increased potential for severe bleeding.

Based on reasonable changes in clot lysis when implementing order-of-magnitude variations of multiple parameters, the model for free streptokinase appears adequate based on these assumptions. Clot lysis results from in vitro studies using free streptokinase in Chapter 4 were utilized to gauge the model's accuracy at one initial pressure drop. Comparing the simulation to FREE SK lysis of plasma clots at an initial pressure drop of 10 mmHg/cm-clot (0.095 cm/min), the model seems to perform properly (Figure 5.3). However, the model deviates from experimental values when the pressure drop (i.e. velocity) is increased to 15 mmHg/cm-clot (0.118 cm/min).

Microencapsulated Streptokinase

Based on reasonable results with the FREE SK simulation, the MESK model was examined for changes in clot lysis upon variation of parameters such as diffusion coefficient, adsorption/desorption to fibrin, and particle dissolution rate. As in the free streptokinase model, velocity was calculated from in vitro experiments in Chapter 4 and held constant. Thrombus porosity was also held constant. Lastly, drug release rate was calculated from the Hixon-Crowell model based on in vitro release of bovine serum albumin from microparticles and estimated at 0.022 min^{-1}.

For this first-generation simulation, the diffusion coefficient of the microparticles was held constant despite the decreasing size of the particles as they erode. An average diffusion coefficient was calculated as described in Appendix B and installed in the

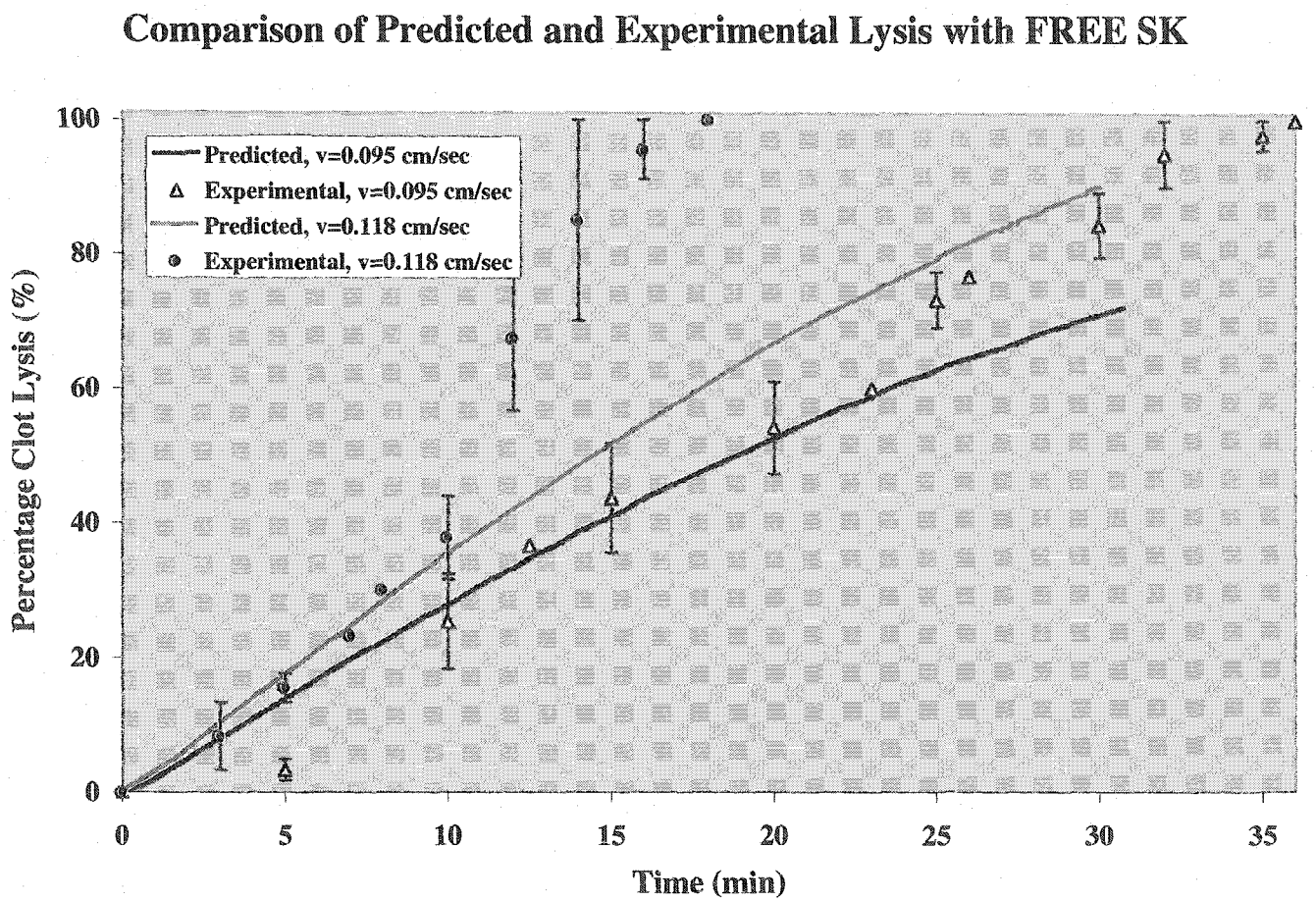

Figure 5.3: Comparison of model results to experimental lysis of plasma clots with FREE SK at 10 and 15 mmHg/cm-clot.

simulation with a value of 5.0E-8 cm^2/min. Using the Stokes-Einstein relationship, diffusion coefficient corresponds inversely to particle size. As particle diameter increases, the diffusion coefficient will get smaller. This enables the simulation to examine variations in particle size. Sensitivity of clot lysis to changes in diffusion coefficient was examined with values of 5.0E-6, 5.0E-8, and 5.0E-10 cm^2/sec. Simulation results in Figure 5.4 (top) demonstrate that clot lysis is not sensitive to changes in diffusion coefficient for this model. However, the time to achieve clot lysis is accelerated to just under 8 min with MESK at any diffusion coefficient with the prescribed $k_{dissolve}$=0.022 min^{-1}.

The effect of streptokinase release was examined by varying the release rate ($k_{dissolve}$) of drug from the microparticles at 0.0022, 0.022, and 0.22 min^{-1}. It is expected that faster release of streptokinase from the microparticles would translate into faster clot digestion. Results from variations in drug release rate are shown in Figure 5.4 (bottom). As expected, increases in release rate from result in significantly faster clot digestion. Based on the faster dissolution than that observed in vitro, it is suspected that the initial calculation of $k_{dissolve}$ is too large.

To quantify the effect of adsorption of the microparticles to the thrombus, order-of-magnitude variations in these values were simulated. As there are no previously determined values, it was assumed that the rates are dramatically slower than free streptokinase, as polyethylene glycol is known to resist adsorption. For this reason, this phenomenon was investigated at k_{ads} values on the order of E-1, E-3, and E-5 μmol/(L*min). The rate of adsorption was varied with higher and lower release rates than 0.022 min^{-1}, and no significant changes were observed in clot lysis with this model.

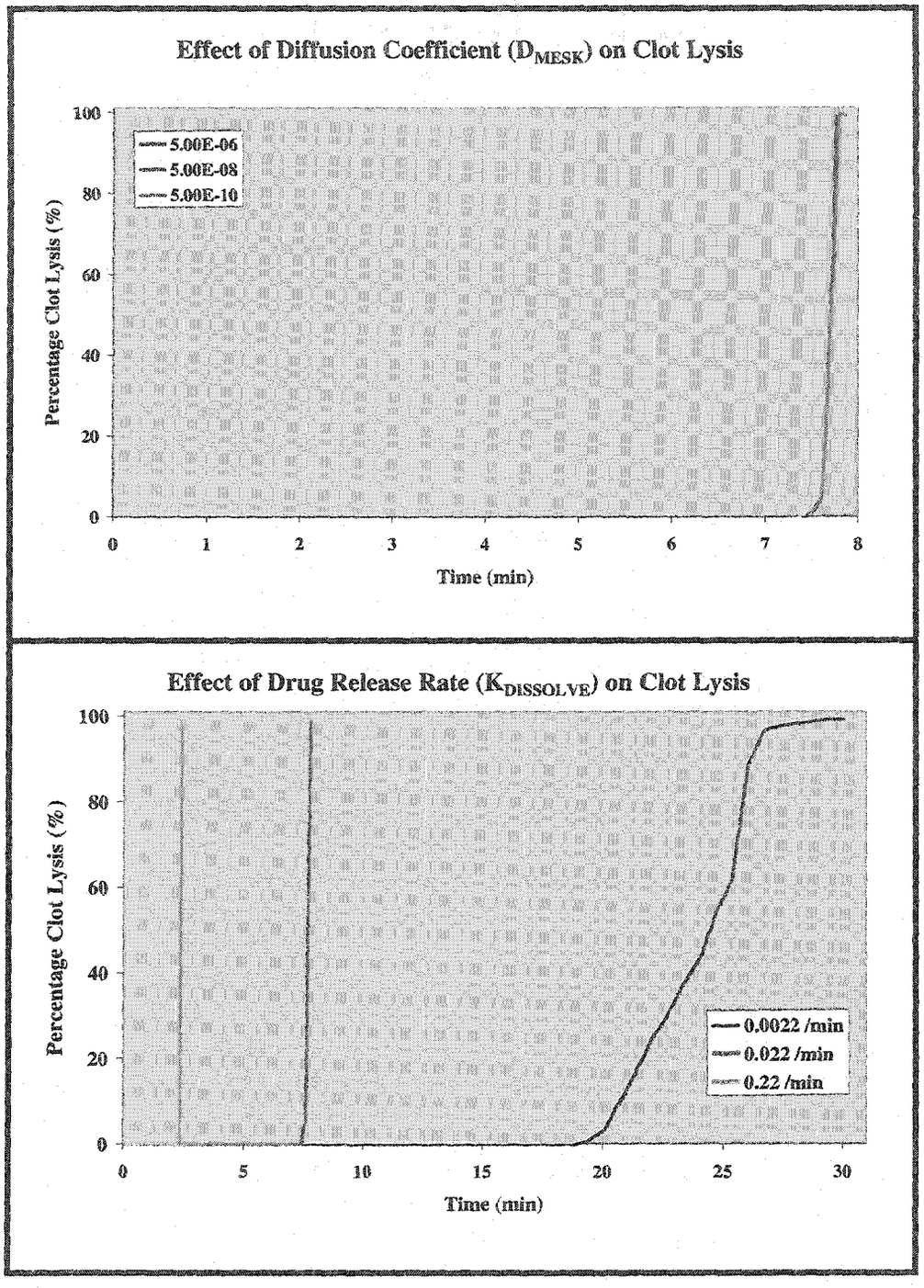

Figure 5.4: Clot lysis with MESK is not affected by changes in microparticle diffusion coefficient (top), but is significantly affected by drug release rate (bottom).

The lack of sensitivity to rates of adsorption and desorption was unexpected. In short, the MESK model is currently only sensitive to changes in velocity, streptokinase concentration, and release rate. However, increased distribution of streptokinase throughout the clot has been observed experimentally using confocal microscopy with MESK (Figure 4.5) compared to free streptokinase, where most of the agent is adsorbed to the leading edge. Simulations examining the movement of free streptokinase show increasing distribution of the agent over time throughout the clot (Figure 5.5, top), a result in stark contrast to FREE SK simulations. Depressions in fibrin concentration within the thrombus approximate the void formations observed in vitro in Chapter 4 (Figure 5.5, bottom). Due to increased spatial distribution of drug, it is likely that the limitations and assumptions of this model are prohibiting substantial differences from becoming obvious.

Clot lysis results from in vitro studies using MESK in Chapter 4 were utilized to gauge the model's accuracy at one initial pressure drop. Comparing the simulation to MESK lysis of plasma clots at an initial pressure drop of 10 mmHg/cm-clot (0.08 cm/min), the model seems to perform adequately at later times provided the release rate constant is changed to 0.0033 min^{-1} (Figure 5.6). Due to the definition of clot dissolution (fibrin reduction of 2/3), the model does not compare well at early times.

DISCUSSION

A first-generation model of clot lysis with free and microencapsulated streptokinase has been developed based on the convection-diffusion-reaction mass transport equation. The model of free streptokinase is sensitive to initial streptokinase

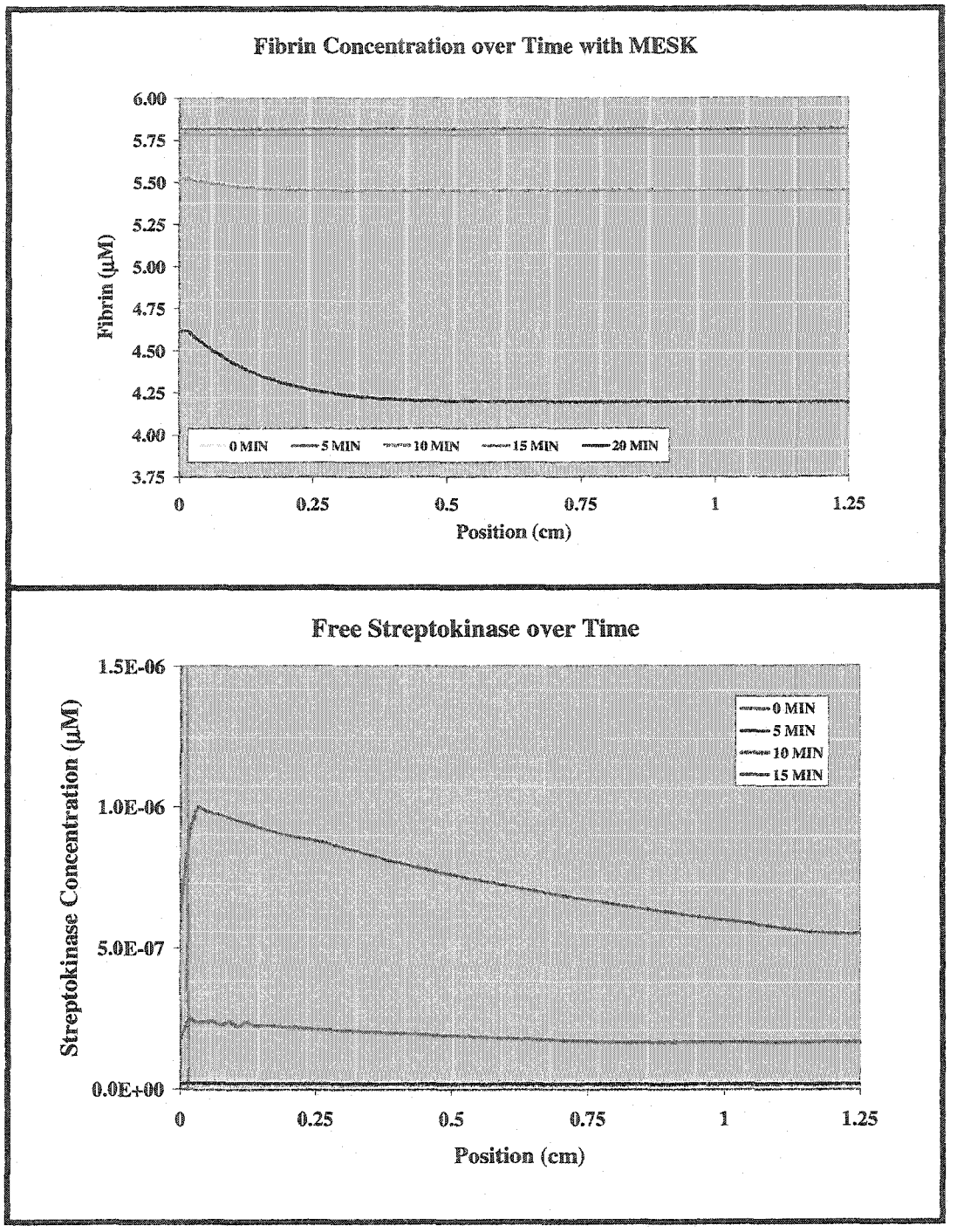

Figure 5.5: Despite the lack of sensitivity to changes in $k_{ADS,}$ fibrin concentration is reduced in the interior of the clot compared to the leading edge (top), while streptokinase concentration increases over time (bottom).

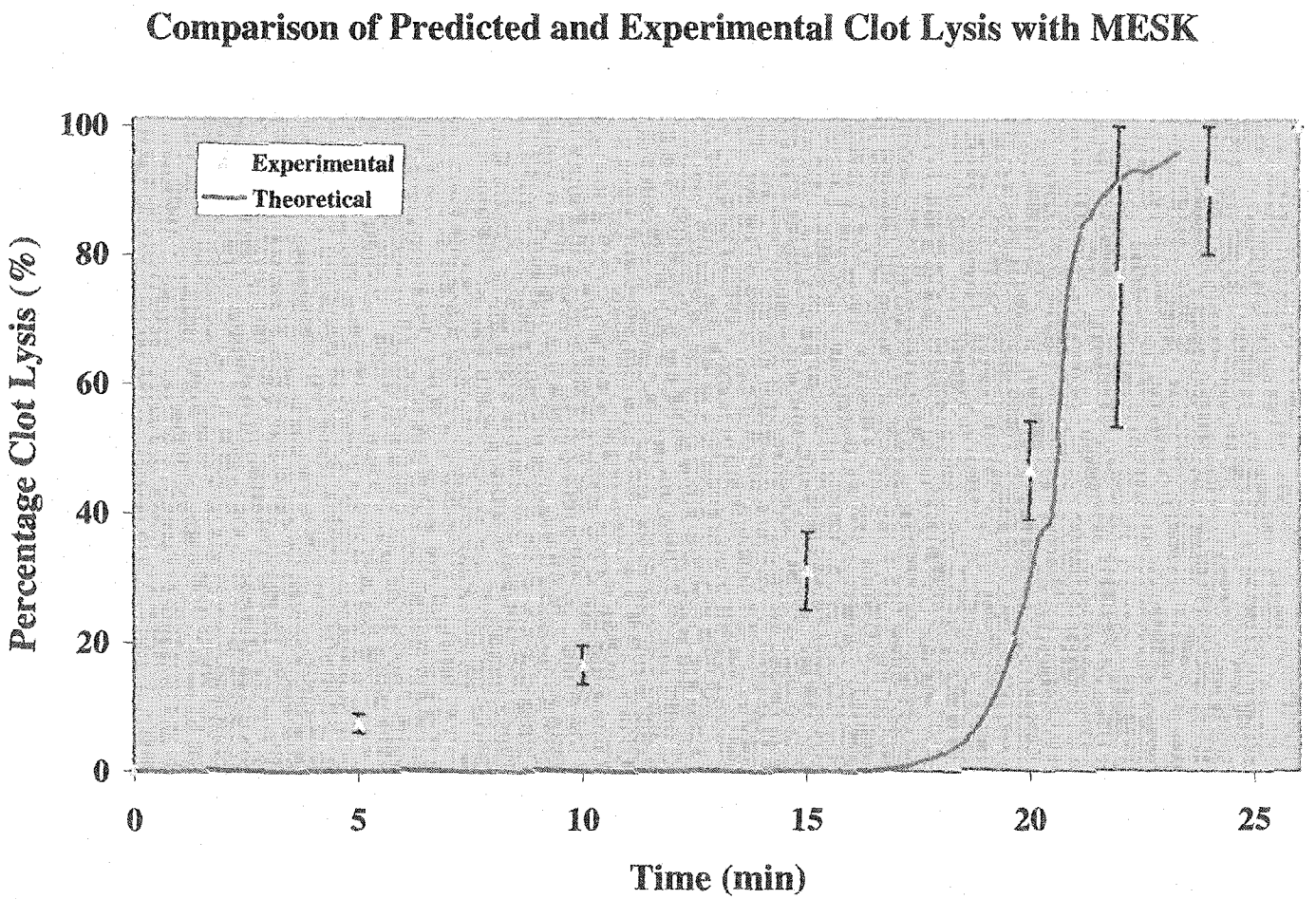

Figure 5.6: Comparison of MESK lysis of plasma clots at 10 mmHg/cm-clot to simulation with release rate of $K_{dissolve}$= 0.0033 min^{-1}.

concentration, porosity, and velocity. The simulation approximates experimental data of plasma clot lysis at 10 mmHg/cm-clot (Figure 5.3).

The model of lysis with MESK is presently sensitive only to velocity, initial streptokinase concentration, and release rate. Based on observations using confocal microscopy, it was expected that the model would be sensitive to changes in rates of adsorption to fibrin. However, no significant changes were observed when the adsorption rate of MESK was varied over four orders of magnitude.

The lack of sensitivity to changes in adsorption is likely due to the assumptions and idealization of the model. First, Darcy's law was not utilized. A constant velocity was calculated instead from in vitro studies and installed in the simulation. As clots shorten during lysis, the velocity of interstitial flow accelerates, thereby carrying more plasminogen activator into the thrombus and speeding digestion. Secondly, the release of streptokinase from the microparticles is assumed to be a first order reaction in which streptokinase is either entrapped within the polymer or is in the free circulation. It is possible that the microparticles release the drug in a different fashion with an intermediate. The intermediate complex would consist of streptokinase with attached polyethylene glycol. The conjugated PEG would limit adsorption to the thrombus as seen with confocal microscopy (Figure 4.5, left). Perhaps the addition of more reactions might capture the effect of adsorption. As mentioned above, the solution is not iterated. Therefore, more precise values would likely be achieved following an iterative solution technique. Lastly, the presence of clot is represented in this model as an "on/off" option, in which fibrin concentration must be above 3.882 μM (2/3 of the baseline value) or else

there is no clot. In reality, fibrin remains at lower concentrations and persists due to other variables that have not been considered in this model.

The computer simulation as currently written suffers from a deficiency in its solution method. Initially, the problem was attempted using an explicit method of solution which solves for the concentrations at the next time step using only known concentrations from previous time steps. This is an effective way to solve such a problem, but it requires very small times steps in order to maintain stability. In short, there is a large charge in computation time when using the explicit method. The hardware and software utilized to solve this simulation with the explicit method was not sufficient to maintain stability and collect output.

The implicit method, as utilized in this model, requires information from future time steps to solve for concentrations at future time points. Concentrations for the entire system are calculated and the system is solved iteratively until differences between concentrations are within a prescribed and acceptable error. Implicit techniques are inherently more stable than explicit techniques with a similar charge in computation time.

In this model, an iterative solution technique has not been implemented due to the available resources and time to achieve measurable changes in species concentrations. Output files, when dumped into a spreadsheet for manipulation, routinely exceed 70 megabyte in size. This results in a significant strain on computer resources. Secondly, iterative calculations are often continued when the change in concentrations is within a certain acceptable error. For this system, the change in species concentrations is minute for long periods of time, making the traditional guidelines for iterative calculations unfeasible.

In conclusion, this first-generation model requires much greater hardware and software resources to utilize an explicit method or an interative solution technique with improved hardware resources using an implicit method. Addition of either suggestion should provide improved results with this first-generation model.

ASSESSMENT OF THROMBOLYTIC EFFICACY OF MICROENCAPSULATED STREPTOKINASE IN A CANINE MODEL OF CORONARY ARTERY THROMBOSIS

CHAPTER 6

This chapter has been submitted in large part for publication.

INTRODUCTION

In order to determine the potential benefit of clot-busting formulations in a clinically relevant situation, canine models of coronary artery thrombosis have been utilized to investigate the efficacy of thrombolytic formulations for treatment of acute myocardial infarction [22, 23]. Canines are a particularly good model to examine fibrinolytic agents for two reasons. First, the vessels of the canine are much larger than other animals which might be used for such investigations, namely rabbits, rats, and mice. This makes the surgical procedures more technically feasible for investigators. Perhaps more importantly, canines develop collateral vessels in the early course of their life, much like older humans. The collateralization phenomenon allows one to study fibrinolytic agents in a healthy specimen instead of the older or diseased specimens in

other species which would be required to simulate humans. While the results obtained with encapsulated streptokinase in the rabbit model of carotid artery thrombosis are promising (Chapter 3), there is a need to examine this formulation in a more clinically relevant model.

Due to the large size of the animal, small modifications in the preparation of MESK were required in order to encapsulate enough streptokinase in a tolerable mass of polymer. To achieve this level of encapsulation, a larger initial concentration of lyophilized concentration was added to the polymer solution (250,000 IU/ 2 gm PEG) compared to that used in the rabbit studies (80,000 IU/1 gm PEG). Additionally, the effect of streptokinase concentration per particle was investigated in two small groups by preparing both high and low concentration per particle dosage forms relative to the formulation used in the majority of canine MESK studies. The variations in concentration per particle were achieved by varying the initial mass of PEG while keeping the initial streptokinase concentration constant.

MATERIALS AND METHODS

Preparation of MESK

Streptokinase (SK) was entrapped in a water-soluble polymer through a modified double emulsion method [70, 71]. Briefly, polyethylene glycol (PEG, 2 gm, 20,000 MW; Polysciences, Inc.) was dissolved at room temperature in 3 mL chloroform (Fisher Scientific, Pittsburgh, PA) and SK (Streptase®, 250,000 IU, Behringwerke AG) was dissolved separately in 0.4 mL distilled water. Aqueous protein solution was added to the polymer solution and immediately dispersed by sonication at 40-50 W for 1 min. The

protein/polymer solution was mixed with 8 mL of 2% (w/v) polyvinyl alcohol (PVA, average MW 30,000-70,000, Sigma Chemical) and vortexed 20 s. The mixture was placed in a freezer at -80°C for 2 h and then lyophilized for 15-20 h. The lyophilized product was washed with hexane in a centrifuge at 600 *x g* for 10 min at 4°C, and then filtered under vacuum through 0.45 μm membranes to remove any externally bound protein, and dried in a fume hood for 1 h. The preparation process was repeated to insure the availability of an adequate dosage for the experiment. Final product composed of porous, mostly spherical particles with an average diameter of 0.810 ± 0.054 μm (n=13; mean ± SD) was then stored at room temperature until use. Particle size was determined by light scattering studies using a Coulter LS Particle Size Analyzer. A blank formulation was prepared by replacing the SK solution with an identical volume of distilled water and completed as described above. High concentration per particle formulations (HI MESK; n=3) were prepared by adding 250,000 IU streptokinase to 4 gm PEG, while LO MESK (n=3) was prepared by adding the same concentration of streptokinase to 8 gm PEG.

Encapsulation efficiency

SK content in the microparticles was quantified by verifying protein concentration and protein activity. The concentration of encapsulated protein was determined with a commercial protein assay using bicinchoninic acid (Pierce Chemical) after dissolving the microparticles (10 mg in 1 mL PBS) completely. SK concentration in each sample was determined spectrophotometrically using a standard curve of SK with blank microparticles at identical concentrations.

Activity of entrapped SK was determined using a commercial chromogenic assay (Helena Laboratories). Microparticles (5 mg in 1 mL PBS) were allowed to dissolve completely in distilled water for at least 45 min. Samples were collected, incubated with plasma, and the kinetics of plasminogen-SK complex formation were determined spectrophotometrically at 405 nm as a measure of SK activity by following the release of *p*-nitroaniline from the chromogenic substrate. Samples were compared to that of a standard curve of SK with blank microparticles.

Animal model

The fibrinolytic activities of various formulations of free and encapsulated streptokinase were examined in a canine model of coronary thrombosis (Figure 6.1) originally described by Kopia and co-workers [90] and modified in our laboratory [22]. Animals were treated under procedures that were examined and approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center and the University of Oklahoma, Norman. Male dogs (15-25 kg) were anesthetized and maintained with intravenous sodium pentobarbital (30 mg/kg). An endotracheal tube was inserted to respire the animal with room air using a Harvard ventilator. Cannulae were inserted into the left and right femoral veins for drug administration. An electrocardiogram (V-2) was measured from the body surface for the duration of the experiment.

A left thoracotomy was performed in the fourth intercostal space and the heart exposed. The left anterior descending coronary artery was isolated at the tip of the left atrial appendage. Coronary artery flow was measured with a 20-MHz pulsed Doppler

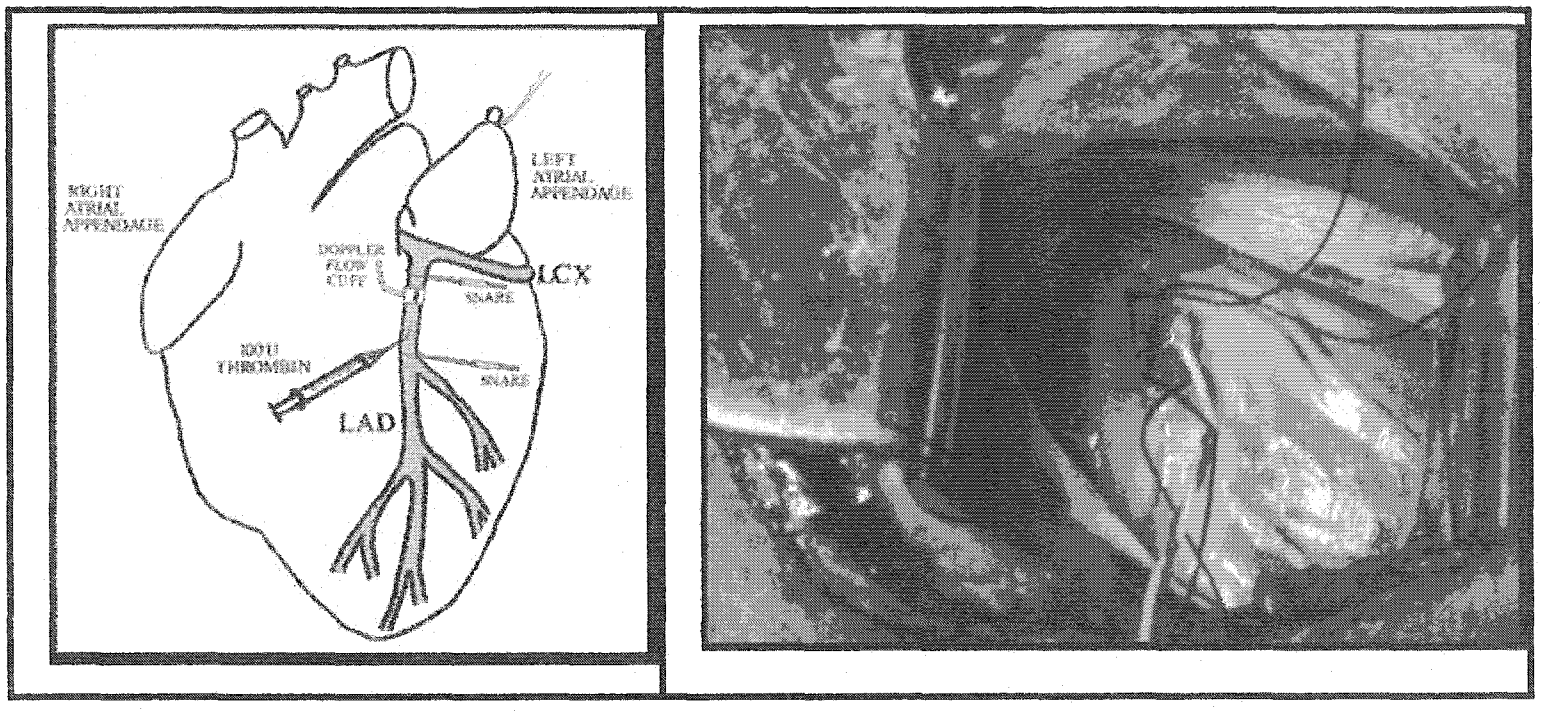

Figure 6.1: Canine model of coronary artery thrombosis. On the left is a cartoon diagramming the placement of the occlusive thrombus within the left anterior descending coronary artery, as well as the placement of the Doppler flow cuff to monitor velocity of flow. On the right is an image of a canine heart used in this in vivo study. Cartoon is courtesy of Dr. Eugene Patterson.

probe. Autologous whole blood (0.1 mL) was withdrawn slowly over 10 s from the coronary artery into a glass syringe containing approximately 100 units thrombin (Sigma Chemical). The autologous blood with thrombin was injected into an ensnared 5-10 mm long segment of the left anterior descending coronary artery. After 10 min, the proximal ligature was released, as was the distal ligature after an additional 5 min. The thrombus was allowed to mature for an additional 30 min prior to administration of the study formulation. The presence of an occlusive thrombus was confirmed in three ways: 1) the cyanotic appearance of the left ventricle; 2) the lack of flow demonstrated with the Doppler flow probe; and 3) S-T-segment changes in the ECG. A mean of 2.7 ± 0.3 injections were required to achieve thrombosis with saline and did not differ significantly between treatment groups.

Dogs were randomly selected to receive one of five infused formulations: saline; blank microcapsules (6 gm, BLANK); free SK (FREE SK, 240,000 IU); free SK/blank microcapsules (FREE SK + BLANK, 240,000 IU); and MESK (240,000 IU). Each preparation was separated into 4 vials with the contents of each vial suspended in 8 mL normal saline and administered consecutively for a total infused volume of 32 mL. An initial bolus of 20,000 IU in the first minute preceded a constant infusion of the remaining formulation over a 2 h period. Animals were observed for 4 h following the initiation of infusion. Reperfusion was documented by a recovery of left descending coronary arterial flow. Sustained reperfusion was defined as flow returning to at least 20% of the baseline flow for the remainder of the experiment without another occlusion event.

Blood loss from the injury sites (femoral vein exposure, chest cavity exposure, and thrombin injection site in the coronary artery) was collected starting with the administration of the study formulation by cotton sponges and measured gravimetrically. At the conclusion of the experiment, 2 mL of blood was collected in 3.8% (wt/wt) sodium citrate at a ratio of 9:1 blood:anticoagulant and immediately centrifuged to obtain plasma. Samples were frozen for later determination of plasma fibrinogen with a commercial assay (Sigma Chemical). Clotting times were obtained in duplicate for each sample and compared to a standard curve of human plasma.

At the conclusion of the experiment, the heart was removed and the left anterior descending coronary artery was dissected. Residual thrombus mass was determined gravimetrically. The heart was sectioned into 5-mm slices parallel to the AV groove and stained with triphenyltetrazolium chloride, a histological stain forming a brick-red precipitate in the presence of intracellular dehydrogenases, delineating vital myocardium. Infarcted myocardium was distinguished by the absence of staining. After storage overnight in 10% buffered formalin, infarcted tissue was separated from surviving myocardium with a scalpel blade and expressed as percentage of total left ventricular mass.

Statistics

Using the Student-Newman-Keuls Multiple Comparisons Test, p values < 0.05 were considered significant. Unless otherwise stated, all results are expressed as mean $\pm$ standard error.

RESULTS

Encapsulation efficiency

Encapsulation efficiency was determined with commercial assays both by the concentration of protein present in the microparticles as well as the activity of the protein. Recovery of materials in the formation of microparticles (PEG + SK + PVA) was 91 ± 1% (n=14). The efficiency of protein entrapment based on concentration was 64 ± 7 %. However, administration dosages were determined using the activity-based encapsulation efficiency test. Efficiency based on activity averaged 32 ± 2%. It is likely that SK activity is lost during the preparation process, a common observation when encapsulating proteins in microparticles using this method [65].

Reperfusion

As expected, no reperfusion occurred by treatment with saline alone or blank microcapsules suspended in saline. MESK produced a significantly reduced mean initial reperfusion time of 24 ± 4 min compared to FREE SK ($p<0.01$) and FREE SK + BLANK ($p<0.05$) with similar mean reperfusion times of approximately 50 min (Figure 6.2). Sustained reperfusion time was defined as continuous flow through the once-occluded vessel of at least 20% of baseline for the remainder of the experiment. One MESK-treated animal did not reperfuse, while another did not achieve sustained reperfusion after an initial reperfusion time of 23 min. FREE SK reperfused 77% of those animals treated (11/14) while FREE SK + BLANK resulted in a 92% reperfusion rate (12/13). Comparable dosages of MESK resulted in a significant reduction in the average time to

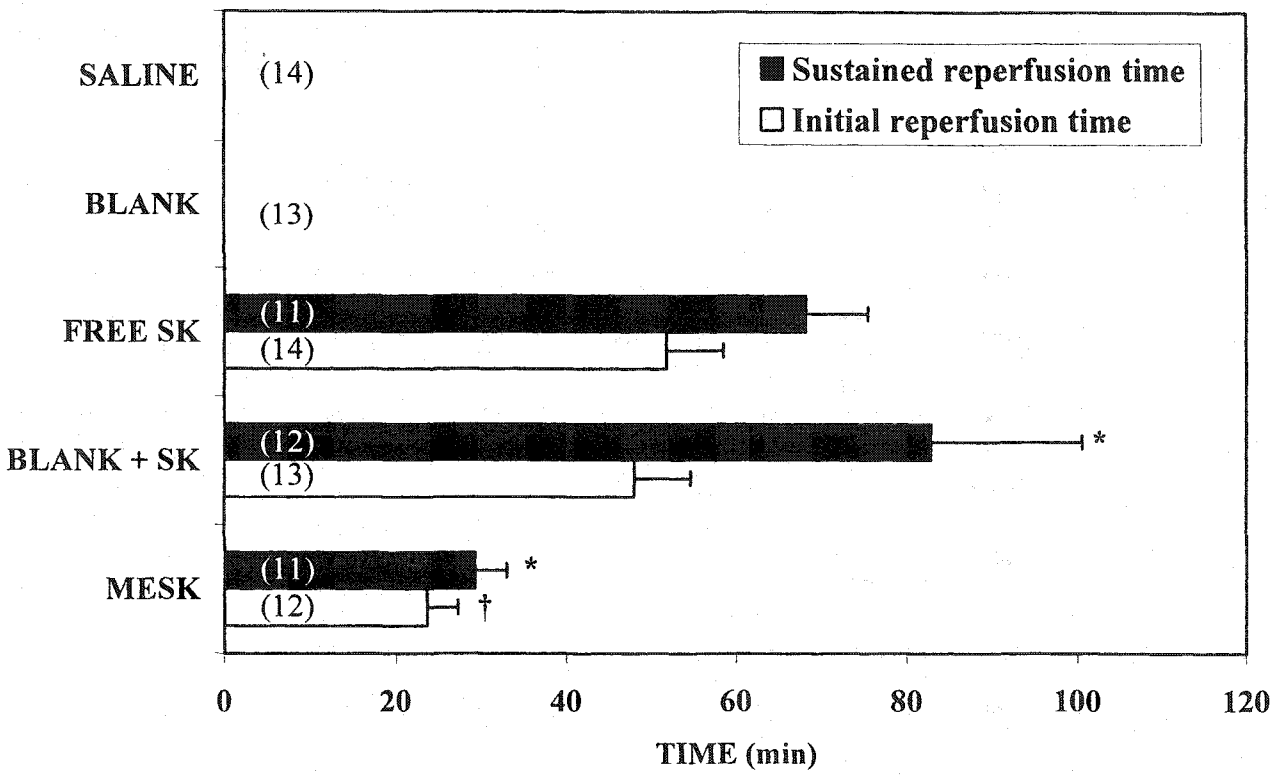

Figure 6.2: Average initial and sustained reperfusion times for each treatment formulation.

* $p<0.05$ vs. FREE SK; † $p<0.01$ vs. FREE SK

achieve sustained reperfusion (29 ± 4 min vs. 68 ± 7 min for FREE SK and 83 ± 18 min for FREE SK + BLANK).

Initial and sustained reperfusion times were comparable for MESK as a result of the reduced frequency of reocclusion episodes (Table 6.1). MESK-treated canines experienced 1.0 ± 0.5 reocclusion events per animal during the 4 h period following treatment onset, while FREE SK- and FREE SK + BLANK-treated animals demonstrated reocclusion more frequently. Occluded coronary vessels lysed with MESK tended to stay open, while those vessels lysed with either FREE SK formulation experienced more episodes of reocclusion following initial restoration of flow (Table 6.1).

Infarct mass

Infarct mass is expressed as a percentage of total left ventricular (LV) mass (Table 6.1). Controls of blank microcapsules added to either saline or FREE SK yielded infarct mass equal to the respective preparations without microcapsules. The infusion of any SK formulation demonstrated significant decreases in infarcted tissue, with both FREE SK formulations producing relatively equal infarct values and MESK resulting in a nearly 50% reduction in infarct mass compared to FREE SK formulations ($p<0.05$ vs. FREE SK/FREE SK + BLANK).

Bleeding

Bleeding is known to be a common problem with streptokinase due to the systemic lytic state invoked by the agent. Additionally, circulating residual plasmin degrades several plasma proteins, particularly fibrinogen, Factor V, and Factor VIII [8].

	Residual Clot Mass [mg]	Reocclusion Episodes	Infarct Mass [% LV]	Blood Loss [g]	Fibrinogen [μmol/L]
SALINE	30.2 ± 4.1 (14)	--	19.2 ± 2.9 (13)	4.7 ± 1.4 (4)	14.4 ± 0.8 (5)
BLANK	38.0 ± 5.5 (13)	--	19.1 ± 2.2 (13)	8.9 ± 4.7 (4)	14.6 ± 0.5 (4)
FREE SK	12.8 ± 2.3 (14)	2.0 ± 0.4 (14)	12.3 ± 2.4 (13)	36.9 ± 7.6 (4)	6.7 ± 0.4 (4)
FREE SK + BLANK	9.5 ± 3.8 (13)	2.9 ± 1.0 (13)	12.2 ± 2.0 (13)	52.6 ± 18.9 (5)	5.0 ± 0.3 (4)
MESK	7.2 ± 3.2 (13)	1.0 ± 0.5 (13)	6.5 ± 1.5 (12)	11.5 ± 6.0 (3)	12.4 ± 1.4 (4)

Table 6.1: Residual clot mass, reocclusion episodes, infarct mass, and bleeding complications in a canine model of coronary thrombosis.

To quantify bleeding complications accompanying each agent, blood released from the injury sites after the initiation of drug therapy was absorbed by multiple sponges and weighed (Table 6.1). Animals treated with saline alone or blank microcapsules demonstrated a lack of bleeding from all sites of injury. As expected, animals receiving FREE SK or FREE SK + BLANK showed more bleeding than saline-treated dogs. In stark contrast, MESK treatment resulted in significantly reduced blood loss compared to FREE SK. As another means to quantify bleeding potential, plasma fibrinogen levels were examined for each treatment modality (Table 6.1). Dramatic drops in circulating fibrinogen levels determined in non-SK controls were observed only with FREE SK formulations (60% on average), while MESK-animals retained significantly higher levels of the clotting factor (only 15% drop). Graphical comparisons of blood loss and fibrinogen levels are presented in Figure 6.3.

DISCUSSION

Rapid restoration of blood flow to ischemic tissue is paramount to improve survival and organ function. Although multiple techniques are available to remove the occlusive thrombus and restore flow, the administration of plasminogen activators is a practical and effective method that can be utilized despite the presence of highly trained personnel and equipment.

The early open artery hypothesis proposes that faster reperfusion results in increased salvage of ischemic myocardium at risk for infarction, improved preservation of ventricular function, and improved survival. The extent of myocardial infarction in a canine model has been directly linked to the duration of occlusion [74]. Newby and

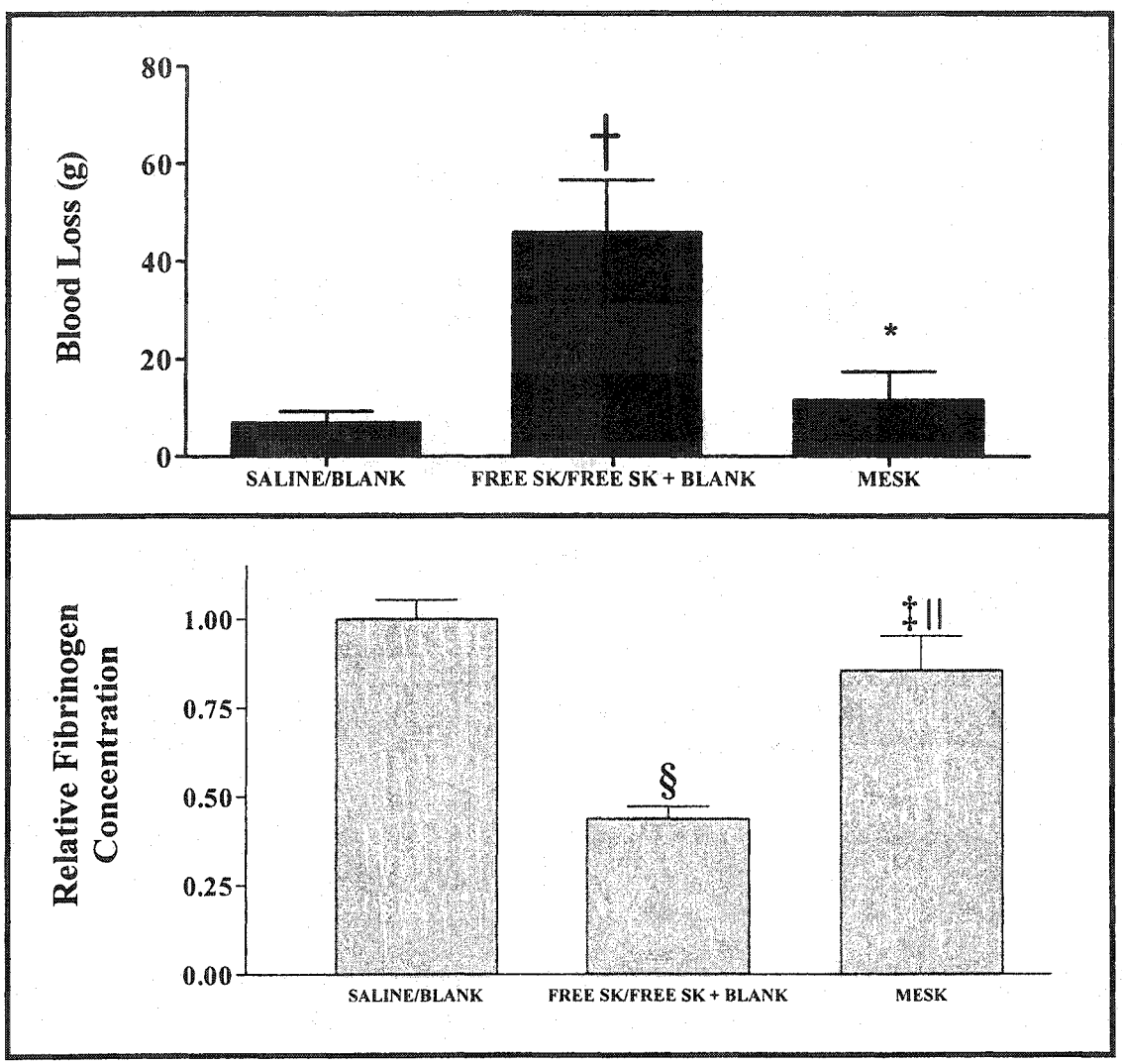

Figure 6.3: Bleeding complications for grouped thrombolytic formulations comparing blood loss (top) and fibrinogen concentrations relative to saline (bottom). * $p<0.05$ vs. FREE SK/FREE SK + BLANK; † $p<0.01$ vs. SALINE/BLANK; ‡ $p<0.001$ vs. FREE SK/FREE SK + BLANK; § $p<0.001$ vs. SALINE/BLANK; ‖ $p<0.05$ vs. SALINE/BLANK

others have demonstrated that increased mortality is observed clinically for every hour of treatment delay [75, 91, 94, 95]. Therefore, it is desirable to affect reperfusion therapy as soon as possible and restore blood flow to deprived tissues.

Drug encapsulation systems have typically been applied for controlled or timed release, targeted delivery of agent, and/or mitigation of toxic side effects [63]. In the case of plasminogen activators, drug delivery vehicles block premature reaction to reduce systemic activation and depletion of key components of the fibrinolytic system while also accelerating reperfusion and increasing the extent of thrombus removal. Encapsulated plasminogen activators clearly show promise both in enhancing performance and in addressing concerns related to their underutilization. Data from animal models confirm maintained fibrinogen levels, reduced blood loss, and fewer incidences of reocclusion.

In addition, encapsulation theoretically tends toward favoring convective delivery of agent over diffusion, so that (say) a blocked coronary artery should receive preferential delivery over a hemostatic plug in a small vessel wall or blocked capillary. Dissolution of hemostatic plugs is a highly diffusion dependent phenomena. This favoring of convection over diffusion can be realized by examining the Mass Flux Equation:

$$N_A = -D_{AB}\nabla C_A + x_A(N_A + N_B)$$

This equation shows that the flux of a chemical species (N_A) generally depends on diffusion ($-D_{AB} \nabla C_A$) and convection ($x_A(N_A+N_B)$). Encapsulation decreases diffusivity to the benefit of convective sites. The reduction in diffusivity can be appreciated from the Stokes-Einstein equation:

$$D_{AB} = \frac{kT}{6\pi * r * \mu_B}$$

where kT indicates the native thermal energy, r represents the size of the species and μ_B represents the viscous resistance to motion. It is seen that increases in the species radius decrease the diffusivity of the agent. Encapsulation of streptokinase increases its radius by several orders of magnitude (from 3 to 800 nm with this formulation). Sites with significant convection, such as an occlusion in a coronary artery near a bifurcation, should be favored over an occluded capillary or a hemostatic plug on a vessel wall as the diffusivity is greatly reduced. Possible synergism between molecular advances in plasminogen activators and drug delivery systems could help these agents realize their potential for rapid intervention in cardiovascular crises.

In the present study, the intravenous infusion of MESK resulted in a greater than 50% reduction in the time required to achieve sustained reperfusion (Figure 6.2) accompanied by a reduction in the incidences of reocclusion (Table 6.1). Reocclusion is a common phenomenon with fibrinolytic agents that undoubtedly affects the level of infarct and mortality. Faster and more complete reperfusion also translates into smaller residual clot masses in the coronary artery. A smaller residual thrombus should act to reduce shear stress levels and the incidence of reocclusion.

More rapid reperfusion and fewer reocclusion events translate to less loss of myocardium. A relatively consistent infarct size (approximately 19% of LV mass) is produced in this model when thrombolytic treatment is not administered (Table 6.1). Reperfusion following FREE SK or FREE SK + BLANK administration reduced infarct size to approximately 12% of LV mass. The more rapid reperfusion observed with a comparable dosage of MESK further reduced the infarct size to 6.5%. While larger reductions in infarct size have been observed under conditions of more aggressive

thrombolytic therapy [93], this study demonstrates that increased myocardial salvage may be achieved with an encapsulated fibrinolytic formulation without additional bleeding risk.

The threat of uncontrolled hemorrhaging is always a concern when using plasminogen activators because clotting function is often compromised by the depletion of circulating fibrinogen levels subsequent to their administration [9] In particular, streptokinase, a relatively non-specific agent, activates both clot-bound and circulating plasminogen indiscriminately. In this canine model, each of the three SK formulations depleted circulating fibrinogen when samples were taken at the conclusion of the experiment (Figure 6.3, bottom). However, MESK maintained higher fibrinogen concentrations at mean values of 12.4 ± 1.4 μmol/L (420 ± 47 mg/dl) compared to 5.9 ± 0.4 μmol/L (199 ± 12 mg/dl) for the two FREE SK-formulations ($p<0.001$). Li found similarly that utilizing liposomal encapsulated urokinase led to higher fibrinogen concentrations than in canines treated with an identical dose of free urokinase [7.4 ± 0.2 μmol/L (250 ± 5 mg/dL) versus 4.7 ± 0.1 μmol/L (160 ± 4 mg/dL), respectively, $p<0.01$] [23]. By reducing the total exposure time of streptokinase to circulating plasma proteins through sequestration in microcapsules, encapsulation avoids the systemic depletion of fibrinogen. The benefit of maintaining higher fibrinogen levels during thrombolytic therapy can be seen in the mass of blood lost throughout the experiment with each formulation (Figure 6.3, top). The infusion of MESK resulted in blood loss comparable to that observed without fibrinolytic treatment (11.5 ± 6.0 gm), while the administration of FREE SK-formulations demonstrated significantly increased bleeding (45.6 ± 13.9 gm, $p<0.05$).

Liposomal encapsulated fibrinolytic agents have demonstrated enhanced thrombolysis over freely infused agents *in vivo* and *in vitro* with faster reperfusion times and reduced clot masses [22-25, 70] Issues of limited stability and prolonged preparation time, however, limit their commercial appeal as thrombolytic agents. The use of polymer-encapsulated streptokinase has now produced comparable results to those observed with liposomal encapsulation in an identical *in vivo* model [22] as well as additional positive findings on infarct size, reocclusion, and bleeding. Unlike aqueous liposomal formulations, this preparation has an extended shelf life. It has been observed that these microparticles retain original levels of activity with storage at room temperature for at least one year.

Compared to FREE SK, MESK improved thrombolysis for each endpoint in this study. MESK produced faster initial and sustained reperfusion times, reduced residual clot masses, fewer reocclusion episodes, smaller infarct mass, and reduced bleeding complications. Aggressive therapy in a clinical setting, made possible with encapsulated thrombolytic agents, could result in greater myocardial salvage.

CONCLUSIONS

CHAPTER 7

Current treatment for patients presenting with dramatically reduced blood flow to tissue due to occlusive thrombi is less than optimal. Although several methods are available for recanalization of the blocked vessel, each has drawbacks that limit their use and ultimately deprives the patient of an optimal chance of full recovery. Angioplasty, although effective, is not widely available. Additionally, time-consuming screening and a specialized environment is required. Plasminogen activators are widely available for use, but there are issues with uncontrolled hemorrhaging and arriving for treatment during the prescribed window. It is widely accepted that, due to the ease of administration, thrombolysis could be more effective if used for all eligible patients. Patient arrival outside the treatment window is a contraindication for thrombolysis. Faster clot lysis with encapsulated agents could widen the treatment window and allow for more patients to receive thrombolytic therapy. Encapsulation of plasminogen activators has previously shown efficacy in numerous models to lyse occlusive thrombi more effectively than identical concentrations of free plasminogen activators. Despite

varying models and agents, one theme is consistent throughout: encapsulated agents digest thrombi faster.

Previous in vivo studies utilizing liposomal encapsulated agents demonstrated faster reperfusion times with smaller residual clot masses compared to free agents. More complete clot digestion reduces the potential for restenosis or embolization. Despite the improvement over free activators, liposomal encapsulated activators have been slow to reach the clinic due to inherent difficulties in preparation and long-term stability. For this reason, a polymer encapsulated plasminogen activator has been investigated for its potential as a fibrinolytic formulation. As a solid dosage form, microencapsulated plasminogen activators possess extended storage potential that should improve the commercial appeal of this agent.

In this study, administration of MESK during in vivo experiments using a rabbit model of carotid thrombosis to approximate stroke and a canine model of coronary thrombosis as an approximation of myocardial infarction have each clearly demonstrated thrombolytic superiority over identical dosages of free streptokinase. Liposomal encapsulated streptokinase was also significantly better than free streptokinase in the rabbit model. Improved lysis capability was demonstrated by MESK in vitro as well. Based on the results of three occlusive models in this study (1 in vitro, 2 in vivo) along with studies performed by other laboratories worldwide, it should be evident that encapsulated plasminogen activators are more effective than free plasminogen activators.

In vitro studies have helped determine the mechanism of clot lysis with MESK. As seen in microscopic images, clot digestion occurs very differently with MESK than FREE SK. While FREE SK tends to bind to the leading edge and promote localized

reactions at the surface, MESK is able to penetrate the clot front so as to more effectively distribute the encapsulated streptokinase. With improved spatial distribution of streptokinase comes distributed intraclot thrombolysis, and therefore faster and more complete clot digestion.

Administrations of MESK in two in vivo models have provided strikingly different results than free streptokinase. In the rabbit model of carotid thrombosis, reperfusion times and residual clot masses were dramatically reduced while a greater return of flow was observed. More extensive studies in a canine model of coronary thrombosis returned significant reductions in reperfusion time, along with a host of other improved endpoints such as reduced bleeding, fewer reocclusion events, and decreased infarct size.

Despite the promising results achieved with encapsulated streptokinase, the microencapsulation of other plasminogen activators must now be explored. The reputation of streptokinase has become sullied over the years as an agent that results in uncontrollable bleeding with no true upside. In reality, it is the systemic degradation of fibrinogen that might be responsible for limiting the restenosis events so often reported with other plasminogen activators. The comparison of MESK to other microencapsulated agents is a question that arises whenever this research is presented, and the studies must be performed in order to answer this inquiry. More fibrin-specific agents such as alteplase or tenecteplase should be encapsulated to contrast results with that of streptokinase, a plasminogen activator with relatively little fibrin specificity.

As mentioned in Chapter 2, there have been recent studies describing the pegylation of plasminogen activators to increase circulation time and half-life. However,

the thrombolytic potential of these formulations have not been examined. As polyethylene glycol is known to resist protein adsorption, it would be prudent to pegylate streptokinase and test its efficacy using confocal microscopy. This series of experiments could bolster the evidence of improved transport to the interior of the clot.

While 10,000 and 20,000 MW polyethylene glycol has shown promise for this application, other polymers should be examined that might provide greater improvement. For example, natural polymers such as dextran or hyaluronic acid which possess high water-solubility could be comparable with fewer physiological limitations. Copolymers of polyethylene glycol with polyethylene oxide might also provide greater control over release rate and further increase circulation times.

Although the current administration of MESK has demonstrated significant benefits, other administration methods should be investigated in future studies. For instance, a bolus infusion of a mixture of smaller and larger microparticles would provide a range of release rates once in the circulation while limiting the infusion to a single infusion task not requiring constant supervision. This undoubtedly would limit potential administration errors by clinical personnel which could result in untimely and unexpected deaths. Perhaps more importantly, the examination of administration regiments could demonstrate that a significantly reduced dosage of MESK is required to effectively restore and maintain vessel patency.

Results from the numerical model demonstrate the tremendous effect that drug release rate has on clot lysis. Release rate, among other properties, can easily be manipulated by varying production parameters while using the double emulsion

technique. Optimization of the production process should be investigated as a means to enhance clot lysis with MESK.

In conclusion, the infusion of encapsulated streptokinase for treatment of occlusion is superior to free streptokinase, as shown in two in vivo models, an in vitro model, and a numerical simulation. MESK restores flow faster, more completely, and safer than FREE SK. Considering the mechanism for efficacy, it is not a leap to believe that comparable results could be obtained with other plasminogen activators. Comparable results with another agent such as t-PA or the newer tenecteplase could vault this technology to the clinical setting.

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APPENDIX A

DESCRIPTION OF ASSAY TECHNIQUES

Pierce BCA (Bicinchoninic Acid) Protein Assay Protocol

Dissolve 10 mg of each sample in 1.0 mL of water in microcentrifuge tubes. Place in refrigerator until ready to use.

Prepare a fresh set of protein standards by diluting a 2.0 mg/mL Albumin (Alb) stock in the same diluent as your sample (i.e. phosphate buffered saline should be used as diluent when dissolving sample as well as standards). A list of standard dilutions follows below:

Final Alb Concentration	Volume of Alb to add	Volume of Diluent to Add
2.0 mg/mL	300 µL **(stock)**	0 µL
1.0 mg/mL **(A)**	325 µL **(stock)**	325 µL
0.5 mg/mL **(B)**	325 µL **(A)**	325 µL
0.25 mg/mL **(C)**	325 µL **(B)**	325 µL
0.125 mg/mL **(D)**	325 µL **(C)**	325 µL
0.025 mg/mL **(E)**	100 µL **(D)**	400 µL
0.0 mg/mL	0 µL	300 µL

Take care to mix each tube sufficiently prior to using it in a subsequent dilution. In order to obtain 325 µL, it is suggested to pipette 2 x 100 µL, then 2 x 62.5 µL. This procedure can be carried out with the white Eppendorff pipette only. This should allow for more consistent results by not switching between instruments.

Prepare fresh BCA Working Reagent (WR) by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. Prepare sufficient volume of WR based upon the number of tests to be done. Each test tube sample requires 2.0 mL of the WR. For example, if you

have 12 samples (including standards), you will need 24 mL of WR. Therefore, mix 25 mL of Reagent A with 0.5 mL of Reagent B.

Label disposable glass test tubes with the appropriate concentration of standard or identity of sample. Add 2.0 mL of the WR to each tube. Use the purple-plunger pipette (200-1000 μL) and pipette 4 x 500 μL in order to prevent excessive uptake. Add 100 μL of each standard and sample to the appropriately labeled test tube. Mix well with vortex mixer. Place tubes in test tube rack in incubator at 37°C for 30 minutes. Turn on spectrophotometer in D212 to a wavelength of 562 nm.

Remove tubes and allow cooling to room temperature (~ 5 minutes). Measure the absorbance at 562 nm of each tube vs. a water reference. **ALL** readings must be complete within 10 minutes to ensure continuity of the results.

Protocol for Helena Laboratories Chrom-Z Plasminogen Assay (Activity Test)

Dissolve 5 mg of sample in 1.0 mL water. Place in refrigerator until ready to use.

Dissolve 30 mg of Blank MESK in 6 mL DI water for use in standard curve preparation (polymer solution).

Prepare Plasminogen Buffer (PLG-buffer)--

To 1.0 mL of PLG-buffer stock (green-capped vial), add 9.0 mL of DI water. Mix well.

Prepare reference plasma (SARP)--

Dissolve the stock (purple-capped vial) in 1.0 mL water. Mix well. First prepare a 100% dilution, then a 50% dilution.

100% SARP: Mix 100 µL stock + 500 µL PLG-buffer.

50% SARP: Mix 200 µL of the 100% SARP + 200 µL PLG buffer

Place in refrigerator until ready to use.

Prepare fresh standards using the following dilutions:

500 IU/mL: 35 µL of 10,000 IU/mL SK + 665 µL diluent

400 IU/mL: 320 µL of 500 IU/mL + 80 µL diluent

300 IU/mL: 120 µL of 500 IU/mL + 80 µL diluent

200 IU/mL: 200 µL of 400 IU/mL + 200 µL diluent

100 IU/mL: 100 µL of 200 IU/mL + 100 µL diluent

0 IU/mL: 300 µL diluent

Prepare 2 sets of standards: one with water as diluent and another with the polymer solution as diluent.

Prepare Plasmin Substrate (yellow-capped vial)

Add 2.0 mL distilled water and mix thoroughly. Refrigerate until use.

The kinetic analyses should be completed with a 96-well plate reader at 405 nm over 20 minutes.

To each well, add:

1. 30 μL of PLG-buffer
2. 3 μL of 50% SARP. Incubate at 37°C for 2-4 minutes.
3. 30 μL of standard or sample. Incubate at 37°C for 1 minute.
4. 30 μL of plasmin substrate quickly
5. Read at 405 nm for 20 min; automix once; V_{max} OD, 86 readings

Protocol for Sigma Chemical Fibrinogen Assay

Prepare reagents –

1. Reconstitute Fibrinogen Reference with 1.0 mL DI water. Restopper vial and allow to stand until dissolution is complete. Invert gently to mix. DO NOT SHAKE.
2. Reconstitute Thrombin Reagent with 6.0 mL DI water. Restopper vial and allow to stand until dissolution is complete. Invert periodically. DO NOT SHAKE.
3. Imidazole Buffer is provided as a liquid and requires no preparation prior to use.
4. Store all reagents at 4°C until use.

Specimen collection—

1. Collect venous blood in 3.8% sodium citrate at a ratio of 9 parts blood to 1 part anticoagulant (1:10 ratio).
2. Isolate plasma by centrifuging at 2500 x g for 15 minutes. Test immediately or freeze at -80°C until use.

Prepare fibrinogen reference dilutions—

Dilution	Fibrinogen Reference (mL)	Imidazole Buffer (mL)	Dilution Factor
1:5	0.5	2.0	2
1:10	0.5 of 1:5 dilution (Tube #1)	0.5	1
1:15	0.4 of 1:5 dilution	0.8	0.66
1:20	0.3 of 1:5 dilution	0.9	0.5
1:25	0.2 of 1:5 dilution	0.8	0.4
1:30	0.2 of 1:5 dilution	1.0	0.33

Specimen preparation—

Test plasmas are prepared by diluting plasma 1:10 with Imidazole Buffer in plastic tubes. Store dilutions at 4°C.

Sample testing—

1. Reconstitute Thrombin reagent and maintain at room temperature during testing.
2. To plastic tube, add 0.2 mL of the diluted plasma sample.
3. Incubate 1-3 min at 37°C (not longer than 5 min).
4. Following incubation, rapidly expel 0.1 mL of Thrombin reagent into the tube while simultaneously starting a timer. Record clotting time in seconds.
5. Repeat each sample and take the average clotting time.
6. Extrapolate concentration from calibration curve.

APPENDIX B

DERIVATION OF NUMERICAL SIMULATION

Key assumptions:

1. The concentrations of clot-bound plasminogen and antiplasmin remain constant within the clot, as any degraded clot-bound plasminogen is instantaneously replaced by plasminogen from the flowing fluid (Blinc et al., 1991).
2. Structural properties of the thrombus (i.e. porosity, permeability) do not change over time.
3. Velocity of interstitial flow is assumed constant (Blinc et al., 1991).
4. Diffusivity of each free species and MESK are uniform and constant throughout the clot (Blinc et al., 1991). In other words, a constant particle size of MESK is assumed, and it will travel with the same velocity as the fluid and not be sieved out through the clot.
5. Adsorption and desorption rate constants are constant for each species throughout clot digestion.
6. Streptokinase is either encapsulated or released, but there is no intermediate complex that might have lingering PEG molecules attached to the protein.
7. Release of streptokinase from the microparticles follows the Hixson-Crowell model.
8. Each node within the clot is digested when the fibrin concentration drops to 2/3 of its initial value [3.882 μM] (Blinc et al., 1991).

Derivation of Model Parameters:

DIFFUSIVITY

An average diffusion coefficient is assumed for MESK (D_{MESK}) by using the Stokes-Einstein relationship for diffusivity of a particle within a fluid:

$$D = \frac{k \cdot T}{6\pi r \cdot \mu}$$

where k*T indicates the thermal energy, r is the size of the species, and μ represents the viscous resistance to motion.

Upon assuming a constant diffusivity of free species of 3e-6 cm^2/min [from Diamond's numerous publications and knowing the radius of streptokinase (3.6 nm) [Damaschun et al., 1992; Wilkins et al., 1999] and assuming an average particle radius based on particle size measurements in our laboratory (0.2 μm; data not shown), a ratio of the equations is used to calculate the diffusivity of MESK.

$$\frac{D_{SK} = 3 \times 10^{-6} \frac{cm^2}{min} = \frac{k \cdot T}{6\pi \cdot \mu \cdot (3.6\,nm)}}{D_{MESK} = x \cdot \frac{cm^2}{min} = \frac{k \cdot T}{6\pi\, \mu (200\,nm)}}$$

$$D_{MESK} = 5 \times 10^{-8} \frac{cm^2}{min}$$

FLUID VELOCITY

An average fluid velocity is determined based upon flowrate data collected when digesting thrombi formed of plasma and thrombin in glass capillary tubes (Chapter 4). The flow (cm^3) was determined for multiple time steps, and averaged over the time of clot digestion. Velocity was then calculated by dividing the average flowrate by the cross-sectional area of the pipet. The result is shown below.

	FREE SK		MESK
Q	0.02678 cm^3	Q=	0.022604 cm^3
A	0.282743 cm^2	A=	0.282743 cm^2
V=	0.094715 cm/min	V=	0.079946 cm/sec

POROSITY OF THROMBUS

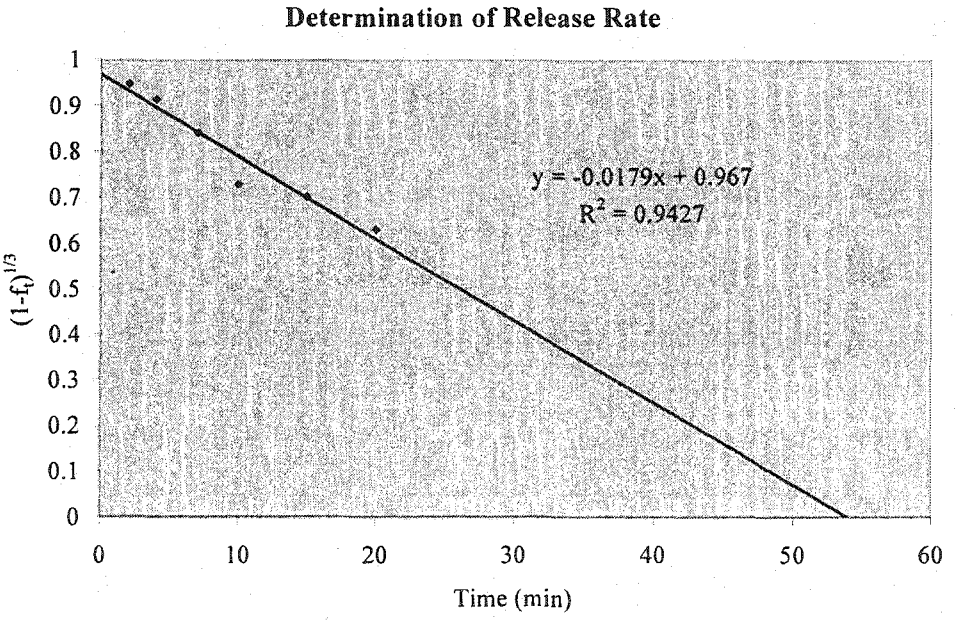
A value for an average thrombus porosity velocity was selected from Anand, Wu, and Diamond's paper [1995] in which the overall porosity for fibrin gels is greater than 0.99. Diamond et al. have consistently used $\varepsilon = V_{VOID}/V_{TOTAL} = 0.9928$.

KINETICS OF ADSORPTION AND DESORPTION

Values for the kinetics of adsorption and desorption were taken from several publications and hypothesized. The kinetic constants are listed in the table in Chapter 6. For the values of adsorption and desorption of MESK, they are merely speculated. As polyethylene glycol is known to resist protein adsorption, it is proposed that the rates of adsorption are at least 2 orders of magnitude smaller than the free streptokinase-plasminogen complex.

ESTIMATE OF MICROPARTICLE DISSOLUTION RATE

Based on numerous experiments monitoring the release rate of a model protein (bovine serum albumin) versus time for multiple preparation techniques, the Hixon-Crowell model was selected as the best empirical fit of the data. An estimate of the polymer dissolution/drug release rate is obtained by plotting the cubic root of the fraction of released drug against time, and the slope represents the release rate.



The value for K to 0.022 min^{-1} was then increased to better approximate the "average" release rate.

FLUID PHASE FREE and BOUND SK:

$$\frac{\partial}{\partial t}C_{SK}^{f} = D_{SK}^{f}\cdot\frac{\partial^2}{\partial x^2}C_{SK}^{f} - \left[v\cdot\left(\frac{\partial}{\partial x}C_{SK}^{f}\right)\right] - \left[\left(\frac{1-\varepsilon}{\varepsilon}\right)\cdot\frac{\partial}{\partial t}C_{SK}^{b}\right] + \left(-k_1\cdot C_{SK}^{f}\cdot C_{PLG}^{f} + k_{M1}\cdot C_{SKPLG}^{f}\right)$$

Initial Condition:

$C_{SK} = C_i$ for all x at t=0

Boundary conditions:

Left side: $\frac{\partial}{\partial x}C_{SK} = 0$ SK in plasma rises rapidly in just 5-10 min and then levels off more or less (Tuliani) Assume C_{SK} at x=0 is constant.

Right side: $\frac{\partial}{\partial x}C_{SK} = 0$

$\frac{\partial}{\partial t}C_{SK}^{b} = 0$ Assume no interaction of SK alone with fibrin!

FLUID PHASE AND BOUND PLASMINOGEN:

$$\frac{\partial}{\partial t}C_{PLG}^{f} = D_{PLG}^{f}\cdot\frac{\partial^2}{\partial x^2}C_{PLG}^{f} - \left[v\cdot\left(\frac{\partial}{\partial x}C_{PLG}^{f}\right)\right] - \left[\left(\frac{1-\varepsilon}{\varepsilon}\right)\cdot\frac{\partial}{\partial t}C_{PLG}^{b}\right] + \left(\left(-k_1\cdot C_{SK}^{f}\cdot C_{PLG}^{f} + k_{M1}\cdot C_{SKPLG}^{f} - k_2\cdot C_{SKPLG}^{f}\cdot C_{PLG}^{f}\right)\right)$$

Initial Condition:

$C_{PLG}^{f} = 2.2\,\frac{\mu mol}{L}$

Boundary conditions:

None required.

Assume that the concentration of plasminogen within the thrombus does not change with time since the activated plasminogen in the thrombus is instantaneously replaced by flowing blood from the blood plasma pool. Therefore, this equation does not need to be included in the solution (Zidansek and Blinc, 1991).

$\frac{\partial}{\partial t}C_{PLG}^{b} = 0$ $C_{PLG}^{b} = 47.618\,\frac{\mu mol}{L}$

FLUID PHASE AND BOUND SK-PLASMINOGEN COMPLEX:

$$\frac{\partial}{\partial t} C_{SKPLG}^{f} = D_{SKPLG}^{f} \cdot \frac{\partial^2}{\partial x^2} C_{SKPLG}^{f} - \left[v \cdot \left(\frac{\partial}{\partial x} C_{SKPLG}^{f} \right) \right] - \left(\frac{1-\varepsilon}{\varepsilon} \right) \frac{\partial}{\partial t} C_{SKPLG}^{b} + \left(k_1 \cdot C_{SK}^{f} \cdot C_{PLG}^{f} - k_{M1} \cdot C_{SKPLG}^{f} - k_2 \cdot C_{SKPLG}^{f} \cdot C_{PLG}^{f} \right)$$

Initial Condition:

$C_{SKPLG} = 0$ for all x at t=0

Boundary conditions:

Left side: $C_{SKPLG} = C_{SK}$ at x=0 for all t Assume equilibrium with high concentration of SK at x=0

Right side: $\frac{\partial}{\partial x} C_{SKPLG} = 0$

$$\frac{\partial}{\partial t} C_{SKPLG}^{b} = k_{ADS}^{SKPLG} \cdot C_{SKPLG}^{f} \cdot \left(\theta_{SKPLG}^{b} - C_{SKPLG}^{b} \right) - k_{DES}^{SKPLG} \cdot C_{SKPLG}^{b}$$

$$\theta_{SKPLG} = 1.5 \frac{\text{sites}}{\text{monomer}} \cdot 0.26 \frac{\mu\text{mol}}{\text{L}}$$

$$k_{ABS}^{SKPLG} = 0.95833e-4 \frac{\mu\text{mol}}{\text{L} \cdot \text{s}}$$

$$k_{DES}^{SKPLG} = 6.658e-5 \cdot \frac{1}{\text{s}}$$

FLUID PHASE and BOUND PLASMIN :

$$\frac{\partial}{\partial t} C_{PLM}^{f} = D_{PLM}^{f} \cdot \frac{\partial^2}{\partial x^2} C_{PLM}^{f} - \left[v \cdot \left(\frac{\partial}{\partial x} C_{PLM}^{f} \right) \right] - \left[\left(\frac{1-\varepsilon}{\varepsilon} \right) \frac{\partial}{\partial t} C_{PLM}^{b} \right] + \left(k_2 \cdot C_{SKPLG}^{f} \cdot C_{PLG}^{f} - k_3 \cdot C_{PLM}^{f} + k_4 \cdot C_{PLM}^{f} \cdot C_{FBN} \right)$$

Initial Condition:

$C_{PLM} = 0$ for all x at t=0

Boundary conditions:

Left side: $C_{PLM} = C_{SKPLG}$ at x=0 for all t

Right side: $\frac{\partial}{\partial x} C_{PLM} = 0$

$$\frac{\partial}{\partial t} C_{PLM}^{b} = k_{ADS}^{PLM} \cdot C_{PLM}^{f} \cdot \left(\theta_{PLM}^{b} - C_{PLM}^{b} \right) - k_{DES}^{PLM} \cdot C_{PLM}^{b}$$

$$\theta_{PLM} = 2.0 \frac{\text{sites}}{\text{monomer}} \cdot 0.5 \frac{\mu\text{mol}}{\text{L}}$$

$$k_{ABS}^{PLM} = 0.5e-3 \frac{\mu\text{mol}}{\text{L} \cdot \text{s}}$$

$$k_{DES}^{PLM} = 5.435e-5 \cdot \frac{1}{\text{s}}$$

BOUND FIBRIN CONCENTRATION:

Using 4th equation above:

$$\frac{\partial}{\partial t} C_{FBN} = -k_4 \cdot C_{PLM}^{b} \cdot C_{FBN}$$

$$C_{FBN}^{initial} = 5.823 \frac{\mu\text{mol}}{\text{L}}$$

APPENDIX C

FORTRAN CODES

Digestion of plasma clots using free streptokinase:

```
      PROGRAM FREESK_1

c     Programmer:  J. Kent Leach
c     Date:  June 26, 2003
c     Location:  University of Oklahoma School of Chemical Engineering
c                                and Materials Science

c     The purpose of this program is to model the digestion of thrombi
c     formed of plasma and thrombin that are treated with streptokinase.
c     The movement of various moieities will be followed
c     with a standard convection-diffusion equation for each species, along
c     with terms for reactions and the prediction of absorption of
c     said species to the thrombus.

c     SKf=streptokinase in fluid phase; PLGf=plasminogen in fluid phase;
c     PLGb=plasminogen in solid phase; SKPLGf=streptokinase/plasminogen complex in
c     fluid phase; SKPLGb= streptokinase/plasminogen complex in solid phase;
c     PLMf=plasmin in fluid phase; PLMb=plasmin in solid phase;
c     FBN=fibrin; PLMAP= plasmin inactivated by alpha-2 antiplasmin
c     FDP=fibrin degradation products

c     Reaction scheme (including constants)
c
c     SK + PLG <---> SKPLG (K1, KM1)
c     SKPLG + PLG ---> PLM + SKPLG (K2)
c     PLM ------> PLMAP  (K3)
c     PLM + FBN ---> PLM + FDP (K4)

      IMPLICIT REAL*8(A-H,L-Z)
      REAL K1,KM1,K2,KM2,K3,K4,KaSKPLGf,KdSKPLGf,KaPLMf,KdPLMf
      PARAMETER (M=200,N=20000)

c     Setting up the arrays for all concentrations of species
      DIMENSION SKf(0:201,0:20001),SKPLGf(0:201,0:20001),
     $          SKPLGb(0:201,0:20001),PLGf(0:201,0:20001),
     $          PLMf(0:201,0:20001),PLMb(0:201,0:20001),
     $     FBN(0:201,0:20001)

      Lclot=3.0d0              !The length of the clot is 3.0 cm
      dx=Lclot/M               !Distance in x-direction is 0.02 cm
      dt=1.66667d-3    !Time difference is 0.1 sec (1.67E-3 MIN)
      lambda=dt/(dx**2.0d0)            !stability quotient
      D=3.0d-6                 !Diffusion coefficient of free species
c                                        assumed constant at 3e-06 cm^2/min
      vx=0.095d0               !Fluid velocity considered constant at 0.095 cm/min
      EPS=0.9928d0   !Void fraction (porosity) of thrombus held constant

c     Rate constants for reactions
      K1=60.0d0                        !min^-1
      KM1=1.8d-3                       !min^-1
      K2=22.02d0                       !min^-1
      K3=0.24d0                        !min^-1
      K4=1500.0d0                      !min^-1
```

```
      PLGb=47.618d0          !umol/L;bound [PLG] remains constant

      KaSKPLGf=5.748d-3      !umol/L*min;Rate of adsorption of SKPLGf to fibrin
      KdSKPLGf=3.9948d-3     !min^-1;Rate of desorption of SKPLGf from fibrin
      ThetaSKPLGb=0.39       !umol/L;concentration of sites available for SKPLGf

      KaPLMf=0.03d0          !umol/L*min; Rate of adsorption of PLMf to fibrin
      KdPLMf=3.264d-3              !min^-1;  Rate of desorption of PLMf to fibrin
      ThetaPLMb=1.0d0              !umol/L; concentration of sites available for PLMf


c     INITIALIZE THE MATRICES WITH VALUES FROM INITIAL CONDITIONS

      DO 1 I=0,M
            SKf(0,0)=0.76d0   !umol/L;initial streptokinase concentration
1     CONTINUE                                    !     bound to leading edge

      DO 2 I=0,M
            SKPLGf(0,0)=SKf(0,0) !umol/L;SKPLG in equilibrium with SK
2     CONTINUE

      DO 3 I=0,M
            PLGf(I,0)=2.2d0   !umol/L;initial plasma plasminogen concentration
3     CONTINUE

      DO 4 I=0,M
            PLMf(I,0)=0.0d0   !umol/L;no plasmin initially present until
4     CONTINUE          !     conversion from plasminogen by SKPLG

      DO 5 I=0,M
            PLMb(I,0)=0.0d0
5     CONTINUE

      DO 6 i=0,M
            FBN(I,0)=5.823d0  !umol/L; initial concentration of fibrin in clot
6     CONTINUE


c     Begin time (J) and position (I) iterations.

39    DO 40 J=0,N

      TIME=J*dt


            IF (j.LE.N) THEN

                        DO 50 i=0,M


                        IF (i.EQ.0) THEN
                              GOTO 11
                        ELSE IF (i.GT.0) THEN
                              GOTO 15
                        ELSE IF (i.EQ.M) THEN
                              GOTO 20
                        END IF

c     Fill matrix with values of concentration for each species based on the convection-diffusion
c     equation with reaction and absorption influences.
```

```
c       For streptokinase in the fluid phase (SKf):
15      SKf(i,j+1)= SKf(i,j) + dt*D*0.5d0*((SKf(i+1,j+1)-2.0d0*SKf(i,j+1)
     $                                +SKf(i-1,j+1))/(dx**2.0d0)
     $                                +(SKf(i+1,j)-2.0d0*SKf(i,j)+SKf(i-1,j))/(dx**2.0d0))
     $                        - dt*(vx*(SKf(i+1,j)-SKf(i-1,j))/(2.0d0*dx))
     $                        - dt*(((1.0d0-EPS)/EPS)*0.0)
     $                        + dt*(-K1*SKf(i,j)*PLGf(i,j) + KM1*SKPLGf(i,j))

c       For plasminogen in the fluid phase (PLGf):
        PLGf(i,j+1)= PLGf(i,j) + dt*D*0.5d0*((PLGf(i+1,j+1)-2.0d0*PLGf(i,j+1)
     $                                + PLGf(i-1,j+1))/(dx**2.0d0)
     $                                + (PLGf(i+1,j)-2.0d0*PLGf(i,j)+PLGf(i-1,j))/(dx**2.0d0))
     $                - dt*(vx*(PLGf(i+1,j)-PLGf(i-1,j))/(2.0d0*dx))
     $                - dt*(((1.0d0-EPS)/EPS)*0.0)
     $                + dt*(-K1*SKf(i,j)*PLGf(i,j) + KM1*SKPLGf(i,j)
     $           - K2*PLGf(i,j)*SKPLGf(i,j))

c       For streptokinase-plasminogen complex in the fluid phase (SKPLGf):
        SKPLGf(i,j+1)= SKPLGf(i,j) + dt*D*0.5d0*((SKPLGf(i+1,j+1)
     $                                - 2.0d0*SKPLGf(i,j+1) + SKPLGf(i-1,j+1))/(dx**2.0d0)
     $                                + (SKPLGf(i+1,j)-2.0d0*SKPLGf(i,j)
     $                                + SKPLGf(i-1,j))/(dx**2.0d0))
     $                        - dt*(vx*(SKPLGf(i+1,j)-SKPLGf(i-1,j))/(2.0d0*dx))
     $                        - dt*(((1.0d0-EPS)/EPS)*SKPLGb(i,j))
     $                        + dt*(K1*SKf(i,j)*PLGf(i,j)
     $                                        - KM1*SKPLGf(i,j)
     $                                        - K2*PLGf(i,j)*SKPLGf(i,j))

c       For plasmin in the fluid phase (PLMf):
16      PLMf(i,j+1)= PLMf(i,j) + dt*D*0.5d0*((PLMf(i+1,j+1)
     $                                - 2.0d0*PLMf(i,j+1) + PLMf(i-1,j+1))/(dx**2.0d0)
     $                                + (PLMf(i+1,j)-2.0d0*PLMf(i,j)
     $                                + PLMf(i-1,j))/(dx**2.0d0))
     $                        - dt*(vx*(PLMf(i+1,j)-PLMf(i-1,j))/(2.0d0*dx))
     $                        - dt*(((1.0d0 - EPS)/EPS)*PLMb(i,j))
     $                        + dt*(-K3*PLMf(i,j) + K2*PLGf(i,j)*SKPLGf(i,j))

c       STREPTOKINASE IN THE FLUID PHASE
c       Left boundary condition for SK in fluid phase
11      SKf(0,j+1)=SKf(0,j) + dt*D*0.5d0*((2.0d0*SKf(i+1,j+1)-2.0d0
     $                                *SKf(i,j+1))/(dx**2.0d0) +
     $                                (2.0d0*SKf(i+1,j)-2.0d0*SKf(i,j))/(dx**2.0d0))
     $                        + dt*(-K1*SKf(0,j)*PLGf(0,j) + KM1*SKPLGf(0,j))

c       PLASMINOGEN IN THE FLUID PHASE
c       Left boundary condition for PLG in fluid phase
        PLGf(0,j+1) = PLGf(0,j) + dt*D*0.5d0*((2.0d0*PLGf(i+1,j+1)-2.0d0
     $                                *PLGf(i,j+1))/(dx**2.0d0) +
     $                                (2.0d0*PLGf(i+1,j)-2.0d0*PLGf(i,j))/(dx**2.0d0))
     $                        + dt*(-K1*SKf(0,j)*PLGf(0,j) + KM1*SKPLGf(0,j)
     $                                + -K2*SKPLGf(0,j)*PLGf(0,j))

c       STREPTOKINASE/PLASMINOGEN COMPLEX IN THE FLUID PHASE
c       Left boundary condition for SKPLG in the fluid phase
        SKPLGf(0,j+1)=SKf(0,j)

c       PLASMIN IN THE FLUID PHASE
c       Left boundary condition for PLM in the fluid phase
        PLMf(0,j+1)= PLMf(1,j)
     $                        - dt*((1.0d0-EPS)/EPS)*PLMb(0,j)
     $                                + dt*(-K3*PLMf(M,j) + K2*PLGf(M,j)*SKPLGf(M,j))
```

```
c       For plasmin in the solid phase (PLMb):
17      PLMb(i,j+1)= PLMb(i,j)
   $                              + dt*(KaPLMf*PLMf(i,j)*(ThetaPLMb
   $                              - PLMb(i,j)) - (KdPLMf*PLMb(i,j)))

c       For streptokinase-plasminogen complex in the solid phase (SKPLGb):
        SKPLGb(i,j+1)= SKPLGb(i,j)
   $                               + dt*(KaSKPLGf*SKPLGf(i,j)
   $                               *(ThetaSKPLGb-SKPLGb(i,j))-KdSKPLGf*SKPLGb(i,j))


20      IF (i.EQ.M) THEN
c       STREPTOKINASE IN FLUID Right side (M):
        SKf(M,j+1)= SKf(M,j) + dt*D*(0.5d0*(2.0d0*SKf(M-1,j+1)-2.0d0
   $                              *SKf(M,j+1))/(dx**2.0d0)
   $                              + (2.0d0*SKf(M-1,j)-2.0d0*SKf(M,j))/(dx**2.0d0))
   $                      + dt*(-K1*SKf(M,j)*PLGf(M,j) + KM1*SKPLGf(M,j))

c       PLASMINOGEN IN FLUID Right side (M):
        PLGf(M,j+1) = PLGf(M,j)
   $                              + dt*D*0.5d0*((2.0d0*PLGf(M-1,j+1)-2.0d0
   $                              *PLGf(M,j+1))/(dx**2.0d0)
   $                              + (2.0d0*PLGf(M-1,j)-2.0d0*PLGf(M,j))/(dx**2.0d0))
   $                      + dt*(-K1*SKf(M,j)*PLGf(M,j) + KM1*SKPLGf(M,j)
   $                              - K2*SKPLGf(M,j)*PLGf(M,j))

c       STREPTOKINASE-PLASMINOGEN COMPLEX IN FLUID Right side (M):
        SKPLGf(M,j+1)= SKPLGf(M,j) + dt*D*0.5d0*((2.0d0*SKPLGf(M-1,j+1)
   $                              - 2.0d0*SKPLGf(M,j+1))/(dx**2.0d0)
   $                              + (2.0d0*SKPLGf(M-1,j)-2.0d0*SKPLGf(i,j))/(dx**2.0d0))
   $                       - dt*((1.0d0-EPS)/EPS)*SKPLGb(M,j)
   $                       + dt*(K1*SKf(M,j)*PLGf(M,j)-KM1*SKPLGf(M,j)
   $                              - K2*SKPLGf(M,j)*PLGf(M,j))

c       PLASMIN IN FLUID Right side (M):
        PLMf(M,j+1)= PLMf(M,j) + dt*D*0.5d0*((2.0d0*PLMf(M-1,j+1)
   $                              - 2.0d0*PLMf(M,j+1))/(dx**2.0d0)
   $                              + (2.0d0*PLMf(M-1,j)-2.0d0*PLMf(i,j))/(dx**2.0d0))
   $                      - ((1.0d0-EPS)/EPS)*PLMb(M,j)
   $                      + dt*(-K3*PLMf(M,j) + K2*PLGf(M,j)*SKPLGf(M,j))

        END IF

c       FIBRIN IN THE SOLID PHASE (FBN):
18      FBN(i,j+1)= FBN(i,j) + dt*(-K4*PLMb(i,j)*FBN(i,j))

        write (*,*) i,j

c       Put the desired results in a file titled "CNResultsSK"

    OPEN (unit=6,file='CNResultsSK.out')


                                IF (SKf(i,j).LT.1.0d-10) THEN
                                        SKf(i,j)=0.0d0
                                END IF
                                IF (SKPLGf(i,j).LT.1.0d-10) THEN
                                        SKPLGf(i,j)=0.0d0
                                END IF
                                IF (SKPLGb(i,j).LT.1.0d-10) THEN
                                        SKPLGb(i,j)=0.0d0
                                END IF
```

```
                                IF (PLGf(i,j).LT.1.0d-10) THEN
                                        PLGf(i,j)=0.0d0
                                END IF
                                IF (PLMf(i,j).LT.1.0d-10) THEN
                                        PLMf(i,j)=0.0d0
                                END IF
                                IF (PLMb(i,j).LT.1.0d-10) THEN
                                        PLMb(i,j)=0.0d0
                                END IF
                                IF (FBN(i,j).LT.3.882d0) THEN
                                        FBN(i,j)=0.0d0
                                END IF


50                              CONTINUE
                        END IF


   WRITE (6,100) SKf(0:200,J)
c       WRITE (6,100) PLGf(0:200,j)
c       WRITE (6,100) SKPLGf(0:200,j)
c       WRITE (6,100) PLMf(0:200,j)
c       WRITE (6,100) PLMb(0:200,j)
c       WRITE (6,100) FBN(0:200,j)

40   CONTINUE

100     FORMAT (e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $            e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4)

        WRITE (*,*) "DONE"
```

```
      STOP

      END PROGRAM FREESK_1
```

Digestion of plasma clots using microencapsulated streptokinase:

```
      PROGRAM MESK_1

c     Programmer:  J. Kent Leach
c     Date:  July 10, 2003
c     Location:  University of Oklahoma School of Chemical Engineering
c                                and Materials Science

c     The purpose of this program is to model the digestion of thrombi
c     treated with microencapsulated streptokinase.  Compared to free
c     streptokinase, MESK has demonstrated in vitro improved distribution
c     of clot lysis.  The movement of various moieities will be followed
c     with a standard convection-diffusion equation for each species, along
c     with terms for reactions and the prediction of absorption of
c     said species to the thrombus.

c     SKf=streptokinase in fluid phase; PLGf=plasminogen in fluid phase;
c     PLGb=plasminogen in solid phase; SKPLGf=streptokinase/plasminogen complex in
c     fluid phase; SKPLGb= streptokinase/plasminogen complex in solid phase;
c     PLMf=plasmin in fluid phase; PLMb=plasmin in solid phase;
c     FBN=fibrin; PLMAP= plasmin inactivated by alpha-2 antiplasmin
c     FDP=fibrin degradation products

c     Reaction scheme (including constants)
c
c     MESK ----> SK + PEG  (Kdissolve)
c     SK + PLG <---> SKPLG (K1, KM1)
c     SKPLG + PLG ---> PLM + SKPLG (K2)
c     PLM ------> PLMAP  (K3)
c     PLM + FBN ---> PLM + FDP (K4)

      IMPLICIT REAL*8(A-H,L-Z)
      REAL K1,KM1,K2,KM2,K3,K4,KaSKPLGf,KdSKPLGf,KaPLMf,KdPLMf
      REAL KaMESKf,KdMESKf,Kdissolve
      PARAMETER (M=200,N=18000)

c     Setting up the arrays for all concentrations of species
      DIMENSION SKf(0:201,0:20001),SKPLGf(0:201,0:20001),
     $          SKPLGb(0:201,0:20001),PLGf(0:201,0:20001),
     $          PLMf(0:201,0:20001),PLMb(0:201,0:20001),
     $          MESKf(0:201,0:20001),MESKb(0:201,0:20001),
     $     FBN(0:201,0:20001)

      Lclot=3.0d0              !The length of the clot is 3.0 cm
      dx=Lclot/M               !Distance in x-direction is 0.02 cm
      dt=1.66667d-3   !Time difference is 0.1 sec (1.67E-3 MIN)
      D=3.0d-6                 !Diffusion coefficient of free species
c                                    assumed constant at 3e-06 cm^2/min
      lambda=D*dt/(dx**2.0d0)          !stability quotient
      DMESK=5.0d-8   !Average diffusion coefficient of microparticles
c                                    from Stokes-Einstein ratio (cm^2/min)
      vx=0.08d0                !Fluid velocity considered constant at 0.08 cm/min
```

```
      EPS=0.9928d0    !Void fraction (porosity) of thrombus held constant

c     Rate constants for reactions
      K1=60.0d0                    !min^-1
      KM1=1.8d-3                   !min^-1
      K2=22.02d0                   !min^-1
      K3=0.24d0                    !min^-1
      K4=1500.0d0                  !min^-1
      Kdissolve=0.022d0       !min^-1

      PLGb=47.618d0           !umol/L;bound [PLG] remains constant

      KaSKPLGf=5.748d-3       !umol/L*min;Rate of adsorption of SKPLGf to fibrin
      KdSKPLGf=3.9948d-3      !min^-1;Rate of desorption of SKPLGf from fibrin
      ThetaSKPLGb=0.39        !umol/L;concentration of sites available for SKPLGf

      KaPLMf=0.03d0           !umol/L*min; Rate of adsorption of PLMf to fibrin
      KdPLMf=3.264d-3               !min^-1;  Rate of desorption of PLMf to fibrin
      ThetaPLMb=1.0d0               !umol/L; concentration of sites available for PLMf

      KaMESKf=5.748d-5        !umol/L*min;Rate of adsorption of MESKf to fibrin
      KdMESKf=3.9948d-5       !min^-1;Rate of desorption of MESKf from fibrin
      ThetaMESKb=0.39               !umol/L;concentration of sites available for SKPLGf


c     INITIALIZE THE MATRICES WITH VALUES FROM INITIAL CONDITIONS

      DO 1 I=0,M
            SKf(0,0)=1.38d-5        !umol/L;initial streptokinase concentration
1     CONTINUE                              !     bound to leading edge

      DO 2 I=0,M
            MESKf(0,0)=1.1d-3       !umol/L;initial PEG concentration
2     CONTINUE

      DO 3 I=0,M
            SKPLGf(0,0)=SKf(0,0)  !umol/L;SKPLG in equilibrium with SK
3     CONTINUE

      DO 4 I=0,M
            PLGf(I,0)=2.2d0         !umol/L;initial plasma plasminogen concentration
4     CONTINUE

      DO 5 I=0,M
            PLMf(I,0)=0.0d0         !umol/L;no plasmin initially present until
5     CONTINUE              !     conversion from plasminogen by SKPLG

      DO 6 I=0,M
            PLMb(I,0)=0.0d0
6     CONTINUE

      DO 7 i=0,M
            FBN(I,0)=5.823d0!umol/L; initial concentration of fibrin in clot
7     CONTINUE

      DO 8 i=0,M
            MESKb(I,0)=0.0d0        !umol/L; initial concentration of MESK in clot
8     CONTINUE


c     Begin time (J) and position (I) iterations.
```

```
39      DO 40 J=0,N

        TIME=J*dt

              IF (j.LE.N) THEN

                                    DO 50 i=0,M


                                    IF (i.EQ.0) THEN
                                          GOTO 11
                                    ELSE IF (i.GT.0) THEN
                                          GOTO 15
                                    ELSE IF (i.EQ.M) THEN
                                          GOTO 20
                                    END IF

c       Fill matrix with values of concentration for each species based on the convection-diffusion
c       equation with reaction and absorption influences.

c       For MESK in the fluid phase (SKf):
15      MESKf(i,j+1)= MESKf(i,j) + dt*DMESK*0.5d0*((MESKf(i+1,j+1)
     $                                    -2.0d0*MESKf(i,j+1)
     $                              +MESKf(i-1,j+1))/(dx**2.0d0)
     $                              +(MESKf(i+1,j)-2.0d0*MESKf(i,j)+MESKf(i-1,j))/(dx**2.0d0))
     $                  - dt*(vx*(MESKf(i+1,j)-MESKf(i-1,j))/(2.0d0*dx))
     $                  - (1.1d-3*(Kdissolve*TIME)**3.0d0)
     $                  - dt*(((1.0d0-EPS)/EPS)*MESKb(i,j))
     $                  + dt*(-Kdissolve*MESKf(i,j))

c       For streptokinase in the fluid phase (SKf):
        SKf(i,j+1)= SKf(i,j) + dt*D*0.5d0*((SKf(i+1,j+1)-2.0d0*SKf(i,j+1)
     $                              +SKf(i-1,j+1))/(dx**2.0d0)
     $                              +(SKf(i+1,j)-2.0d0*SKf(i,j)+SKf(i-1,j))/(dx**2.0d0))
     $                  - dt*(vx*(SKf(i+1,j)-SKf(i-1,j))/(2.0d0*dx))
     $                  - dt*(((1.0d0-EPS)/EPS)*0.0)
     $                  + dt*(-K1*SKf(i,j)*PLGf(i,j) + KM1*SKPLGf(i,j)
     $                              + Kdissolve*MESKf(i,j))
     $                  + (1.1d-3*(Kdissolve*TIME)**3.0d0)


c       For plasminogen in the fluid phase (PLGf):
        PLGf(i,j+1)= PLGf(i,j) + dt*D*0.5d0*((PLGf(i+1,j+1)-2.0d0*PLGf(i,j+1)
     $                              + PLGf(i-1,j+1))/(dx**2.0d0)
     $                              + (PLGf(i+1,j)-2.0d0*PLGf(i,j)+PLGf(i-1,j))/(dx**2.0d0))
     $            - dt*(vx*(PLGf(i+1,j)-PLGf(i-1,j))/(2.0d0*dx))
     $            - dt*(((1.0d0-EPS)/EPS)*0.0)
     $            + dt*(-K1*SKf(i,j)*PLGf(i,j) + KM1*SKPLGf(i,j)
     $       - K2*PLGf(i,j)*SKPLGf(i,j))

c       For streptokinase-plasminogen complex in the fluid phase (SKPLGf):
        SKPLGf(i,j+1)= SKPLGf(i,j) + dt*D*0.5d0*((SKPLGf(i+1,j+1)
     $                              - 2.0d0*SKPLGf(i,j+1) + SKPLGf(i-1,j+1))/(dx**2.0d0)
     $                              + (SKPLGf(i+1,j)-2.0d0*SKPLGf(i,j)
     $                              + SKPLGf(i-1,j))/(dx**2.0d0))
     $                  - dt*(vx*(SKPLGf(i+1,j)-SKPLGf(i-1,j))/(2.0d0*dx))
     $                  - dt*(((1.0d0-EPS)/EPS)*SKPLGb(i,j))
     $                  + dt*(K1*SKf(i,j)*PLGf(i,j) - KM1*SKPLGf(i,j)
     $                              - K2*PLGf(i,j)*SKPLGf(i,j))

c       For plasmin in the fluid phase (PLMf):
16      PLMf(i,j+1)= PLMf(i,j) + dt*D*0.5d0*((PLMf(i+1,j+1)
```

```
     $                              - 2.0d0*PLMf(i,j+1) + PLMf(i-1,j+1))/(dx**2.0d0)
     $                              + (PLMf(i+1,j)-2.0d0*PLMf(i,j)
     $                              + PLMf(i-1,j))/(dx**2.0d0))
     $                        - dt*(vx*(PLMf(i+1,j)-PLMf(i-1,j))/(2.0d0*dx))
     $                        - dt*(((1.0d0 - EPS)/EPS)*PLMb(i,j))
     $                        + dt*(-K3*PLMf(i,j) + K2*PLGf(i,j)*SKPLGf(i,j))

c         MESK IN THE FLUID PHASE
c         Left boundary condition for MESK in fluid phase
11        MESKf(0,j+1)=MESKf(i,j) + dt*DMESK*0.5d0*((2.0d0*MESKf(i+1,j+1)
     $                                    -2.0d0*MESKf(i,j+1))/(dx**2.0d0)
     $                        + (2.0d0*MESKf(i+1,j)-2.0d0*MESKf(i,j))/(dx**2.0d0))
     $                                                  - dt*(vx*(MESKf(i+1,j)-MESKf(i,j))/(2.0d0*dx))
     $              - (1.1d-3*(Kdissolve*TIME)**3.0d0)
     $                        + dt*(-Kdissolve*MESKf(0,j))

c         STREPTOKINASE IN THE FLUID PHASE
c         Left boundary condition for SK in fluid phase
          SKf(0,j+1)=SKf(i,j) + dt*D*0.5d0*((2.0d0*SKf(i+1,j+1)-2.0d0
     $                              * SKf(i,j+1))/(dx**2.0d0)
     $                              + (2.0d0*SKf(i+1,j)-2.0d0*SKf(i,j))/(dx**2.0d0))
     $                        + dt*(-K1*SKf(0,j)*PLGf(0,j) + KM1*SKPLGf(0,j)
     $                              + Kdissolve*MESKf(0,j))
     $                        + (1.1d-3*(Kdissolve*TIME)**3.0d0)

c         PLASMINOGEN IN THE FLUID PHASE
c         Left boundary condition for PLG in fluid phase
          PLGf(0,j+1) = PLGf(0,j) + dt*D*0.5d0*((2.0d0*PLGf(i+1,j+1)-2.0d0
     $                              *PLGf(i,j+1))/(dx**2.0d0) +
     $                              (2.0d0*PLGf(i+1,j)-2.0d0*PLGf(i,j))/(dx**2.0d0))
     $                        + dt*(-K1*SKf(0,j)*PLGf(0,j) + KM1*SKPLGf(0,j)
     $                              + -K2*SKPLGf(0,j)*PLGf(0,j))

c         STREPTOKINASE/PLASMINOGEN COMPLEX IN THE FLUID PHASE
c         Left boundary condition for SKPLG in the fluid phase
          SKPLGf(0,j+1)=SKf(0,j)

c         PLASMIN IN THE FLUID PHASE
c         Left boundary condition for PLM in the fluid phase
          PLMf(0,j+1)= PLMf(1,j)
     $                        - dt*((1.0d0-EPS)/EPS)*PLMb(0,j)
     $                              + dt*(-K3*PLMf(M,j) + K2*PLGf(M,j)*SKPLGf(M,j))


c         For plasmin in the solid phase (PLMb):
17        PLMb(i,j+1)= PLMb(i,j)
     $                              + dt*(KaPLMf*PLMf(i,j)*(ThetaPLMb
     $                              - PLMb(i,j)) - (KdPLMf*PLMb(i,j)))

c         For streptokinase-plasminogen complex in the solid phase (SKPLGb):
          SKPLGb(i,j+1)= SKPLGb(i,j)
     $                               + dt*(KaSKPLGf*SKPLGf(i,j)
     $                               *(ThetaSKPLGb-SKPLGb(i,j))-KdSKPLGf*SKPLGb(i,j))

c         For MESK in the solid phase (MESKb):
          MESKb(i,j+1)= MESKb(i,j)
     $                              + dt*(KaMESKf*MESKf(i,j)*(ThetaMESKb
     $                              - MESKb(i,j)) - (KdMESKf*MESKb(i,j)))


20                IF (i.EQ.M) THEN
```

```
c       MESK IN FLUID Right side (M):
        MESKf(M,j+1)= MESKf(M,j) + dt*DMESK*(0.5d0*(2.0d0*MESKf(M-1,j+1)
     $                                    -2.0d0*MESKf(M,j+1))/(dx**2.0d0)
     $                              + (2.0d0*MESKf(M-1,j)-2.0d0*MESKf(M,j))/(dx**2.0d0))
     $                    - (1.1d-3*(Kdissolve*TIME)**3.0d0)
     $                    + dt*(-K1*SKf(M,j)*PLGf(M,j) + KM1*SKPLGf(M,j)
     $                              - Kdissolve*MESKf(M,j))

c       STREPTOKINASE IN FLUID Right side (M):
        SKf(M,j+1)= SKf(M,j) + dt*D*(0.5d0*(2.0d0*SKf(M-1,j+1)-2.0d0
     $                              *SKf(M,j+1))/(dx**2.0d0)
     $                              + (2.0d0*SKf(M-1,j)-2.0d0*SKf(M,j))/(dx**2.0d0))
     $                    + dt*(-K1*SKf(M,j)*PLGf(M,j) + KM1*SKPLGf(M,j)
     $                              + Kdissolve*MESKf(M,j))
     $                    + (1.1d-3*(Kdissolve*TIME)**3.0d0)

c       PLASMINOGEN IN FLUID Right side (M):
        PLGf(M,j+1) = PLGf(M,j)
     $                              + dt*D*0.5d0*((2.0d0*PLGf(M-1,j+1)-2.0d0
     $                              *PLGf(M,j+1))/(dx**2.0d0)
     $                              + (2.0d0*PLGf(M-1,j)-2.0d0*PLGf(M,j))/(dx**2.0d0))
     $                    + dt*(-K1*SKf(M,j)*PLGf(M,j) + KM1*SKPLGf(M,j)
     $                              - K2*SKPLGf(M,j)*PLGf(M,j))

c       STREPTOKINASE-PLASMINOGEN COMPLEX IN FLUID Right side (M):
        SKPLGf(M,j+1)= SKPLGf(M,j) + dt*D*0.5d0*((2.0d0*SKPLGf(M-1,j+1)
     $                              - 2.0d0*SKPLGf(M,j+1))/(dx**2.0d0)
     $                              + (2.0d0*SKPLGf(M-1,j)-2.0d0*SKPLGf(i,j))/(dx**2.0d0))
     $                     - dt*((1.0d0-EPS)/EPS)*SKPLGb(M,j)
     $                     + dt*(K1*SKf(M,j)*PLGf(M,j)-KM1*SKPLGf(M,j)
     $                              - K2*SKPLGf(M,j)*PLGf(M,j))

c       PLASMIN IN FLUID Right side (M):
        PLMf(M,j+1)= PLMf(M,j) + dt*D*0.5d0*((2.0d0*PLMf(M-1,j+1)
     $                              - 2.0d0*PLMf(M,j+1))/(dx**2.0d0)
     $                              + (2.0d0*PLMf(M-1,j)-2.0d0*PLMf(i,j))/(dx**2.0d0))
     $                    - ((1.0d0-EPS)/EPS)*PLMb(M,j)
     $                    + dt*(-K3*PLMf(M,j) + K2*PLGf(M,j)*SKPLGf(M,j))

              END IF

c       FIBRIN IN THE SOLID PHASE (FBN):
18      FBN(i,j+1)= FBN(i,j) + dt*(-K4*PLMb(i,j)*FBN(i,j))

        write (*,*) i,j

c       Put the desired results in a file titled "CNResultsMESK"

    OPEN (unit=6,file='CNResultsMESK.xls')

                                    IF (SKf(i,j).LT.1.0d-10) THEN
                                          SKf(i,j)=0.0d0
                                    END IF
                                    IF (SKPLGf(i,j).LT.1.0d-20) THEN
                                          SKPLGf(i,j)=0.0d0
                                    END IF
                                    IF (SKPLGb(i,j).LT.1.0d-20) THEN
                                          SKPLGb(i,j)=0.0d0
                                    END IF
                                    IF (PLGf(i,j).LT.1.0d-20) THEN
                                          PLGf(i,j)=0.0d0
                                    END IF
```

```
                              IF (PLMf(i,j).LT.1.0d-20) THEN
                                     PLMf(i,j)=0.0d0
                              END IF
                              IF (PLMb(i,j).LT.1.0d-20) THEN
                                     PLMb(i,j)=0.0d0
                              END IF
                              IF (MESKf(i,j).LT.1d-30) THEN
                                     MESKf(i,j)=0.0
                              END IF
                              IF (FBN(i,j).LT.3.882d0) THEN
                                     FBN(i,j)=0.0d0
                              END IF

50                            CONTINUE
                       END IF

c     WRITE (6,100) SKf(0:200,j)
c        WRITE (6,100) MESKf(0:200,j)
c        WRITE (6,100) MESKb(0:200,j)
c        WRITE (6,100) PLGf(0:200,j)
c        WRITE (6,100) SKPLGf(0:200,j)
c        WRITE (6,100) PLMf(0:200,j)
c        WRITE (6,100) PLMb(0:200,j)
         WRITE (6,100) FBN(0:200,j)

40     CONTINUE

100      FORMAT (e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,
   $           e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4,e15.4)

         WRITE (*,*) "DONE"

         STOP

         END PROGRAM MESK_1
```