CHARACTERIZATION AND PROTEIN-DRUG RELEASE STUDIES OF A NOVEL COPOLYMER OF HEMA

Characterization and Protein-Drug Release Studies of a Novel Copolymer of HEMA

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ABSTRACT

The use of biomedical materials for cardiovascular applications is becoming more widespread as they can ideally be non-immunogenic, cheaper, and more biocompatible than autografts, allografts, and xenografts. In addition to these advantages, they can be mass produced. These materials are typically employed as substitutes for damaged vasculature. Yet major problems including restenosis and thromboemboli associated with these materials persist. Creation of a completely thromboresistant biomaterial has not yet been achieved, and thrombolytic materials have consequently been pursued.

In the present work, novel copolymers formed from 2-hydroxyethyl methacrylate (HEMA) and 2-methacryloyloxyethyl *n*-butylcarbamate (MBC) were used as protein-releasing fibrinolytic biomaterials. The MBC component has been shown to improve the mechanical properties of poly(HEMA) due to its urethane character. Monomers were combined (HEMA:MBC molar ratio 50:50 to 100:0) with either 2,2'-azo-*bis*-isobutyronitrile or benzoyl peroxide as initiators in an aqueous solution containing a small amount of tetrahydrofuran (THF), and free radical polymerization was initiated by UV irradiation (Mequanint and Sheardown, 2005). Solid copolymer disks 3/16" in diameter and 1.5 mm thick were subsequently punched out of the products. Confirmation of the HEMA:MBC synthesis was obtained using ^{13}C NMR, which revealed stronger and sharper MBC peaks as the MBC content in the copolymers increased. Glass transition temperatures (T_g) of the polymers were determined via DSC, and showed no substantial difference between dry 100:0 and 80:20 HEMA:MBC samples, although the 80:20

HEMA:MBC material had a higher T_g than the 100:0 HEMA:MBC sample when hydrated – this was likely due to contributions from both the imbibed water and the hydrophobic MBC. For all materials, only one T_g was seen between 20°C and 200°C, confirming that MBC was incorporated as a comonomer, and not as a separate homopolymer.

Sessile drop water contact angles increased with increasing MBC content of the copolymers, demonstrating the more hydrophobic character of the MBC-containing polymers. The uptake of various solvents by the HEMA:MBC copolymers revealed that higher MBC content also led to reduced aqueous solvent uptake, but increased organic solvent uptake as expected. Furthermore, decreased copolymer uptake of solvent and swelling were associated with increases in solvent polarity. The choice of solvent and the MBC content can consequently affect the quantity of solution imbibed by the copolymers.

It is highly probable that the quantity of drug imbibed by the copolymers can be influenced by adjusting the amount of solvent loaded into them. This idea is supported by the observation that by increasing the MBC content of the copolymers, which affects solvent uptake, the amount of r-tPA taken up could be reduced.

It was also found that the amount of recombinant tissue-type plasminogen activator (rt-PA) released from the biomaterial could be controlled by altering the drug concentration in the imbibition solution. SDS-PAGE confirmed that the rt-PA released from the copolymers remained structurally intact, while a S2251 assay showed that the released rt-PA possessed fibrinolytic activity. However, the enzymatic activity of released rt-PA appeared to decrease with increasing MBC content of the copolymers.

Studies of drug release kinetics suggested that a first-order process could describe release. The release profiles plateaued before ~60 h following an initial burst effect, and the cumulative protein release was shown to be dependent on the MBC content of the biomaterial. Results for the release of rt-PA and human serum albumin, used as a model protein of similar molecular weight to rt-PA, from the copolymers showed similar trends; however there were differences in the amounts of protein taken up and released, demonstrating that molecular weight is only one factor in determining protein release characteristics. When HEMA:MBC samples were immersed in a 2 mg/mL rt-PA solution, the cumulative amounts of rt-PA released ranged from 5.2 μg to 6.2 μg rt-PA, as the MBC content decreased. Whether these amounts are sufficient for efficient thrombolysis to occur *in vivo*, as opposed to *in vitro*, must still be determined.

The potential for these HEMA:MBC copolymers for the release of rt-PA appears promising. Ideally, this copolymer would be employed as a coating on other biomedical polyurethanes to enhance their antithrombogenicity, and thus their clinical applicability.

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5-TAMRA SE	5-carboxytetramethylrhodamine succinimidyl ester
AIBN	2,2'-azo-*bis*-isobutyronitrile
BI	*n*-butyl isocyanate
BP	benzoyl peroxide
CV	cardiovascular
DMF	N,N-dimethylformamide
DMSO	dimethyl sulfoxide
DSC	differential scanning calorimetry
EGDMA	ethylene glycol dimethacrylate
FTIR	Fourier transform infrared spectroscopy
HEMA	2-hydroxyethyl methacrylate
HSA	human serum albumin
Iodo-gen®	1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril
L-Arg	L-arginine
MBC	2-methacryloyloxyethyl *n*-butylcarbamate
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
pHEMA	poly(2-hydroxyethyl methacrylate)
RAFT	reversible addition-fragmentation chain transfer
rt-PA	recombinant tissue-type plasminogen activator
TBS	tris buffered saline

TF	tissue factor
T_g	glass transition temperature
t-PA	tissue-type plasminogen activator

1 INTRODUCTION

Cardiovascular (CV) diseases rank as the leading cause of death in developed nations, and is predicted to be the leading cause of death in developing countries by 2010 (WHO, 2005). Globally, 16.7 million people died in 2003 as a result of CV-related illness according to the WHO, and in 2004 the American Heart Association found that the USA alone spent US$368.4 billion treating CV complications. Obviously, there are significant therapeutic and economic incentives for developing effective treatments that can inhibit CV-related problems.

Of the broad range of CV complications, the most devastating with respect to human morbidity and death are those that are due to vascular occlusions, with the most common and life-threatening of these occurring in the critical vessels of the heart and brain. Commonly used treatment options, particularly in the heart, include the creation of alternative blood flow routes typically through bypass surgeries and angioplasties. Such treatments are increasingly associated with the usage of *in vivo* biomedical devices including stents, vascular grafts and heart valves. The objective of these devices is to preclude further CV problems, especially restenosis, yet the vast majority fail to achieve this. For example bare metal stents have restenosis rates of 32-53% when used to percutaneously treat chronic total occlusions (Sirnes et al., 1996; Suttorp et al., 1998). No synthetic small diameter vascular prosthesis (< 5 mm) is currently available; even autograft- and allograft-based saphenous vein grafts and mammary artery grafts have

autograft- and allograft-based saphenous vein grafts and mammary artery grafts have patency rates at 10 years of only 61% and 85%, respectively (Goldman et al., 2004). This is because biomaterials, which are natural and/or artificial components of biomedical devices that are meant to replace or augment an organ or bodily function (Ratner et al., 1996), are generally prothrombotic in the context of blood-contacting applications (Dawids, 1993; Didisheim and Watson, 1996; Hanson, 1998) due to interactions with the cellular and proteinaceous components of blood. The resulting thrombi can result in occlusion of the vessel or can embolize, leading to ischemia and other complications.

Much research has gone into the study of how to minimize or eliminate thrombus formation on biomaterial surfaces. The research has largely focused on the design of synthetic materials and surfaces since natural biomaterials are often less abundant, less durable, less customizable (i.e. in terms of shape), and more immunogenic than their synthetic counterparts. The development of blood contacting materials accompanied by drug treatments has also been intensively studied, as this method offers an active pharmacological approach to minimizing thrombotic challenges. These drug therapies can be categorized into two categories: 1) systemic treatment and, 2) local treatment.

Systemic drug treatment has conventionally been the therapy of choice since it is typically viewed as being more predictable and safer than localized drug treatment. It can target both known thrombi and undetected/nondetectable thrombi and emboli dispersed throughout the entire body. However, high doses and thus costs are often required to achieve and maintain therapeutically-relevant drug levels. Advances in fibrinolytic drugs and biomaterials synthesis, and combinations thereof have made

localized and controllable drug delivery more attractive and this treatment is now being more aggressively pursued. Using this method, a controlled dose of drug is delivered from a biomaterial directly to the site of action which should result in lower requisite drug doses and costs, in addition to more rapid therapeutic effects.

The variety of drugs that have been studied and commercialized for anticoagulant effects is extensive. They include heparin, hirudin, nitric oxide, warfarin, aspirin, urokinase plasminogen activator (u-PA), streptokinase, tissue-type plasminogen activator (t-PA), and variants thereof. Of all known anticoagulants, none has succeeded fully in yielding antithrombotic effects without undesired hemorrhaging (Da Silva and Sobel, 2002). Nevertheless, there are some drugs that remain preferable for therapeutic use such as the widely prescribed fibrinolytic protease, t-PA. The popularity of t-PA can be attributed to its many unique characteristics including 1) specificity for blood clots unlike undirected agents (e.g. heparin, warfarin, aspirin) that induce greater hemorrhagic effects by affecting upstream members of the coagulation cascade, 2) it is a compound in humans that is naturally thrombolytic, and 3) it has a 24 year history of predictable and effective clinical usage (Collen, 1986; Genentech, 1999). Furthermore, t-PA has not encountered problems such as the potential to transmit infectious agents, which resulted in the withdrawal of u-PA from the market by the U.S. Food and Drug Administration (FDA) in 1999, or streptokinase's well-known antigenic response that has limited its use to once per patient (Reyes et al., 2004).

Various materials are potentially useful as drug delivery vehicles. Among these, poly(2-hydroxyethyl methacrylate), p(HEMA), has been extensively investigated due to

its widespread use in biomaterials applications ranging from soft contact lenses to nasal cartilage replacements (Voldrich et al., 1975). The reason for pHEMA's pervasiveness is largely because of this synthetic polymer's hydrogel nature. By being a hydrogel, pHEMA exhibits a high water content, a soft consistency, and low surface friction -- all of which reflect the physical and biomechanical properties of natural soft tissues (Ferruti et al., 2004; Saltzman, 2001). Furthermore, because pHEMA has a very porous three-dimensional network, it can potentially take up and release via diffusion, substantial amounts of molecules (i.e. drugs) carried in any absorbed liquid. Despite all of these advantages, the significant decrease in the mechanical properties of pHEMA with increasing water uptake represents a major disadvantage.

In this work, a novel copolymer of HEMA and 2-methacryloyloxyethyl *N*-butylcarbamate (MBC) (Mequanint and Sheardown, 2005), was investigated as a vehicle for the controlled delivery of t-PA. The incorporation of the hydrophobic MBC component has already been shown to increase HEMA's tensile strength. A range of HEMA:MBC copolymers (HEMA:MBC molar ratio 50:50 to 100:0) were synthesized to generate smooth homogenous hydrogel products. The resultant gels were characterized using differential scanning calorimetry and water contact angles. Protein-drug uptake and release experiments were performed using human serum albumin (HSA) as a model protein, as well as t-PA itself. Uptake and release were monitored using ^{125}I-labeled protein-drugs, as well as fluorescently-labeled protein-drugs using the fluorophor, 5-carboxytetramethylrhodamine succinimidyl ester (5-TAMRA SE). All release studies were conducted at 37 ± 1°C in PBS (pH 7.4). It is ultimately believed that this

HEMA:MBC copolymer can be used as a coating material on polyurethane (PU) materials such as a fibrinolytic PU surface (McClung et al., 2003) as suggested by the work of Tan (1999).

2 LITERATURE REVIEW

2.1 Biomaterials in the Cardiovascular System

A system of patent coronary, cerebral, and peripheral blood vessels is essential to the maintenance of tissue health. As a result, there has been an abundance of research surrounding antithrombotic and fibrinolytic materials, which are essential elements of *in vivo* biomedical devices such as vascular grafts. Natural materials that have been evaluated and commonly employed in such applications include allografts, xenografts (e.g. porcine and bovine sources), and autografts such as saphenous veins. Unfortunately, their lack of availability, their limited resilience at arterial blood pressures, and their immunogenic tendencies have provoked vast interest in synthetic biomaterials. Ideally, synthetic biomaterials can be mass produced, are relatively non-antigenic, and have reduced production and storage costs relative to natural materials. However, they are generally prothrombotic and must be modified to improve blood compatibility.

2.2 Biological Interactions with Blood Contacting Biomaterials

Blood interactions with artificial surfaces are characterized by a variety of different responses, as shown in Figure 2-1. Of these, platelet reactions, intrinsic coagulation, fibrinolytic activity, erythrocyte and leukocyte interactions, as well as complement activation, are all preceded by the adsorption of plasma proteins (Courtney

et al., 1994). Protein adsorption and the consequent coagulation effects are specifically reviewed herein.

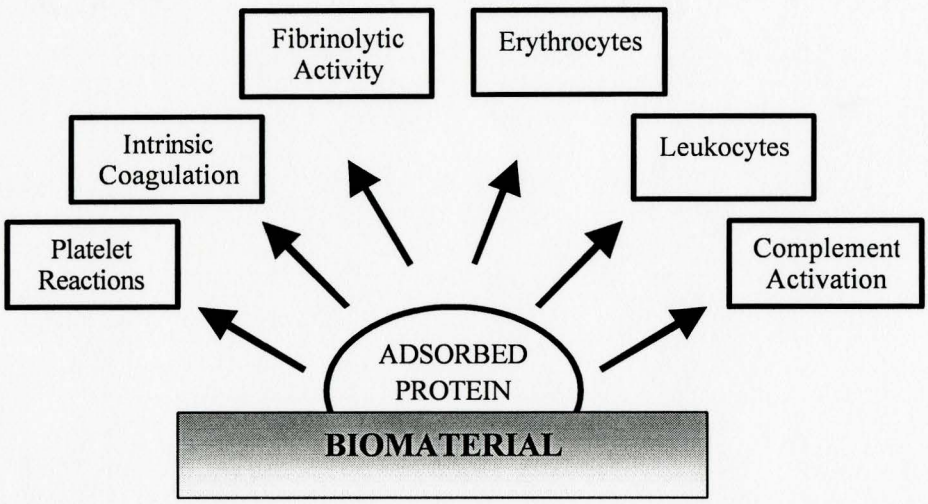


Figure 2-1 Responses resulting from blood-biomaterial interactions (Courtney et al., 1994)

2.2.1 Events Leading to Thrombus Formation

Blood coagulation naturally occurs in response to ruptured vessels, minimizing the loss of vital body fluids. These hemostatic mechanisms have evolved to become self-amplifying, rapid, and efficient. Nevertheless, they are also responsible for increasing the risk for thrombotic complications associated with biomaterial use.

The mechanisms regulating coagulation are complex and involve the interactions of a number of substances including proteins, enzymes, cells, and signaling molecules. However, the activation mechanisms may be generalized into two pathways: the intrinsic and the extrinsic pathways, as seen in Figure 2-2. The extrinsic pathway is mediated by

the presence of tissue factor (TF), a transmembrane protein that is exposed to the blood during tissue injury (Cotran et al., 1999) Its endpoint with respect to thrombus generation is the stimulation of platelets and the formation of fibrin networks, through thrombin production. Similar to the extrinsic pathway, the intrinsic pathway auto-catalytically functions to produce thrombi-related constituents. It is initiated through contact activation, which refers to reactions provoked by the adsorption of contact factors onto a surface (Colman et al., 2000). The intrinsic pathway is the coagulation pathway associated with biomaterials use *in vivo.*

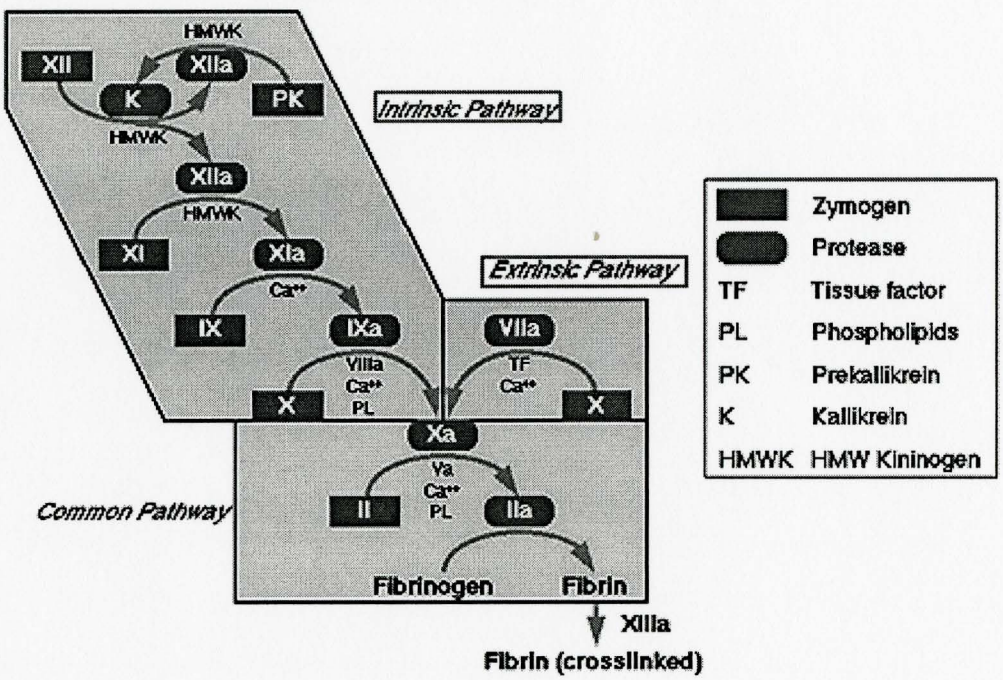


Figure 2-2 Depiction of the intrinsic and extrinsic pathways involved in blood coagulation (Tollefsen, 2004)

2.2.1.1 Protein Adsorption

Artificial biomaterials activate the intrinsic pathway of blood coagulation immediately upon contact with blood. The initial event is the adsorption of plasma proteins to the biomaterial surface which is driven by bulk concentration and intrinsic surface activity. As a result of the wide assortment of amino acid residues and sequences, the size, charge, and the shape of proteins differs significantly and it is therefore not surprising that the outcome of the competitive adsorption of proteins onto biomaterials is quite varied. The resultant adsorbed protein layer mediates subsequent blood-biomaterial interactions (Courtney et al., 1994; Brash, 1991).

2.2.1.2 Platelet Reactions

Thrombi are often distinguished as being either red or white, depending on the relative number of erythrocytes present in their composition; red thrombi possess relatively high quantities of red blood cells, while white thrombi contain few red blood cells relative to other archetypal constituents such as fibrin, leukocytes, and platelets (Lehmann et al., 1997). Although platelets do not dramatically affect the colour of thrombi, they are significant in that they adhere to biomaterial surfaces via interactions with the adsorbed protein layer. Subsequent signaling molecules (e.g. ADP) that are released from the internal granules of platelets result in platelet aggregation and additional mass accretion, which in turn contribute largely to the resulting granular shape and increasing size of thrombi (Cotran et al., 1999).

2.2.1.3 Fibrin Formation

During coagulation, protein adsorption plays a role in the activation and accumulation of blood clot components. These components contribute to the cascade of reactions present in the extrinsic and intrinsic pathways, which ultimately lead to thrombin formation (Factor IIa, Figure 2-2). Thrombin is a serine protease that aids in regulating coagulation. This is achieved either by amplification of intrinsic pathway molecules via stimulation of Factors V, VIII, and XI, or by inhibiting the intrinsic pathway by promoting the formation of the anticoagulant known as activated protein C (APC). Once formed, APC targets Factors Va and VIIIa of the intrinsic pathway, rendering them inactive by degrading them (Figure 2-3). Thrombin is also critical to the generation of blood clots because it converts soluble plasma fibrinogen into fibrin polymer. This polymer can be crosslinked to yield an insoluble fibrin matrix capable of consolidating platelets and cells into a stable thrombus (Colman et al., 2000; Whalen and Tuman, 1996).

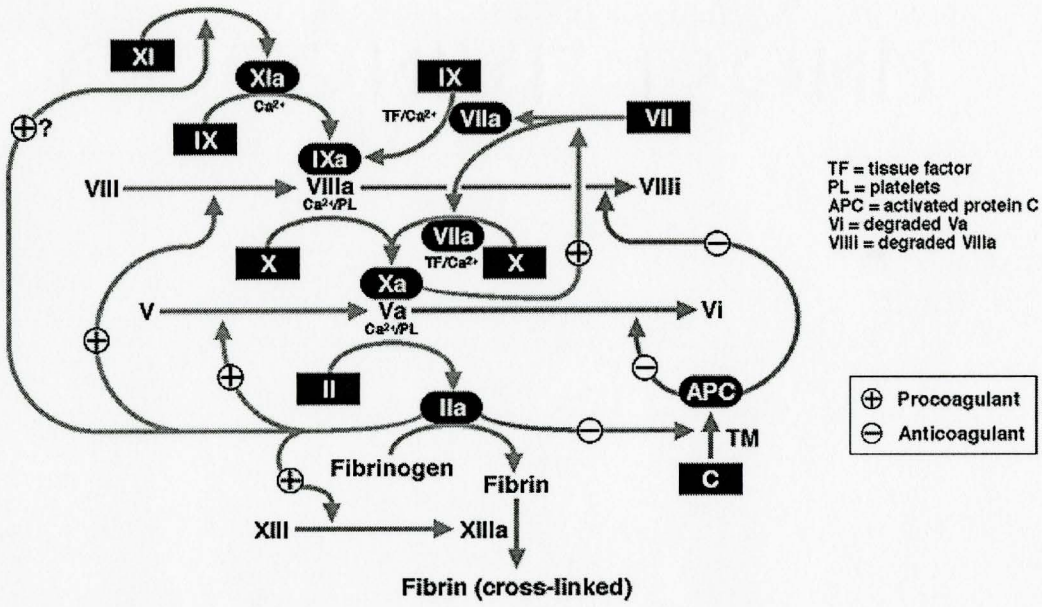


Figure 2-3 Feedback pathways in the extrinsic and intrinsic coagulation cascades (Tollefsen, 2004) Note that IIa = thrombin

2.2.1.4 Summary

The interaction of blood with an artificial material incites, amplifies, and prolongs many naturally-occurring prothrombotic events including the adsorption of protein, platelet adhesion and aggregation, initiatio n of the intrinsic coagulation pathway, and the generation of fibrin networks. All of these processes result in platelet and fibrin deposition, aggregation, and stabilization. They also often attract and entrap red blood cells, which can further contribute to the bulk of a thrombus. The severity of the thrombus formation varies though, depending on factors such as the structure of the biomaterial, the presence of an antithrombotic agent, the patient's health status, and the application of the biomaterial (Courtney et al., 1994). Currently, the long term response to a biomaterial and its dependence on the initial surface events are not well understood.

2.2.2 Commonly Used Thromboresistant Cardiovascular Biomaterials

Although currently in widespread cardiovascular (CV) use, polymers such as polyethylene terephthalate (Dacron) and expanded poly(tetrafluoroethylene) (ePTFE) have been shown to readily interact with blood cells and adsorb plasma proteins within 4 h of exposure – tell-tale signs of thrombus susceptibility (Falkenback et al., 2000; Zarge et al., 1997). Furthermore, when ePTFE is used as graft material for infrapopliteal bypasses, only 43% and 30% patency rates are achieved at one and three years, respectively (Veith et al., 1986). In general, while artificial materials suitable for the replacement of large (> 5 mm) diameter vessels are available, the replacement of small diameter vessels is much more difficult (Bos et al., 1998).

Another popular class of polymers used in CV therapy is polyurethanes, which have been cited as possessing superior thromboresistance (Tiwari et al., 2002), even though many of these polymers have been shown to elicit similar results to Dacron and ePTFE (Lelah and Cooper, 1986; Andrade et al., 1987). Clinical trials involving a wide range of polyurethane biomaterials further provide evidence of the short term problems experienced by this family of polymers during blood-biomaterial interactions; these problems include short and long term graft failures that generally arise from infection and thrombosis (Tiwari et al., 2002) (Table 2-1).

Table 2-1 Clinical trials: patency rates of various conventional polyurethanes used as vascular grafts (Tiwari et al., 2002)

Author	Clinical use	Brand name	No. of grafts	Graft patency
Ota *et al.* (1989) [39]	A–V fistulae	PEU	15	73% at 0.5 year
Ota *et al.* (1991) [69]	A–V fistulae	Modified PEU	34	46% at 1 year
Bull *et al.* (1992) [37]	Femoro-popliteal	Polyurethane	15	47% at 0.5 year
Dereume *et al.* (1993) [40]	Femoro-popliteal	Corethane[a]	57	59% at 0.5 year
Nakagawa *et al.* (1994) [41]	A–V fistulae	PEPU	49	64% at 1 year
Nakagawa *et al.* (1995) [42]	A–V fistulae	PE-PEUG[b]	39	53% at 1 year
Allen *et al.* (1996) [38]	A–V fistulae	Thoratec VAG	145	45% at 1 year

2.2.2.1 Surface Modification Methods for Biomaterials

Biomaterial surface modification is one approach that has been pursued in order to achieve greater material thromboresistance. Polyethylene oxide (PEO) is one compound that has been widely studied, particularly due to its antifouling effects (Amiji and Park, 1993; Malmsten and Muller, 1999; Leckband et al., 1999). It is a hydrophilic, water soluble polymer that has been observed to significantly reduce preliminary prothrombotic events such as protein adsorption and platelet adhesion (Amiji and Park, 1993; McPherson et al., 1995). Many methods have been investigated to incorporate PEO into a biomaterial. Some recent approaches include appending PEO-reactive surface-modifying end groups onto polyurethanes, chemisorbing PEO to biomaterial

surfaces through thiol reactions, incorporating PEO derivatives into polydimethyl siloxane through vulcanization chemistry, and grafting PEO onto surfaces (Chen et al., 2002; Unsworth et al., 2005; Chen et al., 2005; Li and Ruckenstein, 2004; Archambault and Brash, 2004). While these approaches have demonstrated decreased plasma protein interactions, complete inhibition of plasma protein adsorption has yet to be shown. Furthermore, the evaluation of the long-term clinical efficacy of such materials remains scarce. Surface coupling of antithrombogenic agents (e.g. heparin) to achieve thromboresistance has also been studied as a means of enhancing the clinical efficacy of biomaterials. Unfortunately, retention of the covalently attached drugs onto polymers as well as retention of drug activity are major problems encountered with this approach (Bures et al., 2001; Harvey et al., 1989; Brash, 2000; Bamford and Al-Lamee, 1992).

2.2.2.2 Summary of Thromboresistant Materials

Clearly, despite promising *in vitro* data, there are no clinically acceptable thromboresistant materials. An alternative approach has been the development of thrombolytic biomaterials.

2.2.3 Potential of Fibrinolytic Material

Fibrinolytic materials have attracted great interest for use in blood contacting applications (Hwang et al., 2002; Vakkalanka et al., 1996; Asadullin et al., 1993; Sugitachi et al., 1980; Kusserow et al., 1973). The idea of thrombolysis originates from

the idea that if materials cannot be found that resist thrombi formation (thromboresistance), lysing recently formed thrombi before they accrue enough mass to become occlusive (thrombolytic) is a promising alternative. Fibrinolysis is one such thrombolytic therapy, whereby the fibrin meshwork of forming thrombi is lysed to decrease the structural stability of a blood clot, hopefully making it more susceptible to degradation.

Brash and colleagues (McClung et al., 2003; McClung et al., 2000; Woodhouse and Brash, 1992) have developed lysine-containing PU surfaces with exposed ε-amino and carboxyl groups that have been very promising due to their high fibrinolytic activity. Their ability to dissolve fibrin clots has been shown to be the result of the lysine selectively adsorbing plasminogen, with minimal adsorption of other proteins. Plasminogen can readily initiate fibrinolysis in the presence of t-PA. The profibrinolytic behaviour of this modified PU makes it a likely candidate for use in minimizing thrombus formation in blood-contacting devices.

2.3 Thrombolysis

Various mechanisms and molecules exist in humans to regulate blood coagulation so that unwanted complications, such as thromboemboli formation, can be avoided. Endothelial cells, which line the vessel walls, play one of the most important roles in regulating blood clot prevention and degradation. Not only are their surfaces anticoagulant in nature when intact, but these cells also release compounds and express them on their surfaces to prevent the growth of both mural thrombi and thrombi

generated in the bulk. These compounds exert and modulate diverse blood-clot preventative effects including anticoagulant (e.g. thrombomodulin, heparan sulfate), antiplatelet (e.g. prostacyclin, nitric oxide), and fibrinolytic (e.g. tissue-type plasminogen activator (t-PA), urokinase plasminogen activator (u-PA)) effects (Colman et al., 2000). Fibrinolysis, which refers to the enzymatic degradation of fibrin-based clots (Fearnley, 1965), will be examined below.

2.4 Mechanisms of *In Vivo* Fibrinolysis

As previously mentioned, thrombi are composed of aggregates of platelets and blood cells held together and onto surfaces via crosslinked fibrin matrices. By breaking apart these fibrin networks, the stability of these blood clots is compromised and the blood clot degrades. The resulting fibrin degradation products can be subsequently engulfed by phagocytic white blood cells, or readily interact with thrombin to render them incapable of reforming a thrombus (Weitz et al., 1998).

The fibrinolytic system itself is very complex and some of its regulatory mechanisms are still not well understood. One example is the system involved in suspending fibrinolysis in an injured vessel that has naturally occluded to preclude hemorrhaging, until vessel reparations are finished. Nevertheless, the key components of fibrinolysis have been identified, as well as their properties (Appendix A) and interactions with one another (Figure 2-4).

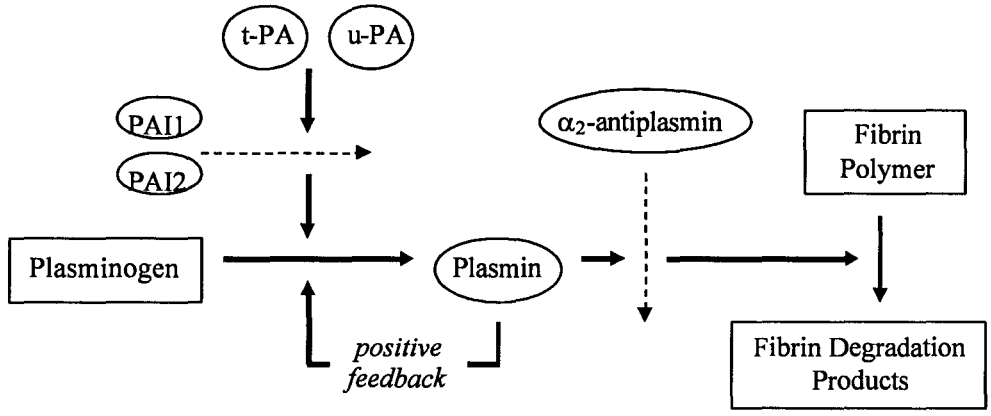


Figure 2-4 The fibrinolytic pathway, which is responsible for fibrin degradation. Note the role of tissue-type plasmingen activator (shown as t-PA); it converts plasminogen into plasmin, and is inhibited by plasminogen activator inhibitors type-1 and type-2 (PAI1, PAI2) (Dobrovolsky and Titaeva, 2002)

As seen in Figure 2-4, the fibrinolytic cascade can be initiated by plasminogen activators such as t-PA or u-PA that enzymatically cleave the proenzyme plasminogen into its active form, plasmin. This resultant serine protease carries out fibrinolysis by cleaving the fibrin in fibrin-based blood clots into fibrin degradation products, which themselves can act as weak anticoagulants (Cotran et al., 1999).

It is also evident from Figure 2-4 that there are multiple deterrents to fibrinolytic activity. These include plasminogen activator inhibitors (e.g. PAI1 and PAI2) that irreversibly bind to t-PA and u-PA to render them inactive, as well as plasmin inhibitors (e.g. α_2-antiplasmin and α_2-macroglobulin) that exist in plasma and neutralize any freely circulating or excessive plasmin. Other molecules also exist that hinder fibrinolysis, such

as thrombin activatable fibrinolysis inhibitor (TAFI) (Colman et al., 2000; Nesheim et al., 1997).

2.5 Tissue-type Plasminogen Activator (t-PA) and Fibrinolysis

The mechanism of efficient intravascular fibrinolysis can be best understood by focusing on its major physiological activator, t-PA. This enzyme is extremely effective at dissolving blood clots when naturally secreted into the vasculature, or when administered as a therapeutic drug to treat myocardial infarctions and strokes. It is a naturally-occurring serine protease that was first identified as a fibrinolytic agent by Astrup and Permin (1947). Composed of 530 amino acids (Figure 2-5, Appendix A), it is constitutively synthesized and secreted in an active single-chain t-PA (sct-PA) form throughout the circulatory system by endothelial cells (Lijnen and Collen, 2001).

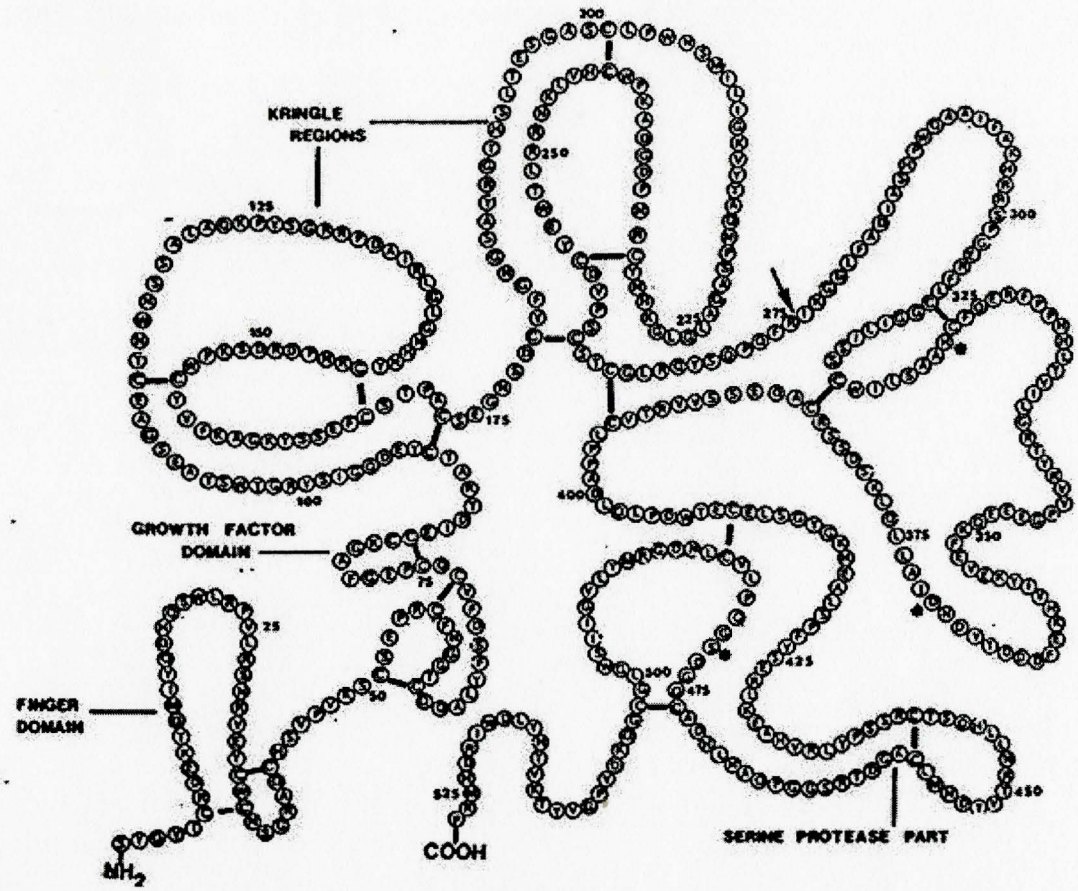


Figure 2-5 Depiction of the primary structure of tissue-type plasminogen activator (t-PA), linked by disulfide bonds (black bars) (with permission from (Verstraete, 1995)). Note that the arrow indicates where single-chain t-PA is cleaved into two-chain t-PA (Arg^{275}-Ile^{276}), and the asterisks indicate active site residues (His^{322}, Asp^{371}, Ser^{478})

2.5.1 t-PA: structure-function relationships

Following its secretion from endothelial cells, sct-PA's Arg^{275}-Ile^{276} peptide bond can be hydrolytically cleaved by enzymes such as plasmin, kallikrein, or Factor Xa to form two-chain t-PA (tct-PA), whose light and heavy chains are coupled by a single disulfide bond (Tate et al., 1987; Wun, 1988).

t-PA's role in initiating fibrinolysis depends on the activities of this enzyme's five structural domains. As shown in Figure 2-5, the identity and arrangement of these domains begin with the NH_2-terminal domain at one end that is homologous to fibronectin's finger-like domain, then the epidermal growth factor (EGF)-like domain, followed by two kringle-like domains (K1 and K2), and finally the catalytic COOH-terminal end domain that is homologous to trypsin-like proteases (Wun, 1988). The primary roles of each of these domains are summarized in Appendix A.

It is the strong association of t-PA's catalytic domain with its EGF-like and/or finger domains that is believed to give both sct-PA and tct-PA a compact, ellipsoidal shape. Both sct-PA and tct-PA's globular appearance also tends to arise as a result of structural in-folding of t-PA's domains (Lijnen and Collen, 2001).

2.5.2 t-PA: fibrinolytic mechanism

t-PA converts plasminogen into a fibrinolytic enzyme, plasmin, by cleaving plasminogen's Arg^{561}-Val^{562} peptide bond through t-PA's catalytic domain. This process occurs most effectively when t-PA is bound to fibrin, emphasizing t-PA's role in targeting recently formed fibrin-based clots (Brommer, 1984). Its affinity for fibrin is akin to the fibrin affinity of plasminogen, as both possess lysine binding sites that attract terminal lysine residues of fibrin (Pennica et al., 1983). Without t-PA binding to lysine residues, the conversion of plasminogen to plasmin is at least 1000-fold slower (Norrman et al., 1985). Based on kinetic data, it is believed that fibrin's surface structure also contributes to t-PA induced plasmin activation, as it encourages the formation of a cyclic

ternary complex of t-PA and plasminogen (Hoylaerts et al., 1982). Furthermore, in the presence of fibrin cofactor both sct-PA and tct-PA display equal fibrinolytic activity; in the absence of fibrin, tct-PA has a greater plasminogen activation potential than sct-PA (Tate et al., 1987; Wallen et al., 1981).

2.5.3 Advantages of t-PA over other Thrombolytic Drugs

While recombinant t-PA is the most common therapeutic agent used for the treatment of thromboembolic disorders in humans, other non-t-PA-related drugs have also been examined. Streptokinase and urokinase were the first thrombolytics to be therapeutically used, yet the systemic fibrinolysis and consequent uncontrolled hemorrhaging associated with their use have limited their success. This is partly due to the fact that urokinase and streptokinase do not exhibit the fibrin specificity of t-PA (Verstraete, 1995), resulting in slower clot lysis and greater systemic bleeding. Furthermore, streptokinase was found to possess an immunological danger, with an incidence of allergic reaction in up to 4% of patients (Aventis Behring LLC, 2002; Timoney et al., 2002). Streptokinase may also induce the generation of antistreptokinase antibodies that can neutralize the action of successive streptokinase doses and preclude it from repeated usage, which is a significant disadvantage given that thrombosis has a high probability of occurring in patients who have experienced previous CV-related occlusions (Tanswell et al., 2002; PHS, 2003).

2.6 Biomaterial Drug Delivery for the Treatment of Thrombosis

The administration of therapeutic compounds from biomaterials has been an important therapy for treating a variety of diseases including the ever growing prevalence of occlusions and re-occlusions in humans. The main concerns of this treatment modality include delivering compounds: 1) at efficacious doses that minimize side effects, 2) with release durations that are sufficient to combat problems only when they are present, 3) at locations where they are most needed, 3) using procedures that minimize costs, and 4) using procedures that are both convenient for the physician and comfortable for the patient (Langer, 1983).

2.6.1 Systemic Versus Localized Drug Delivery

Systemic delivery of therapeutic agents, including tPA, is commonly used to treat peripheral arterial occlusions (McNamara, 1987). Through systemic administration, it is hoped that thrombotic areas of the body can be targeted, including areas which have previously evaded detection as being thrombotic -- particularly important following implantation of various biomedical device(s) for the treatment of CV disease. However, high concentrations of the drug are required to achieve efficacious doses at the desired sites. In the case of thrombolytics, this leaves the patient vulnerable to uncontrolled haemorrhaging as well as other associated systemic complications (Da Silva and Sobel, 2002). The relatively short half-lives of many of these compounds exacerbates this problem as higher concentrations must be delivered to compensate. This can

substantially elevate the requisite amount of drug(s); therefore the costs required to maintain therapeutic *in vivo* drug concentrations can be significant.

Local treatment refers to drug release that is specifically targeted to problematic areas, often at sites of biomaterial implantation or within thrombotically-susceptible areas. Various methods of localized drug administration exist for the treatment of restenosis and they include site specific injections, catheter-based intra-arterial thrombolysis, minipumps near implanted biomaterials, as well as controlled delivery via drug-releasing polymers (Chorny et al., 2000; Bailey, 1997; Langer, 1990). There are a number of advantages for using controlled delivery methods to provide localized, targeted therapy. Because the drug is released directly at the target site, lower doses are possible, accompanied by lower associated costs and lower potential for complications (Bailey, 1997; Chorny et al., 2000; Kavanagh et al., 2004). Additionally, the need for follow-up care is typically reduced, patient comfort and compliance is improved, and drug-delivering biomaterials may aid in the preservation of the activity of impregnated medications, particularly proteins which tend to have short *in vivo* half lives (Langer, 1990). An occasional drawback to local delivery is its invasiveness, especially when the treatment involves multiple injections, or extra surgeries for insertion of catheters and minipumps.

2.6.2 Systemic Versus Localized Drug Delivery: Tissue-type Plasminogen Activator (t-PA)

The local administration of t-PA is attractive due to the fact that current t-PA treatments, which are all systemic-based, remain quite expensive due to the large quantity of t-PA required for systemic administration (0.9 mg t-PA/kg body weight for treatment of acute ischemic stroke) (Genentech, 1999) that is the result of t-PA possessing such a short half-life (2-6 min). This systemic therapeutic quantity of t-PA greatly exceeds the recommended local 0.45–1.00 μg t-PA/mL of body fluid required to treat a thrombus, which degrades even more quickly when the t-PA concentration is increased (AHFS (American Hospital Formulary Service), 1996; Majerus et al., 1995).

2.6.3 Polymers for Cardiovascular Drug Delivery

Clearly, an implanted nontoxic CV biomaterial that releases an appropriate active CV drug at the implant site has significant potential advantages for the prevention and treatment of CV occlusions. It is believed that these drug-incorporated bio materials can interact with and, in time, become better integrated into their biological environments compared with the bio material itself. By avoiding chemical fixation of the drug to the polymer, activity loss should be precluded and the drug will also have the ability to degrade both surface and intimal thrombi in the case of a polymeric vascular graft (Harvey et al., 1989; Langer, 1990). This prospect has provoked research into the controlled, localized delivery of fibrinolytic drugs from synthetic biomaterials, which

offer prolonged and better drug administration control compared to conventional systemic CV treatments (Kavanagh et al., 2004; Bailey, 1997; Langer, 1990).

The choice of biomaterial for a CV drug delivering device varies widely, depending on factors such as cost, location of implantation, method of release, therapeutic objective (e.g. thrombolytic, thromboresistant), and size. The most successful polymers will be those that can control a drug's release rate, maintain the drug within a therapeutic range, sustain or enhance the activity of the drug upon release, possess the ability to be used in multiple devices and areas of the body, be highly biocompatible and inexpensive, and indefinitely prevent the formation of occlusive thrombi (Langer, 1990).

2.6.4 Examples of Drug Delivering Cardiovascular Biomaterials

A variety of biomaterials have been proposed and analyzed for their CV drug delivering potential, primarily for antithrombotic purposes (Stamler et al., 1998; Winslow et al., 2005; Hanker and Giammara, 1988). For example, using a co-matrix of polylactic acid microspheres within chitosan spheres that releases an anti-proliferative agent, taxol, and an anticoagulant, heparin, respectively, Chandy et al. (2001) found that by modifying the polyethylene glycol (PEG) coating compositions on the microspheres, the *in vitro* diffusive release rate of the drugs could be reduced. Alternatively, synthetic nonbiodegradable copolymer stent coatings of poly-*n*-butyl methacrylate and polyethylene–vinyl acetate that releases another anti-proliferative drug, sirolimus, was investigated by Suzuki et al. (2001) in a porcine coronary model. Their studies found that there was a 50% reduction of in-stent stenosis compared to the non-drug-coated

stents, likely due to drug-induced decline of in-stent neointimal hyperplasia. An example of a hydrolytically degradable copolymer for CV drug delivery is a polylactic acid stent coating releasing a modified thrombin inhibitor, PEG-hirudin, and a platelet aggregation inhibitor, iloprost. This study by Herrmann et al. (1999) showed only minor elevations in coagulation markers and no blood clots in the *ex vivo* human stasis model used, while the bare metal stents were associated with high marker levels and complete thrombus coverage. There are many other polymer-based drug release systems being investigated that are temperature sensitive, pH sensitive, or even potentially 'smart' (closed-loop) micro- and nano-sized polymers that independently regulate and administer extremely minute drug quantities based on autonomous sensors (LaVan et al., 2003).

2.6.5 Tissue-type Plasminogen Activator (t-PA) Delivering Biomaterials

There have been few studies conducted on the polymeric delivery of t-PA. One of these studies involved oxidized regenerated degradable cellulose fabric (mTC7) that was draped over rabbits' hearts to directly deliver recombinant t-PA (rt-PA) to their cardiac surfaces. The purpose of this was to reduce epicardial adhesion arising from impaired pericardial fibrinolytic activity, through localized therapy. Although rt-PA was found to decrease adhesion formation when administered from the mTC7 (4%, n = 4), the appearance of significant postoperative bruising and bleeding compromised the clinical safety of this method (Wiseman et al., 1992). Menzies and Ellis (1991) investigated the use of sodium hyaluronate gels loaded with 1 mg rt-PA/g of inactive gel, for the treatment of adhesion formation in laparotomized rabbits. Both initial and recurrent

adhesion were reduced with the rt-PA releasing gels compared to rabbits using non-drug loaded gels and no gels. Results also showed that wound strength, colonic anastomotic healing, and hemorrhagic events were comparable among all rabbits, indicating, at least for the rabbit model used, that intra-abdominal administration of rt-PA is safe.

Hydrogels have also shown promise for t-PA delivery. In a study by Hill-West et al. (1995), rt-PA was administered via a biodegradable PEG-lactic acid oligomer-based hydrogel, into a rat uterine horn devascularization and serosal model, for the purpose of reducing postsurgical adhesion formatio n. The rt-PA was incorporated into the hydrogel during the photopolymerization of a t-PA-containing precursor solution at the site of injury. When compared to hydrogels that did not carry a drug, the rt-PA delivering hydrogels showed a significant decrease in adhesion formation (4% vs 22%). Compared to rt-PA systemically administered via four intraperitoneal injections, the localized polymeric delivery vehicle was even more effective at reducing adhesion formation (4% vs 49%). u-PA and streptokinase delivery from the same hydrogel was also compared to rt-PA, with comparable and inferior adhesion prevention results, respectively (6%, 45%). These results indicate that local delivery of rt-PA appears advantageous over systemic delivery. As well, rt-PA was found to be fibrinolytically more potent than the streptokinase plasminogen activator. On the other hand, Park et al. (2001) worked with poly(L-glutamic acid) (PLGA) semi-interpenetrating polymer networks (IPN) crosslinked with PEG-methacrylate, and rendered porous with sodium bicarbonate foaming during the chemically-initiated polymerization step. The results of the work showed that by

adjusting the porosity of the material, or the content of the crosslinker, PLGA and/or t-PA, *in vitro* t-PA release from the semi-IPN hydrogel could be regulated.

2.7 Hydrogels

Hydrogels are strongly hydrophilic polymer networks that exhibit a unique ability to swell significantly when placed in water. This absorption of water can be up to thousands of times the dry weight of the polymer (Hoffman, 2002). Many types of hydrogels have been discovered. They vary with respect to their chemical structure, their origin (e.g. natural, synthetic, combination), their macromolecular structure (e.g. linear homopolymer, block or graft copolymer, IPN), their method of stabilization (e.g. physical and/or chemical crosslinking), and their physical form (e.g. solid, pressed powder matrix, coating, membrane, liquid) (Hoffman, 2002).

2.7.1 "Reversible/Physical" vs. "Permanent/Chemical"

One of the most important differences between hydrogels is the stability of the gel network. This stability is determined by the nature of the crosslinks in the hydrogel, and can affect a hydrogel's degradability as well as its solubility in different solvents. Concerning drug delivery, the swellability of the hydrogel, and subsequently the drug uptake and release capacities, are significantly dependent on the crosslink densities and structure (Hoffman, 2002).

When polymeric hydrogel networks are stabilized due to molecular entanglements and/or secondary forces, they are termed pseudogels, or "reversible or "physical" hydrogels. When the networks contain covalent crosslinks, due to the presence of crosslinking agents during the polymerization process, they are termed true gels, or "permanent" or "chemical" hydrogels (Hoffman, 2002).

2.7.2 Hydrogel Preparation

Hydrogels can be prepared by a variety of different methods. Hoffman (2002) has compiled a list of the more common methods for producing both physical and chemical hydrogels, as seen in Table 2-2.

Table 2-2 Various routes used to synthesize physical and chemical hydrogels (Hoffman, 2002)

Physical Gels
• Warm a polymer solution to form a gel (e.g. PEO-PPO-PEO block copolymers in water)
• Cool a polymer solution to form a gel (e.g. agarose or gelatin in water)
• 'Crosslink' a polymer in aqueous solution, using freeze–thaw cycles to form polymer microcrystals (e.g. freeze–thaw PVA in aqueous solution)
• Lower pH to form a hydrogen-bonded gel between two different polymers in the same aqueous solution (e.g. PEO and PAAc)
• Mix solutions of a polyanion and a polycation to form a complex coacervate gel (e.g. sodium alginate plus polylysine)
• Gel a polyelectrolyte solution with a *multi*valent ion of opposite charge (e.g. Na^+ alginate$^-$ + Ca^{+2} + $2Cl^-$)
Chemical Gels
• Crosslink polymers in the solid state or in solution with: Radiation (e.g. irradiate PEO in water) Chemical crosslinkers (e.g. treat collagen with glutaraldehyde or a bis-epoxide) Multi-functional reactive compounds (e.g. PEG + diisocyanate = PU hydrogel)
• Copolymerize a monomer + crosslinker in solution (e.g. HEMA + EGDMA)
• Copolymerize a monomer + a multifunctional macromer (e.g. bis-methacrylate terminated PLA-PEO-PLA + photosensitizer + visible light radiation)
• Polymerize a monomer within a different solid polymer to form an IPN gel (e.g. PAN + starch)
• Chemically convert a hydrophobic polymer to a hydrogel (e.g. partially hydrolyze PVAc to PVA)

Abbreviations: PEO = poly(ethylene oxide), PPO = poly(propylene oxide), PVA = poly(vinyl alcohol), PAAc = poly(acrylic acid), PEG = poly(ethylene glycol), PLA = poly(lactic acid), PAN = poly(acrylonitrile), PVAc = poly(vinyl acetate)

2.7.3 Hydrogels as Biomaterials

Hydrogels offer many unique advantages in biomaterials applications, leading to their popularity in various (academic and commercial) devices, such as tissue engineering matrices, drug delivery devices, and both permanent and biodegradable implants. This spectrum of applications is due to the highly tunable chemical and physical properties of

these materials, and their highly biocompatible nature. Their biocompatibility results from their high water content, soft rubbery consistency, and low interfacial tension when placed in physiological fluids, all of which allow them to suitably mimic the physical and biomechanical properties of natural soft tissues (Ferruti et al., 2004; Saltzman, 2001). Additionally, their hydrophilic nature allows undesirable reaction by-products to be easily washed out before implantation. They can also be easily sterilized with ultraviolet radiation and fabricated into various geometric shapes (Hoffman, 2002).

Hydrogels are commonly used in drug delivery applications. One of the most important features of hydrogels with respect to drug delivery is their porous three-dimensional network. This network provides them with the potential to take up substantial quantities of molecules (i.e. drugs) through both adsorption and absorption via imbibition of drug-containing aqueous solvents, and to release these drugs when placed in aqueous, physiological solutions.

2.7.4 Poly(2-hydroxyethyl methacrylate) (p(HEMA)) as a Biomaterial

2.7.4.1 pHEMA Chemistry

pHEMA is a hydrogel belonging to the family of methacrylate polymers. Because there is always trace amounts of ethylene glycol dimethacrylate (EGDMA) in HEMA monomer solutions, pHEMA contains some permanent crosslinks that are indicative of chemical hydrogels. The presence of additional exogenous crosslinker can be used to further stabilize the resultant gel. pHEMA was originally synthesized in 1960

by Wichterle and Lim (1960), and is most widely known as the biomaterial that spawned the soft-contact lens industry. This polymer's structure is composed of repeating units of a HEMA monomer, resulting in a polymeric structure as seen in Figure 2-6.

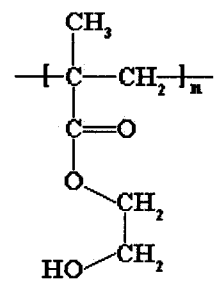


Figure 2-6 Chemical structure of poly(2-hydroxyethyl methacrylate)

The presence of the pendant hydroxyl groups in the polymer's structure imparts many beneficial properties to pHEMA. These include making it highly hydrophilic, allowing it to form internal physical crosslinks via hydrogen bonding, and allowing functionalization of the monomer or polymer to occur through the primary alcohol groups.

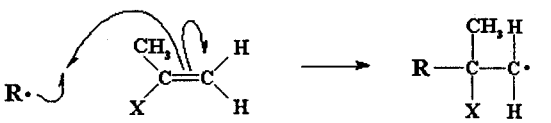
2.7.4.2 Polymerization Methods

In the polymerization of HEMA monomers, crosslinkers are often used to create a permanent hydrogel. pHEMA gels may also be formed without added crosslinkers, primarily due to the inevitable presence of EGDMA impurities in the HEMA monomer.

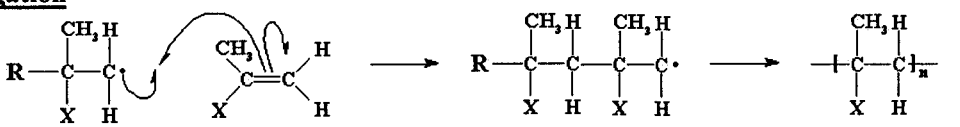
HEMA is usually polymerized by free radical polymerization (Scheme 2.1). This process is typically achieved using a free radical initiator (e.g. 2,2'-azo-*bis*-

isobutyrylnitrile (AIBN), benzoyl peroxide (BP)), high-energy radiation, or reversible addition-fragmentation chain transfer (RAFT) agents, as described below.

Initiation



Propagation

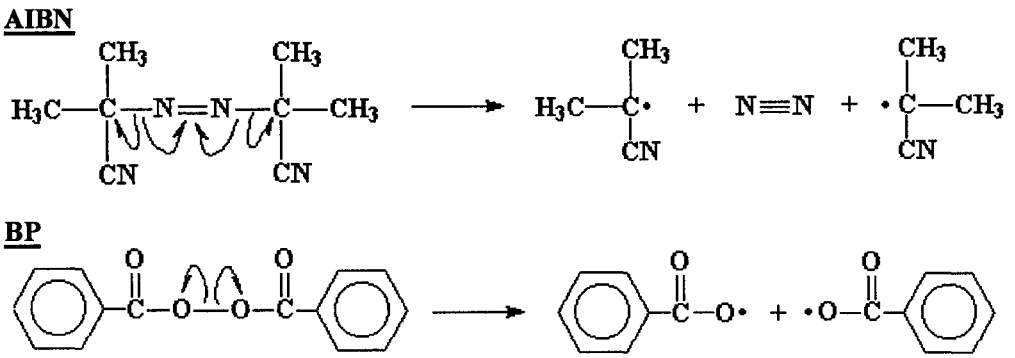


Scheme 2.1 Polymerization of 2-hydroxyethyl methacrylate (HEMA) into poly(HEMA) via free radical polymerization. R = free radical initiator/HEMA, X = $-CO_2(CH_2)_2OH$

(1) Free radical initiation relies on the ability of initiators to uniquely degrade upon exposure to heat or ultraviolet (UV) radiation. Both methods may only need to be applied for a few minutes to induce polymerization, and the intensities of the heat and UV can be as low as 40°C and 1.22 mW/cm^2 at 365 nm, respectively, for HEMA polymerization to occur (Ferruti et al., 2004; Garrett et al., 2000). A shared disadvantage of both heat and UV exposure is potential thermal and photo damage to drugs that may reside in a drug-loaded pHEMA polymer. Heat-induced polymerization also typically requires longer polymerization times than UV-induced polymerization.

When the initiator degrades from heat or UV exposure, as shown in Scheme 2.2, two initiator fragments arise that each possess one unpaired electron. These product molecules are termed free radicals, and they readily attack the electron pairs of pi bonds,

such as those found in the carbon-carbon double bonds of HEMA monomers. These "attacks", which covalently link initiators to monomers, result in the conversion of monomers into free radicals themselves, allowing them to subsequently attack and link to other monomers. The end result of the chain reaction addition of monomer molecules to the free radical ends is a polymer, as seen in Scheme 2.1.



Scheme 2.2 Degradation of 2,2'-azo-*bis*-isobutyronitrile (AIBN; top) and benzoyl peroxide (BP; bottom) into initiator fragments containing one free electron

A chemically-initiated free-radical polymerization requires all reagents to be soluble in the same medium. Although it is the most widely used polymerization method for hydrogels, one disadvantage of this process, particularly if the pHEMA is to be used in a biological setting, is that initiator fragments may reside in the polymer, only to be released via diffusion upon swelling (Hill et al., 1999).

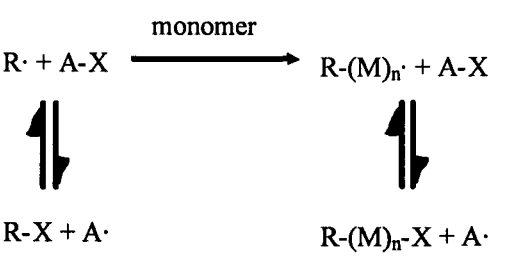
(2) High-energy radiation generates polymers from monomers by bombarding monomer solutions with electromagnetic radiation such as X-rays or gamma rays (typically from a ^{60}C source). This bombardment causes several monomers to convert into free radicals, by breaking their carbon-carbon double bonds. Once the free radicals evolve, chain propagation will occur to form polymers, according to Scheme 2.1.

Zhuang et al.'s (2005) work with a copolymeric hydrogel of *N*-isopropylacrylamide and 2-acrylamide-2-methylpropane sulfonate, found that a gamma radiation dose-rate of 1 kGy/h, and a total radiation dose of 30–40 kGy, led to hydrogel products with maximum swelling and phase-transition temperatures. Gamma ray doses of 1.1 kGy/h to 9.8 kGy/h (depending on ambient temperatures) and total doses of 17-19 kGy though, have been found to yield sufficiently polymerized pHEMA samples (Hill et al., 1999). Interestingly, gamma radiation has also been found to induce crosslinking in uncrosslinked soluble pHEMA, yielding a gel through a proposed mechanism of radiation-induced radical formation on the methylene units of the HEMA side-chains. (Hill et al., 1996). 40 kV X-ray doses have been adequate to produce copolymers of HEMA and methacrylic acid (Mahkam and Allahverdipoor, 2004). Adjustment of the dose-rate of the radiation, or the total dose of radiation, can alter the crosslinking density of the final polymer (Zhuang et al., 2005). Although radiation-initiated polymerizations allow the polymerization and sterilization of the product in one step, occur at lower temperatures then chemically-initiated polymerizations (room temperature or below), and avoid the presence of leachable initiator remnants, the use of X-rays or gamma rays

creates safety concerns and requires the use of restricted and specialized radiation equipment (Mahkam and Allahverdipoor, 2004; Zhuang et al., 2005).

(3) The reversible addition-fragmentation chain transfer (RAFT, or degenerative transfer) process is a relatively new living radical polymerization that has only recently been used to form pHEMA from HEMA monomers. The primary advantage of this method is that it can yield controlled molecular weight polymers with a very narrow MW distribution (often < 1.2). It is also a very versatile process since it can be carried out in bulk, solution, emulsion, or suspension under standard reaction conditions (Chiefari et al., 1998).

The RAFT process requires the use of initiators (e.g. AIBN) and certain dithio agents. Its mechanism can be seen in Scheme 2.3. Additional information on the free radical-induced polymerization is detailed by Pantalone et al. (2003).



Scheme 2.3 Simplified mechanism of the reversible addition-fragmentation chain transfer process. A-X = transfer agent, R• = initiating radical, R-$(M)_n$• = propagating radical, (R-X, R-$(M)_n$-X) = transfer agents, A• = polymerization initiator

A significant disadvantage of the RAFT is the fact that it is not a well developed technique -- although it has been significantly researched, its methods were only first formally published in 1998 (Chiefari et al., 1998). Furthermore, compared to other processes, it requires additional species to be introduced to the monomer mixture (e.g. dithiobenzoates). Finally, a pronounced discoloration is often apparent in polymers produced by the RAFT (Mestach et al., 2005).

2.7.4.3 pHEMA as a drug-delivery biomaterial

In addition to possessing all of the advantages of hydrogels, pHEMA exhibits additional well-characterized properties that make it advantageous as a drug delivery vehicle. Its porosity and isotropic swelling, which can be upwards of 67% by mass in water alone (Gehrke et al., 1994), can be adjusted to control drug uptake and entrapment within the polymer; this is turn will affect the subsequent quantity of drug released into aqueous medium. pHEMA's hydrophilic nature also offers superficial benefits, since water adsorption on the polymer's surface results in low interfacial tension and a low frictional surface, both of which improve the polymer's biocompatibility. Moreover, its hydrophilic surface deters protein adsorption as well as cell adhesion and spreading, making it suitable as a blood contacting biomaterial. The highly porous structure of pHEMA and its associated large pores enable this polymer to carry and release higher molecular weight species, including protein-drugs. By manipulating the composition of pHEMA through the addition of comonomers, the total amount of drug released and its activity can be customized (Antonsen et al., 1993). Other biocompatible properties

exhibited by pHEMA are that it is permeable to metabolites and oxygen, and it is also insensitive to small fluctuations in pH and ionic strength. As well, it has low toxicity, is immunoneutral, is resistant to degradation *in vivo*, and is easy and inexpensive to produce in various shapes (Saltzman, 2001; Ka'lal, 1984; Montheard et al., 1992).

Despite its many positive characteristics, pHEMA also possesses a few disadvantages that may compromise its role as a stable, durable, material for thrombolytic drug release. The first drawback of this material is that it has been shown to cause the consumption of $(7.9 \pm 2.5) \times 10^8$ platelets/cm^2/day, when grafted onto cannulae that were implanted into baboons (Hanson et al., 1980). This observation could detrimentally affect the overall biocompatibility and effectiveness of the hydrogel. Another significant shortcoming of pHEMA is a sizeable decrease in its mechanical strength as water uptake increases (Hutmacher, 2001). Different methods of resolving this problem have been studied. Arima et al. (1995) found that the strength and Young's modulus of pHEMA-based polymers could be increased by up to about 50% by varying the concentration of different cross-linking agents. The addition of certain additives to the pHEMA structure have also been found to yield improved strength, Young's modulus, and elongation at break, as shown by Young et al.'s (1998) pHEMA composites with various imbedded fiber types. Additionally, Jones et al. (2005) and Lou et al. (1999) have investigated the use of pHEMA-based interpenetrating networks (IPN) using hydrophobic polycaprolactone and even pHEMA itself, respectively. An increase in tensile strength in the resultant IPNs was observed in both materials. Instead of IPNs, other polymer incorporating techniques that have improved pHEMA's mechanical properties without

dissociating its favourable blood compatibility, include the grafting of pHEMA onto polyurethanes, polysiloxanes, and styrene-butadiene-styrene triblock copolymers (Saltzman, 2001; Hsiue et al., 1988). Another material that significantly improved the mechanical strength of pHEMA polymers was developed by Mequanint and Sheardown (2005), and was based on a novel polyurethane-like copolymer of HEMA.

2.7.4.3.1 Drug-delivery from pHEMA

Previous studies using pHEMA or pHEMA copolymers as drug-delivery vehicles are extensive. They include examinations of the release of proteins and model compounds such as lysozyme and alpha-amylase, acid orange 8, timolol, insulin and protamine (Antonsen et al., 1993; Lu and Anseth, 1999; Hiratani and Alvarez-Lorenzo, 2004; Brahim et al., 2003). The results from these experiments have varied with respect to the quantity of molecules released, activity retention in the released molecules, the release patterns, and many other characteristics. Because pHEMA's physical and chemical properties can be comprehensively adjusted, which in turn affects molecular release and suitability for different applications, this clearly indicates that pHEMA and its copolymers are prospective materials for a wide range of drug-delivery applications.

2.7.5 Factors Affecting Protein-drug Loading

Various factors can independently, or synergistically, control both the quantity and the distribution of protein-drugs loaded into hydrogels. Many of these are related to

the composition and crosslink density/type of the hydrogel as shown in Table 2-3. Protein-drug factors can also be significant. Protein size and shape as well as relative hydrophilicity/hydrophobicity, and net and local charge can also influence loading. Other external factors (solvent, presence of other molecules, temperature, pH, and method of loading) can also be altered to change the amount of drug present in the polymer matrix (Saltzman, 2001; Langer, 1990; Hoffman, 2002).

Table 2-3 Hydrogel properties that can affect protein-drug release (Saltzman, 2001)

• volume fraction of water
• composition (e.g. IPN, copolymer, graft polymer, etc.)
• net and local charge (i.e. ionic, polar, apolar functional groups)
• bound water (e.g. at pore 'dead ends', hydrogen-bonded or hydrophobically bound, water located in small diameter areas that are inaccessible to the protein-drugs due to their size, etc.) vs. free water (i.e. availability of 'free' water molecules)
• whether the hydrogel is dried/swollen prior to protein-drug loading via imbibition
• tortuosity (average pore size, pore size distribution, volume fraction of pores, pore interconnections)

2.7.6 Protein-drug Release Mechanisms from Polymers

For an *in vivo* drug delivering material to be effective, it must be able to spatially and temporally maintain the drug in the desired therapeutic range at the implant site. The release mechanisms that occur to achieve this largely depend on the properties of the biomaterial, which dictate either a diffusion controlled, swelling controlled, and/or erosion controlled system of release (Langer, 1983). When dealing with release from

non-biodegradable hydrogels, only diffusion and swelling controlled release mechanisms are considered in understanding and optimizing the controlled delivery system.

2.7.6.1 Diffusion Controlled Release Mechanism

This mechanism is the most common for biomaterial-associated drug delivery, and is based on the principle of a drug migrating from a region of higher to one of lower concentration as a result of random thermal motion. The type of diffusion-controlled system that hydrogels display is that of a monolithic device, where the active agent is uniformly dissolved and/or dispersed throughout a solid polymer matrix (Figure 2-7). This is in contrast to reservoir diffusion systems that are often structurally complex and expensive to manufacture. More importantly, reservoir systems usually employ hydrophobic polymers (e.g. silicone, EVAc) which are readily known to inhibit macromolecule (e.g. proteins such as the protein-drug, t-PA) dissolution and diffusion in the polymer Diffusion through the polymer interior to its surface is the rate-limiting step in any diffusive mechanism of release (Saltzman, 2001).

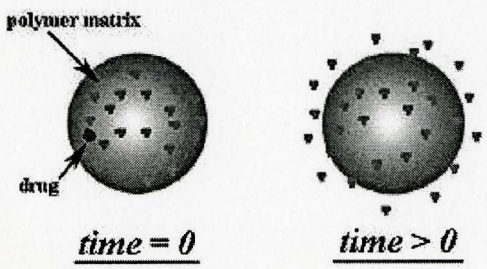


Figure 2-7 Depiction of monolithic (polymer matrix) diffusion of drug

2.7.6.1.1 Desorption of a dissolved drug from a disk

The rate and pattern of drug release from a polymer can be predicted based on parameters such as its geometry and diffusion coefficient. Conversely, by adjusting the surface area-to-volume ratio of the material used, drug release can be manipulated. Equation (2-1) shows a one-dimensional time-dependent diffusion expression derived from Fick's second law of mass conservation in rectangular coordinates.

$$\frac{\partial c}{\partial t} = D_{eff} \frac{\partial^2 c}{\partial x^2} \tag{2-1}$$

where c is the concentration of the drug in the polymer (mol/m^3), t is time (s), x is the spatial position (m), and D_{eff} is the effective diffusion coefficient of molecules dissolved in a complex, porous, polymer (m^5/mol/s). D_{eff} can be determined if the molecular diffusion coefficient of the drug in water (D_o, m^4/mol/s), as well as the tortuosity (t) and shape factor (F, m^{-1}), are known as seen in Equation (2-2).

$$D_{eff} = \frac{D_o}{F\tau} \tag{2-2}$$

Equation (2-1) can be used to derive supplementary equations that allow for the prediction of the cumulative mass of drug released from a pHEMA disk, at different fractional release points (Equation (2-3, 2-4)).

$$M_t = M_\infty \cdot 4\sqrt{\frac{Dt}{\pi l^2}} \quad \text{when } 0 \le \frac{M_t}{M_\infty} \le 0.6 \tag{2-3}$$

$$M_t = M_\infty \left[1 - \frac{8}{\pi^2} \exp \frac{-\pi^2 Dt}{l^2} \right] \quad \text{when } 0.4 \le \frac{M_t}{M_\infty} \le 1.0 \tag{2-4}$$

where M_t is the mass of drug released at time t (mg), M_∞ is the total mass of drug that can be released (mg), l is the thickness of the disk (m), and D is the diffusion coefficient (m^2/s). Equation (2-3) accounts for a drug's initial rapid burst release, while Equation (2-4) accounts for a drug being more slowly released following the burst effect.

The rate of drug release from a disk can also be modeled by taking the time derivatives of Equations (2-3, 2-4), resulting in Equations (2-5, 2-6).

$$\frac{dM_t}{dt} = 2 \cdot M_\infty \sqrt{\frac{D}{\pi l^2 t}} \quad \text{when } 0 \le \frac{M_t}{M_\infty} \le 0.6 \tag{2-5}$$

$$\frac{dM_t}{dt} = \frac{8DM_\infty}{l^2} \exp \frac{-\pi^2 Dt}{l^2} \quad \text{when } 0.4 \le \frac{M_t}{M_\infty} \le 1.0 \tag{2-6}$$

It should be noted that all of these equations pertain to active agents that have been loaded into a polymer at or below their solubility limits (i.e. the drugs are dissolved as opposed to dispersed throughout the polymer matrix). Analogous equations can be derived to describe drug release when it is loaded at concentrations above the solubility limit (Higuchi, 1961 ; Baker, 1987). Furthermore, for Equations (2-3, 2-4, 2-5, 2-6) to be

applicable, the following boundary and initial conditions must be satisfied (see Figure 2-8 for reference) (Saltzman, 2001; Baker, 1987):

1) at $t = 0$, $c = c_o$ for all x ($0 \le x < \frac{\pm l}{2}$), where c_o is the initial drug concentration in the polymer (mol/m^3)

2) $\frac{dc}{dx} = 0$ at $t > 0$ and $x = 0$

3) $c = c_{bulk}$ at $t > 0$ and $x = \frac{\pm l}{2}$, where c_{bulk} is the external drug concentration (mol/m^3)

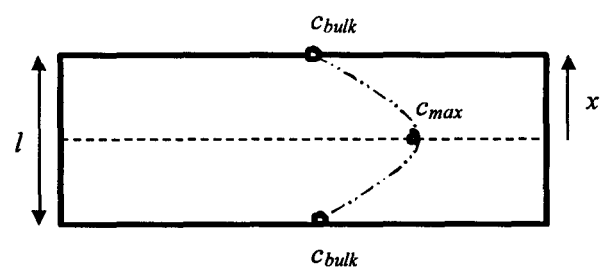


Figure 2-8 Drug concentration distributions in a cross-section of a polymer matrix loaded with dissolved drug, and placed in aqueous solution

2.7.6.2 Swelling Release Mechanism

As previously mentioned, hydrogels are unique in their ability to imbibe large quantities of aqueous solution. The resultant swelling is limited by both osmotic forces and a hydrogel's physical integrity. As solvent penetrates into a hydrogel loaded with drug, substantial swelling occurs as pores fill up and macromolecular polymer chains relax – all events that further provoke the dissolution and permeation of large molecules such as proteins. Dissolution of the hydrogel itself is prevented by both chemical and physical cross-links that maintain its structural integrity. Drugs at the polymer's surface can rapidly dissolve into the surrounding and penetrating medium, accounting for the initial burst release of a drug (Figure 2-9). On the other hand, drugs in the polymer's interior are more slowly transported and released through a network of interconnected pores. This is because the network of interconnected pores effects longer and more tortuous travel routes for the drugs (compared to surface-released drugs), as well as delays drug movement since solvent permeation into the polymer's interior takes time.

The most common pattern of drug release is that seen in Figure 2-9, which demonstrates a first-order drug release. This pattern of drug release is proportional to the square root of time, resulting in a profile that accounts for an initial burst of drug followed by a plateau in the cumulative amount of drug released. It is advantageous since it offsets the burst of host prothrombotic and immune-responses that occur upon biomaterial contact with the body(Saltzman, 2001).

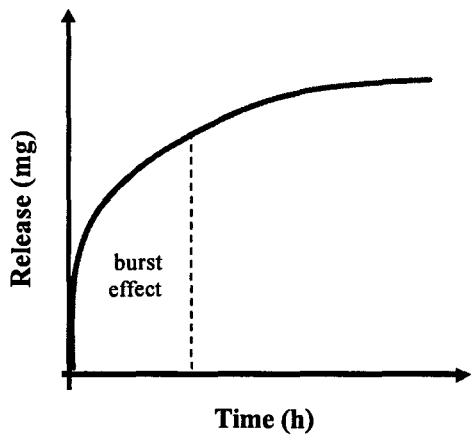


Figure 2-9 First-order drug release

3 OBJECTIVES OF THE PRESENT RESEARCH

It is clear that the thrombogenic responses arising from blood-contacting devices are currently unavoidable events. Various research themes have been pursued to develop more effective biocompatible non-thrombogenic materials, with some success. One of the most promising, yet still understudied, methods involves the use of t-PA releasing synthetic biomaterials in these devices, with the objective of endowing the material with thrombolytic properties.

Therefore, the overall objective of this work was to evaluate and optimize a novel copolymer specifically for the controlled release of t-PA. This copolymer, developed by Mequanint and Sheardown (2005), was composed of the previously mentioned widely used HEMA monomer, and a hydrophobic urethane-based monomer 2-methacryloyloxyethyl *n*-butylcarbamate (MBC). Because of its recent development, further optimization of the synthesis of this copolymer, and its physical and chemical characterization, was required. As well, the preparation of variable HEMA:MBC compositions, ranging from 100:0 to 50:50 (molar ratio of reagents), was necessary to determine the effect of MBC on the copolymer's physical and release properties. Finally, studies on t-PA uptake, release, and stability in association with these different copolymer compositions were undertaken.

The synthesis of the HEMA:MBC copolymer was optimized by selecting reagents and reaction conditions that would yield smooth, uniform samples that would be least

likely to affect any embedded drug. A high-intensity UV-initiated process was selected to catalyze the free-radical reaction required to form the copolymer.

To verify that dissimilar HEMA:MBC copolymers were being produced from the various HEMA:MBC monomer solutions, polymerized samples were compared with respect to their nuclear magnetic resonance (NMR) spectra, water contact angles, masses, and equilibrium solvent uptake. Differential scanning calorimetry was also performed to ensure the existence of copolymer as opposed to homopolymers.

Drug delivery experiments with the HEMA:MBC copolymers were conducted using both human serum albumin (HSA) as a model protein, and the protein-drug, recombinant t-PA. HSA was selected due to the fact that its molecular weight is similar to that of t-PA (HSA: 68 kDa, t-PA: 70 kDa). The method of protein uptake into the copolymer materials was through imbibition. Monitoring of adsorbed, absorbed, and migrating protein (or protein-drug) was carried out using ^{125}I radiolabelling, as well as fluorophore labeling with 5-carboxytetramethylrhodamine succinimidyl ester (5-TAMRA SE). All release studies were conducted at 37 ± 1°C in phosphate buffered saline (PBS, pH 7.4).

Evaluations of the structural stability and activity retention of the released rt-PA, compared to controls, were achieved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the S2251 assay for plasmin. SDS-PAGE can determine whether any structural protein-drug degradation has occurred, while the S2251 assay can indirectly detect the presence of active t-PA by measuring its ability to lyse the S2251 chromogenic substrate.

The data arising from these experiments would hopefully lead to a novel biocompatible material, which could maintain a predetermined rt-PA release rate through the adjustment of parameters such as MBC content, solvent, etc. Because of the copolymer's urethane component, contributed by the MBC monomer, this material could have potential application as a coating on polyurethane-based devices (Tan, 1999), or for the delivery of t-PA from fibrinolytic materials (McClung et al., 2003).

4 Materials and Methods

4.1 HEMA:MBC Copolymer Synthesis

The synthesis of the HEMA:MBC copolymers involved two separate steps: the synthesis of the MBC monomer, followed by the synthesis of the HEMA:MBC copolymer from HEMA and MBC monomer solutions. The procedure used was a modified version of that described by Mequanint and Sheardown (2005).

4.1.1 Synthesis of 2-methacryloyloxyethyl *n*-butylcarbamate (MBC) Monomer

MBC was synthesized from the reagents shown in Figure 4-1, and using the equipment set-up shown in Figure 4-2.

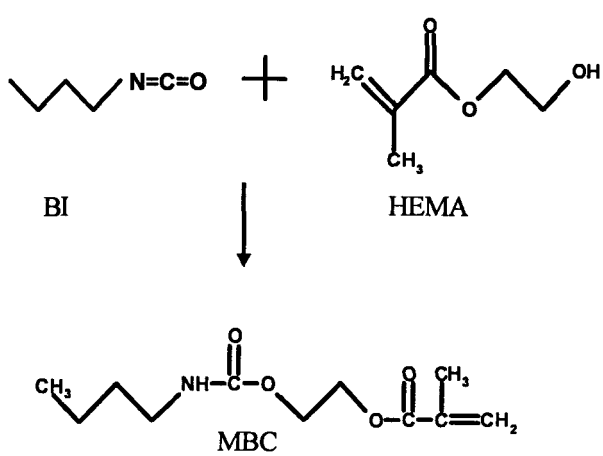


Figure 4-1 Synthesis of 2-methacryloyloxyethyl *n*-butylcarbamate (MBC) monomer from *n*-butyl isocyanate (BI) and 2-hydroxyethyl methacrylate (HEMA)

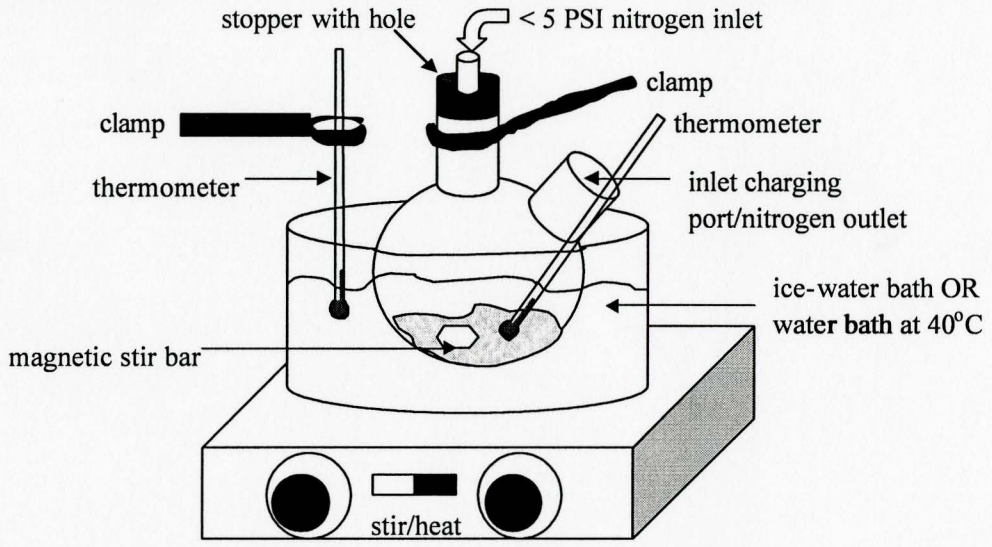


Figure 4-2 Set-up for the synthesis of 2-methacryloyloxyethyl n-butylcarbamate (MBC) monomer

A two-necked 250 mL round bottom flask fitted with a nitrogen inlet, a nitrogen outlet/charging port, and a stir bar, was placed in an ice-water bath (< 5°C). The flask was charged with *n*-butyl isocyanate (BI, 98% pure, Aldrich Chemical Co., Inc., Milwaukee, WI). An equimolar amount of 2-hydroxyethyl methacrylate (HEMA, 99.5+% pure, Monomer-Polymer & Dajac Labs, Inc., Feasterville, PA) was added drop-wise to the BI with stirring under a nitrogen atmosphere. The liquid mixture was subsequently allowed to react at room temperature for 6 h. After 6 h, the ice-water bath was replaced with water, and the temperature of the reaction mixture was slowly increased to, and held at, 40°C for another 6 h to complete the reaction.

The liquid product was collected and passed through an Acrodisc syringe filter with a 0.2 μm Supor membrane (PALL Life Sciences, Ann Arbor, MI) to obtain a clear

liquid free of any suspended matter. Following this, 1/16" Type 4A molecular sieves (Caledon Laboratories Inc., Georgetown, ON, Canada) were added to dehydrate the product overnight, in a refrigerator. The molecular sieves were initially at 100°C, but were cooled prior to introduction to the MBC liquid to prevent heat-induced MBC polymerization. Prior to further use, a Fourier transform infrared spectrum was recorded to ensure that no -NCO peak (2265 cm^{-1}) was detected, indicating the complete reaction of the BI.

4.1.2 Synthesis of HEMA:MBC Copolymer

Monomer solutions of varying HEMA:MBC composition (100:0 to 50:50 HEMA:MBC molar ratio) were used to synthesize HEMA:MBC copolymers, with either 2,2'-azo-*bis*-isobutyrylnitrile (AIBN, 98% pure, Aldrich Chemical Co., Inc., Milwaukee, WI) or benzoyl peroxide (BP, 97% pure, Aldrich Chemical Co., Inc., Milwaukee, WI) as initiators, and Milli-Q water as solvent. To achieve this, the required amounts of HEMA and MBC were added together to make 5 g of monomer feed. This feed was then combined with either 0.025 g of AIBN or 0.0025 g BP, 2.15 mL of Milli-Q water, and 7 drops of tetrahydrofuran (THF) which aided in the dissolution of the initiator. The mixture was then magnetically stirred for 30 minutes at room temperature to ensure that the initiator was completely dissolved, then placed in a refrigerator until needed.

An in-house polymerization mold, shown in Figure 4-3, was used to generate HEMA:MBC copolymers from their respective monomer solutions. A syringe fitted with a 22G1-1/2 needle was used to draw up 1 mL of the HEMA:MBC monomer solution

Following this, glass microscope slides (Goldline plain; VWR Canlab, Mississauga, ON, Canada) and a sandwiched Teflon spacer (1.5 mm thickness) were secured into the Perspex®-based polymerization mold using screws. The solution was then injected through the injection port into the monomer containment area (polymerization window) of the mold until the mold was filled. A Teflon plug was then inserted into the injection port to prevent the monomer solution from escaping.

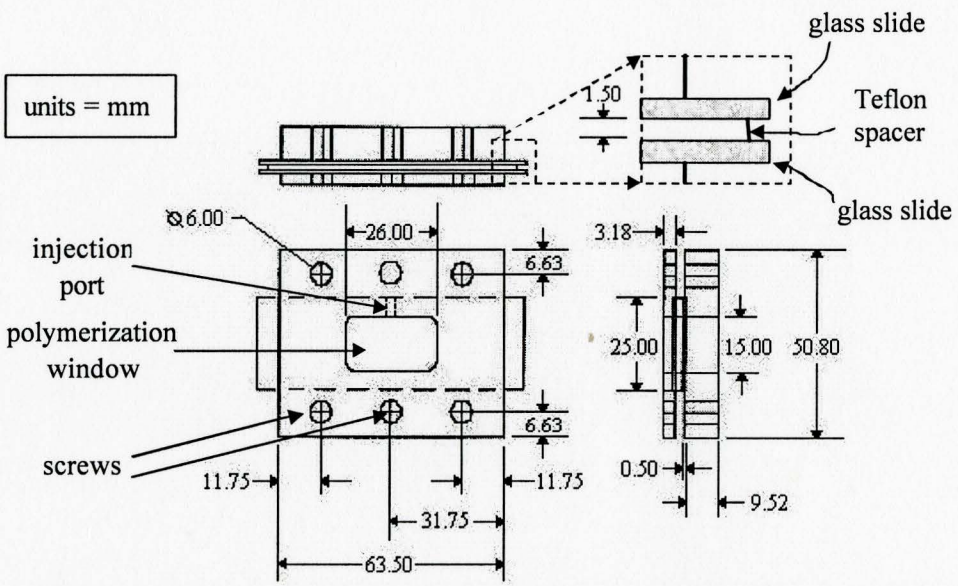


Figure 4-3 Various views of the polymerization mold used to created HEMA:MBC copolymers

The polymerization molds, after being filled with HEMA:MBC monomer solutions, were placed into the polymerization box shown in Figure 4-4. The box contained three sections: the outer two sections contained ice, while the inner section

held up to three polymerization molds. The purpose of the slits in the inner panels of the box was to allow ice-cold water, from the melting ice, to enter into the area containing the polymerization molds. The water level was kept high enough to always submerge the polymerization molds, using an outlet attached to clear laboratory tubing. While allowing the escape of water, the outlet also served to maintain mixing of the water in the box. The purpose of immersing the devices in cold water was to ensure that they were kept below 15°C during the polymerization process, to inhibit thermal protein-drug degradation in the cases where protein-drug was loaded into the monomer solution prior to polymerization.

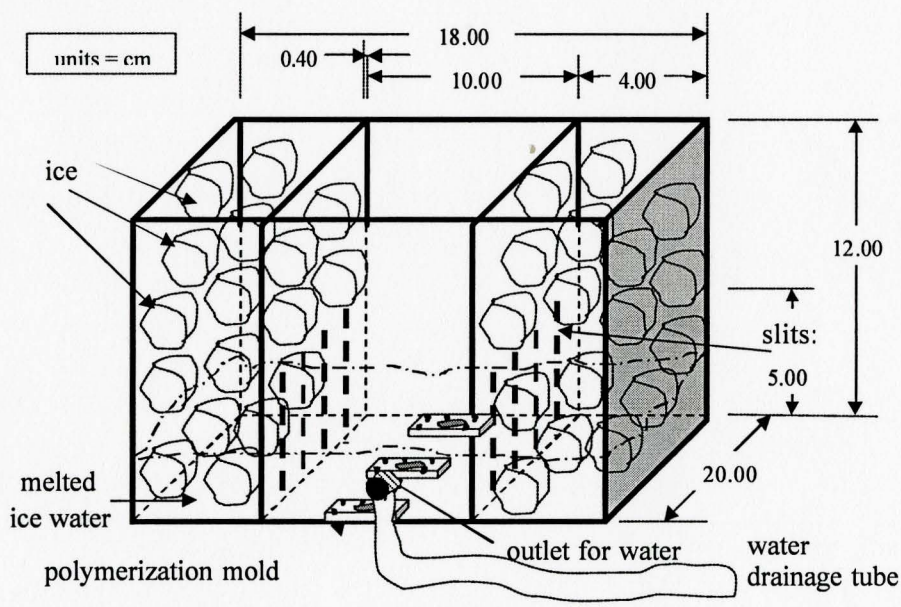


Figure 4-4 Polymerization box used to hold polymerization molds and to keep them at a low temperature during polymerization (not to scale)

The polymerization box was placed inside a Cure Zone 2 ultraviolet (UV) chamber (UV Process Supply, Inc., Chicago, IL), containing a metal hydride lamp generating a light intensity of 80 mW/cm^2. The molds were exposed to two h of irradiation, during which time the box was constantly refilled with ice. Following this, the molds were removed, disassembled, and the polymerized copolymers were removed. The copolymer products were then rinsed with Milli-Q water, and immersed overnight at room temperature (~22°C) in 20 mL of Milli-Q water. This was done to remove any residual unreacted monomer and free radicals that may be cytotoxic (Moreau et al., 1998). After immersion, 1.5 mm thick cylindrical samples were punched out of the copolymer products using a 3/16" metal hole punch, then dried at 37°C for three days.

4.2 Surface Characterization

4.2.1 Sessile Drop Contact Angles

Measurement of sessile drop contact angles on surfaces allow for the rapid quantitative assessment of the relative surface hydrophilicity/hydrophobicity of a material (Andrade, 1985). The values obtained can however be subject to variability as a result of regional differences in surface chemistry, topography, chemical heterogeneity, contamination, and measurement protocol. When polymeric surfaces are examined, it has also been observed that the polymer-liquid interface may rearrange due to water-polymer interactions. Furthermore, large hysteresis has been observed in the advancing and receding contact angles when a hydrogel such as pHEMA is analyzed (Holly and

Refojo, 1975; Merrett et al., 2002; Hermitte et al., 2004). A Milli-Q water drop with a volume no greater than 10 μL was placed on previously swollen HEMA:MBC copolymer surfaces and the advancing and receding water contact angles measured using a Ramé-Hart NRL 100-00 goniometer (Mountain Lakes, NJ).

4.3 Bulk Characterization

4.3.1 Differential Scanning Calorimetry (DSC)

DSC allows for the determination of the thermal properties of a material. By recording the differential heat flow (enthalpy) as a function of temperature, thermal transitions (e.g. glass transitions) indicating changes of state and other phenomena can be observed. Such information can also be useful in confirming whether a material, such as the HEMA:MBC copolymer, is homogeneous or exhibits a heterogeneous composition denoted by the presence of separate glass transition temperatures for each component. To determine the T_g for the HEMA:MBC samples, the temperature of a sample was first increased from 10°C to 200°C at a rate of 15°C/min, and then quenched. This step was done to ensure that the thermal history of the samples was uniform. The temperature was then increased again to 200°C to reveal the T_g's of the different samples. Both dried samples and samples swollen in Milli-Q water were evaluated in the DSC, and all tests were performed under a nitrogen blanket atmosphere. The instrument used was a DSC-2910 (TA Instruments, Inc., Wilmington, DE).

4.3.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

Solid state ^{13}C 1-dimensional NMR was carried out to verify the formation of HEMA:MBC copolymers of varying compositions, as well as to compare their spectra. A Bruker AV300 pulsed NMR spectrometer operating at 120°C, 75.47 Hz, and a 10,000 Hz rotation rate was used for all experiments.

4.3.3 Fourier Transform Infrared Spectroscopy (FTIR)

Because different functional groups react differently to infrared (IR) radiation through vibrations and/or stretching, the analysis of absorbance or transmission spectra arising from materials exposed to IR radiation can reveal the chemical bonds (i.e. functional groups) existing in the materials. In this work, FTIR (Bruker Vector 22, Ettlingen, Germany) was used to confirm the synthesis of MBC.

4.4 Equilibrium Solvent Uptake

A hydrogel material's ability to imbibe solvent is important in drug delivery applications. The amount of protein-drug that can be loaded into a material is directly related to the amount of drug-containing solvent that can penetrate into the material. Previously dried (3 d, 37°C) and weighed samples were placed in Milli-Q water and allowed to equilibrate. The mass of the swollen sample was then determined and the equilibrium solvent content was calculated based on Equation (4-1).

$$Solvent\ Uptake(\%) = \frac{m_s - m_d}{m_d} \times 100\% \tag{4-1}$$

where m_s is the mass of the swollen sample (g) and m_d is the mass of the dried sample (g). Studies were also performed in various solvents to assess alternative loading methods using the same procedure.

4.4.1 Equilibrium Swelling

Evaluation of the amount of equilibrium swelling in various solvents was performed in parallel to the water/solvent uptake experiments. Swelling was determined by measuring the changes in thickness and diameter of the HEMA:MBC sample disks, after each day of immersion, then employing Equation (4-2):

$$Swelling\ (\%) = \frac{\pi\left(\frac{D_s}{2}\right)^2 t_s - \pi\left(\frac{D_d}{2}\right)^2 t_d}{\pi\left(\frac{D_d}{2}\right)^2 t_d} \times 100\% \tag{4-2}$$

where D_d and D_s are the diameters of the dried and swollen samples, and t_d and t_s are the thicknesses of the dried and swollen samples, respectively.

4.5 Protein Labeling

4.5.1 Protein Preparation

Human serum albumin (HSA) was obtained in a lyophilized fraction V, high purity state (Calbiochem, La Jolla, CA), and was used as is. Recombinant tissue-type plasminogen activator (rt-PA), was also obtained in a lyophilized state (Activase® rt-PA, Genentech, Inc., South San Francisco, CA). Once received, the 50 mg vial of Activase® rt-PA was reconstituted in 10 mL of sterile water, following the manufacturer's instructions (Genentech, 1999). The contents were than aliquoted into 250 μL portions, and put into a -70°C freezer until needed.

4.5.2 Labeling Using ^{125}I

Radioiodination of proteins is an established and sensitive method for the precise quantification, and tracking, of small amounts of protein. For the ^{125}I radiolabelling to be effective, the proteins to be labeled must possess amino acids bearing a sulfur atom or an aromatic structure (e.g. cysteine, histidine, methionine, phenylalanine, tryptophan, and tyrosine). Of the available amino acids, tyrosine is the most reactive (Regoeczi, 1984). Human serum albumin contains 19 tyrosine groups, while tissue-type plasminogen activator contains 23 tyrosine residues (Collen, 1986; Meloun et al., 1975), enabling them to readily react with ^{125}I. A gamma-counter (Wallac 1480 Wizard 3" Automatic, Perkin-Elmer Life Sciences, Turku, Finland) was employed to measure the radioactivity of all samples. A Beckman DU 640 UV spectrophotometer (Beckman Coulter, Fullerton, CA)

was used to determine the concentrations of protein by measuring the absorbance of solutions at 280 nm.

4.5.2.1 Protein Radiolabeling Procedure

HSA and t-PA were radiolabeled with $Na^{125}I$ (ICN, Irvine, CA) using either the Iodo-gen method (Pierce Chemicals, Rockford, IL) for labeling small amounts of protein (< 0.6 mg), or the iodine monochloride (ICl) method for labeling larger amounts (> 0.6 mg).

For the Iodo-gen method, 0.2 mg protein was reacted for 15 min with 5 μL of ^{125}I, and 10 μg of Iodo-gen iodination reagent coated onto the sides of a reaction vial with 0.1 mL chloroform. For the ICl method, 2.5 mg protein was reacted for 2 min in a 1:4 molar ratio with ICl reagent, and 5 μL of ^{125}I (Appendix B).

Separation of free isotope from protein-bound isotope was achieved using a column packed with AG 1-X4 resin (Bio-Rad Laboratories, Richmond, CA) for the ICl method, and dialysis with 10,000 MWCO Slidealyzer membranes (Pierce, Rockford, IL) for the Iodo-gen method. Phosphate buffered saline (PBS, 7.4) acted as the solvent/eluate. A free iodide check using a trichloroacetic acid precipitation (Appendix C) was subsequently done to ensure that unbound ^{125}I made up less than 3% of the total solution radioactivity.

4.5.2.2 Protein Adsorption from Buffer

Prior to protein adsorption experiments, HEMA:MBC copolymers were equilibrated in TBS buffer (pH 7.35) overnight. Following this, the samples were placed in the wells of 96 well flat-bottom multi-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) containing 250 μL of 1 mg/mL HSA (10% radiolabeled) and incubated for 2 h at room temperature (~22°C). Adsorption of proteins would be aided by the fact that the *pre-swollen* gels would possess a decreased solvent penetration force when placed into the protein solutions. Because of reduced solvent penetration, this should result in a concomitant decrease in protein penetration/absorption into the gel, particularly during the short 2 h immersion period. The copolymer surfaces were then rinsed three times for 10 min each with fresh TBS (pH 7.35) to remove any loosely-bound protein. Following this, the radioactivity of the samples was measured by gamma counting. Surface radioactivity and corresponding adsorbed protein amounts were determined relative to solution counts that were obtained from samples of the original protein adsorption solutions.

4.5.3 Labeling Using 5-TAMRA SE

The use of fluorophores is another common, albeit less accurate, method of quantifying proteins. Fluorescent labeling is advantageous not only because of its non-toxic reagents, but also because it may be able to determine whether any structural changes have occurred in a fluorophor-conjugated protein, which can directly impact

protein-drug activity. 5-carboxytetramethylrhodamine succinimidyl ester (5-TAMRA SE) is the 527.5 Da fluorescent dye that was used to label proteins in these experiments. It is more photostable and fluorescent than fluorophores such as isothiocyanate derivatives of tetramethylrhodamine, and is also stable between pH 4 and 10 (Molecular Probes, 2003).

5-TAMRA SE reacts with non-protonated aliphatic amine groups (e.g. amine terminus of proteins and ε-amino group of lysine) to produce stable carboxamide bonds (Brunner et al., 1998). Once 5-TAMRA SE has been covalently bound to a protein or peptide, it produces 2 absorption peaks at 520 nm and 550 nm, instead of a single absorption peak at 550 nm that is characteristic of unbound 5-TAMRA SE.

A Fluoroskan Ascent FL fluorimeter (Thermo Electron Corp., Vantaa, Finland), with 544 nm excitation and 590 nm emission filters, was employed to measure the fluorescence of all samples. A Beckman DU 640 UV spectrophotometer (Beckman Coulter, Fullerton, CA) was used to determine the absorbance spectra of solutions to confirm the presence of protein-bound 5-TAMRA SE (two peaks at 520 nm and 550 nm) and to measure protein concentrations (using the 280 nm peak).

4.5.3.1 Evaluation of t-PA Solubility

Lyophilized rt-PA as received (Activase® rt-PA, Genentech, Inc., South San Francisco, CA), is combined with preserving and buffering agents. In particular, the added L-arginine (L-Arg) makes up more than 75% by mass of the lyophilized cake. Because the 5-TAMRA SE fluorophor can, and likely will, readily react with the L-Arg

instead of rt-PA, there was a need to remove the L-Arg from the rt-PA. Consequently, evaluation of the solubility of rt-PA was conducted after removing L-Arg and other agents through dialysis. Various basic compounds such as ε-amino-*n*-caproic acid (Sigma Chemical Co., St. Louis, MO, USA), a derivative of lysine with one blocked amine group known as H-Lys(BOC)-OH (Fluka Biochemika, Buchs, Switzerland), or a derivative of L-Arg that had all of its amine groups blocked known as Nα-*t*-BOC-ω-Nitro-L-Arginine (Sigma Chemical Co., St. Louis, MO, USA), were substituted for the L-Arg component. This was done in order to try to compensate for the fact that L-Arg, a basic amino acid, is required to maintain the pH of rt-PA in solution. The removal of the L-Arg without the addition of another basic agent will result in a decrease in the pH and precipitation of the t-PA.

4.5.3.2 Protein-Fluorophor Labeling Procedure

HSA and rt-PA were fluorescently-labeled with 5-TAMRA SE (Invitrogen Canada, Inc., Burlington, ON, Canada), according to the amine-reactive labeling procedure described by Molecular Probes (Molecular Probes, 2003). Dimethylsulfoxide (Caledon Laboratories, Ltd., Georgetown, ON, Canada) was used as the solvent, and no stop reagent was used. The free 5-TAMRA SE was separated from protein-bound fluorophor using a PD-10 Sephadex G-25M column (Pharmacia Biotech, Piscataway, NJ). A free 5-TAMRA SE check using a trichloroacetic acid precipitation (Appendix C) was subsequently done to ensure that unbound 5-TAMRA SE made up less than 3% of the total solution fluorescence.

For concentration determinations of 5-TAMRA SE-labeled protein, Equations (4-3, 4-4) were used:

$$A_{protein} = A_{280} - A_{max}(CF) \tag{4-3}$$

$$1.4 A_{protein} = 1 \text{ mg/mL protein} \tag{4-4}$$

where CF (5-TAMRA SE) = 0.30, A_{max} is the maximum absorbance value in the protein's spectrum, and Equation (4-4) was assumed (assumption is valid for IgG antibodies).

4.6 Protein Loading in HEMA:MBC Copolymers

4.6.1 Protein Loading into Polymer before Polymerization

If protein is to be loaded into a polymer before polymerization, it should be uniformly dissolved and/or dispersed within the monomer solution. To achieve this, various amounts of HSA were mixed with liquid HEMA:MBC monomer solutions. The distribution of the protein in the monomer was qualitatively evaluated.

4.6.2 Protein Loading into Polymer after Polymerization via Imbibition

Imbibition can be used to load protein into a polymer after polymerization. Prior to protein uptake via imbibition (through adsorption and absorption, i.e. immersion)

experiments, HEMA:MBC copolymers were dried to equilibrium. The samples were then each individually immersed in 250 μL of labeled protein solution for three days, in either Immulon 1 Removawell strips (Dynatech Laboratories, Inc., Chantilly, VA) for ^{125}I labeled proteins, or 96 well flat-bottom plates (Becton Dickinson Labware, Franklin Lakes, NJ) for the 5-TAMRA SE-labeled proteins. Immersion was done at 4°C in the refrigerator to minimize thermal degradation of the proteins. These solutions varied with respect to the concentrations of HSA and rt-PA used. The percentage of radiolabeled protein in all solutions was 10%.

4.7 Protein Release

After the 3/16" diameter HEMA:MBC samples had been immersed in protein solution for three days, they were transferred to fresh sets of Immulon 1 Removawell strips for the radiolabelled samples, or fresh 96 well flat-bottom plates (Becton Dickinson Labware, Franklin Lakes, NJ) for the fluorescently labeled samples. The wells in both the strips and plates contained 250 μL of PBS (pH 7.4), were covered by NUNC polyolefin sealing tape (VWR International, Ltd., Mississauga, ON, Canada), and placed in a 37°C oven. At timed intervals, these samples would be transferred into fresh wells of 37°C PBS (pH 7.4), for a period of up to 5 days. The wells were analyzed to determine the quantities of released protein, based on fluorescent- or ^{125}I-based calibration curves. By analyzing the entire well, the quantity of protein released was accurately determined with no losses due to adsorption on the walls of the wells.

4.7.1 Analysis of Protein Released – Bradford Assay

A Bradford assay, a simple technique used for the quantification of proteins that does not require protein labeling, was used as an alternative method of quantifying the protein released. As such, effects on protein release due to interactions between the label, the protein, and the biomaterial are avoided. This assay uses Coomassie brilliant blue G-250 (CBBG), a dye that can bind to the arginine and aromatic residues of proteins. Once the dye has bound to a protein, it will have an absorbance maximum at 595 nm, whereas the unbound form has an absorbance maximum at 470 nm (Bradford, 1976).

There are two common procedures for the Bradford assay: the standard assay (100-1000 μg/mL sensitivity), and the microprotein assay (10-100 μg/mL sensitivity). These methods are described in detail by Bradford (1976). Briefly, solutions containing proteins are mixed with CBBG, incubated for 5-20 min, then the absorbance is read. A BioRad Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA) with a 595 nm filter was used to analyze the absorbance of the solutions. By relating the absorbance to a calibration curve, released amounts of protein could be determined.

An even more sensitive assay, termed the modified microprotein assay (1-10 μg/mL sensitivity), was also used with highly dilute samples. The main procedural difference is that equal volumes of dye reagent and sample were mixed during the protein-CBBG mixing step. In some of these experiments, sample wells were preadsorbed with milk protein, by immersion the wells in a 1 mg/mL solution of skim milk powder for 10 min, then rinsing twice with Milli-Q water. This minimized protein loss due to the adsorption of the protein of interest onto the surface of the well.

4.8 Protein Stability using SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12% gels) is a technique that is used to separate proteins according to their size. A detailed procedure is presented in Appendix D It was employed to identify rt-PA after it had been released from HEMA:MBC copolymers into solution, and to compare the band patterns to that of fresh rt-PA to verify whether any degradation had occurred due to protein-drug and biomaterial interactions. Colloidal gold staining (Protogold, BioCell Research Laboratories, Cardiff CF2 4AY, UK) was used to amplify the protein banding patterns in the resultant gels. Additional electrophoretic transfer of the bands to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) allowed the banding patterns to be transposed to a more durable and permanent material.

4.9 rt-PA Activity using the S2251 Assay

The S2251 chromogenic assay was used to assess the enzymatic activity of t-PA. Evidence for t-PA retaining its activity after being loaded and released from a biomaterial is essential to validate the HEMA:MBC copolymer as a potential biomaterial for the controlled delivery of t-PA. The loss of significant t-PA activity following loading into the polymer would substantially reduce the applicability of the HEMA-MBC as a delivery vehicle.

If t-PA is active, it will function as shown in Figure 4-5. t-PA activates plasminogen to its active form, plasmin. Plasmin in turn cleaves the S2251 molecule (Chromogenix, Mölndahl, Sweden), H-D-Val-Leu-Lys-*p*-nitroanilide, into H-D-Val-Leu-

Lys-OH and *p*-nitroanilide (pNA). pNA can be measured by spectrophotometry at a wavelength of 405 nm.

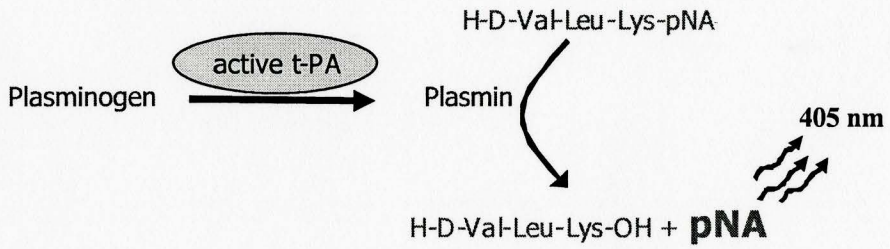


Figure 4-5 Activation of the S2251 substrate through the activity of tissue-type plasminogen activator (t-PA)

In the wells of a 96 well flat-bottom plate (Becton Dickinson Labware, Franklin Lakes, NJ), 10 μg of plasminogen (Enzyme Research Laboratories, Inc., South Bend, IN) and 30 μL of 3.5 mM S2251 were combined with TBS (pH 8.35) into a 250 μL volume. The wells were preheated to 37°C, and then 1 μg of t-PA from each sample (including control samples) was added to each well and the reactants incubated for 30 min. A BioRad Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA) with a 405 nm filter was used to determine the absorbance values of the wells. Controls included rt-PA that had been kept in a -70°C freezer after being reconstituted and aliquoted (-70°C rt-PA) and rt-PA that had been exposed to the same conditions as the rt-PA loaded into the polymers without exposure to the HEMA:MBC (control rt-PA).

5 RESULTS AND DISCUSSION

5.1 HEMA:MBC Copolymer Synthesis

5.1.1 2-methacryloyloxyethyl *n*-butylcarbamate (MBC) Synthesis

n-butyl isocyanate (BI) and 2-hydroxyethyl methacrylate (HEMA) were reacted to yield a clear, colourless to pale yellow, liquid MBC monomer. To confirm that the reaction went to completion, the urethane-containing MBC product was analyzed using FTIR. As seen in the absorbance spectrum of MBC in Figure 5-1, the absence of a –NCO peak at 2265 cm^{-1} confirms complete reaction of the isocyanate. Other peak assignments can be seen in Table 5-1. Of particular note is the peak at 3407.04 cm^{-1}, often indicative of the presence of hydroxyl groups and/or water. This peak was likely a secondary amine group peak that had broadened as a result of hydrogen bonding in the monomer or less likely, due to the presence of hydroxyl groups from unreacted HEMA monomer. The former observation is common in urethane reactions (Lelah and Cooper, 1986). The absorption of water into the system was eliminated as a possible cause of the peak, as FTIR analysis of the MBC product before and after dehydration with Type 4A molecular sieves showed the consistent presence of the ~3420 cm^{-1} peak.

Table 5-1 Major Fourier transform infrared bands and assignments for MBC monomer, based on Lelah and Cooper (1986) a. ν = stretching, ν_a = asymmetric stretching

Wavenumber (cm^{-1})	**Assignment[a]**
3407.04	ν(N-H) – H-bonded N-H
2957.91	$\nu_a(CH_2)$
1715.58	ν(C=O) – H-bonded C=O of urethane or free C=O of urea
1636.81	ν(C=O) – H-bonded C=O of urea

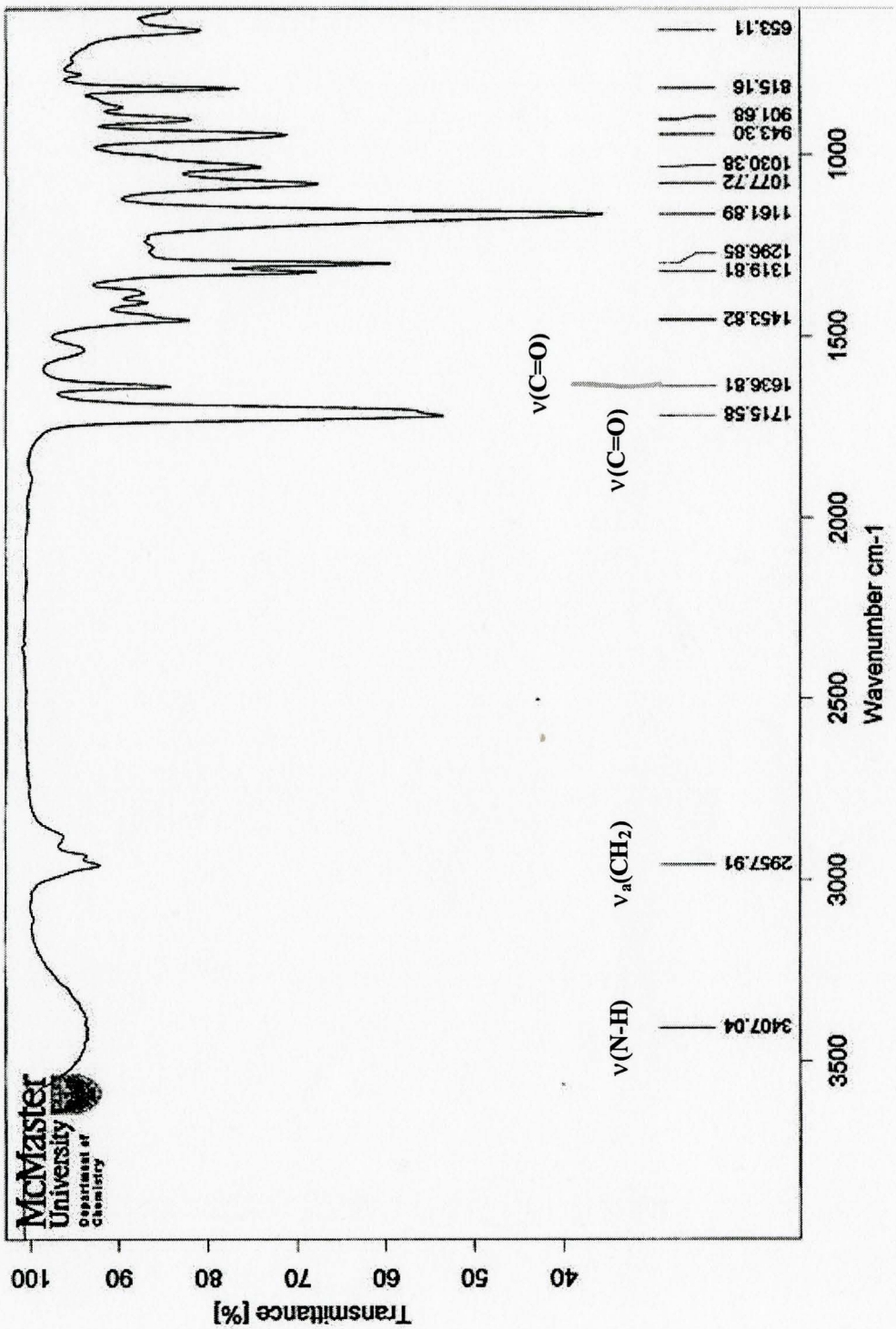


Figure 5-1 Fourier transform infrared spectrum of 2-methacryloyloxyethyl *n*-butylcarbamate (MBC) monomer

5.1.2 HEMA:MBC Copolymer Synthesis

Once synthesized, the MBC monomer was stored at 4°C until needed. At times, solid particles (probably polymerized aggregates of MBC monomer), would appear in the solution. When visible particulates were present, passing the MBC monomer through a 0.2 μm Supor membrane (PALL Life Sciences, Ann Arbor, MI) removed this suspended matter from the liquid prior to use.

Although AIBN was the initiator previously used to synthesize the HEMA:MBC copolymer products (Mequanint and Sheardown, 2005), it was found that the products consistently displayed signs of bubble formation in the products. These bubbles were believed to be due to the gaseous nitrogen byproduct of AIBN activation, as shown in Scheme 2.2. As a result, BP was used in place of AIBN, and smooth physically homogenous HEMA:MBC products resulted.

When MBC monomer was combined with HEMA monomer at very high molar ratios (e.g. 50:50) and Milli-Q water was added, an emulsion-like mixture was often produced. This problem was resolved by ensuring that these HEMA:MBC monomer solutions were well stirred until a homogeneous, clear, colourless to pale-yellow solution resulted (usually 30 min) when the mixtures were made. As well, prior to polymerizing the monomer solutions, these solutions were always stirred for a few minutes to ensure adequate mixing of reagents.

The two hour time period that was chosen for irradiating the monomer solutions was based on qualitative experiments that evaluated whether the products were solid after various durations of irradiation. It was found that at least 1.5 h was required to

polymerize the different HEMA:MBC solutions from a liquid to a solid state; the extra 30 minutes was added to ensure complete polymerization.

The HEMA:MBC products that resulted from these experiments were solid and smooth, with rubber-like consistency and flexibility when in the water-swollen state. It was clear that the higher the amount of MBC reagent in the initial monomer solution (up to 50:50 HEMA:MBC), the more opaque and white the product would be; pure HEMA (100:0), on the other hand, was transparent and colourless. Less solid, residual portions of possibly monomer and polymer were removed from the edges of the final product by washing with Milli-Q water The disks for subsequent experiments were punched from uniform areas, remote from the edges of the polymerized product.

5.2 Surface characterization

5.2.1 Surface Hydrophilicity – Contact Angle Data

Because of the hydrophilic nature of the HEMA:MBC copolymers, water droplets placed on a dry copolymer readily change in shape as water is absorbed (Pokidysheva et al., 2001). Consequently, all copolymer samples that were evaluated were swelled to equilibrium in Milli-Q water prior to contact angle measurements.

In the advancing and receding contact angle data seen in Figure 5-2, as the MBC content in the HEMA:MBC monomer solutions was increased, there was an accompanying increase in the surface hydrophobicity of the resulting copolymers and an associated increase in both the advancing and receding water contact angles. This is in

agreement with observations by Hermitte et al. (2004) who found that the amount of HEMA present in HEMA copolymers is linearly correlated to the measured contact angles. The trend in Figure 5-2 is also expected as MBC is more hydrophobic than HEMA (Mequanint and Sheardown, 2005); this suggests that MBC is being incorporated into the products. While there are differences in the average values of the advancing and receding contact angles for pure pHEMA (100:0) with 30% water content, 39.3° and 27.0° respectively, and those reported in the literature, advancing: 61.0° to 84.3° and receding: 8.8° to 27.3° (Holly and Refojo, 1975; Harkes et al., 1991, Hutcheon et al., 2001), these differences are most likely attributable to other factors such as water content, the pHEMA synthesis process, the environment, and the user (Holly and Refojo, 1975; Merrett et al., 2002).

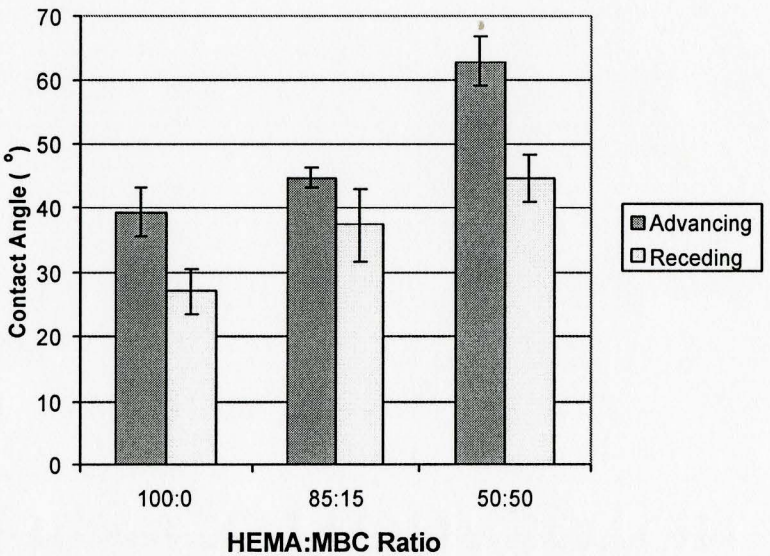


Figure 5-2 Advancing and receding sessile water drop contact angles for various HEMA:MBC copolymers (mean ± SD, n = 3)

5.3 Bulk Characterization

5.3.1 Equilibrium Solvent Uptake

The amounts of solvent that different HEMA:MBC copolymers can take up as well as the kinetics of uptake was important information in establishing the feasibility of using these polymers for controlled delivery, as well as providing information about the relative hydrophilicity/hydrophobicity of the different copolymer compositions. The time required for equilibrium swelling of the polymers in a solvent is useful in determining the time required to achieve equilibrium protein-drug loading into the hydrogel. Knowledge of the change in mass and volume of the copolymers in different solvents can indicate the suitability of these copolymers in various environments as well as aiding in designing a drug loading procedure. The uptake of DMF, 50% (v/v) ethanol (aq), PBS (pH 7.4), and Milli-Q water at room temperature were determined in these solvent uptake studies.

5.3.2 Solvent Uptake Kinetics

Figure 5-3 shows the results of a study to examine the kinetics of solvent uptake. The polymers rapidly absorbed the solvent, in this case 50% ethanol (aq), with no significant further change in solvent uptake being observed after two days of immersion; similar results were obtained with other solvent systems. Results for the loss of solvent from equilibrium-swollen HEMA:MBC samples demonstrated that an equilibrium dry mass was attained after three days of drying in a 37°C oven (data not shown). As such, it was concluded that soaking HEMA:MBC samples in solvents for at least three days, or

drying them in a 37°C oven for at least three days, would ensure that equilibrium sample masses were achieved.

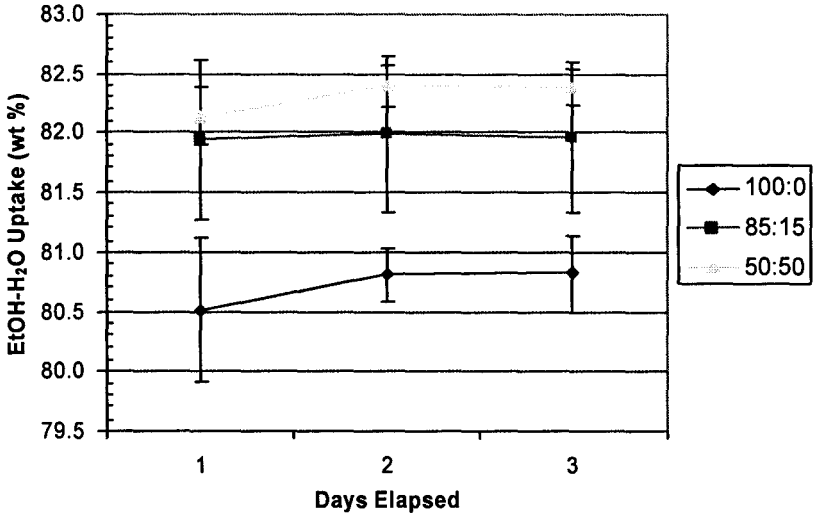


Figure 5-3 Amount of 50% ethanol (aq) taken up by various HEMA:MBC copolymers after various time periods (mean ± SD, n= 3)

5.3.3 Copolymer Solvent Uptake

Trends with respect to the effect of MBC content on equilibrium solvent uptake were apparent. When the solvent was organic, DMF or 50% ethanol (aq), an increase in MBC content resulted in higher equilibrium solvent uptake, as seen in Figure 5-4. When the solvent was aqueous, PBS (pH 7.4) or Milli-Q water, an increase in MBC content resulted in lower equilibrium solvent uptake, as seen in Figure 5-5. The magnitudes of the Milli-Q water up take for 100:0 and 85:15 HEMA:MBC copolymers, 37.6% ± 1.2% and 34.0% ± 1.1%, respectively, differed from the ~42% and ~22% seen by Mequanint

and Sheardown (2005). This can likely be attributed to slight differences in the composition of products due to slightly different fabrication methods.

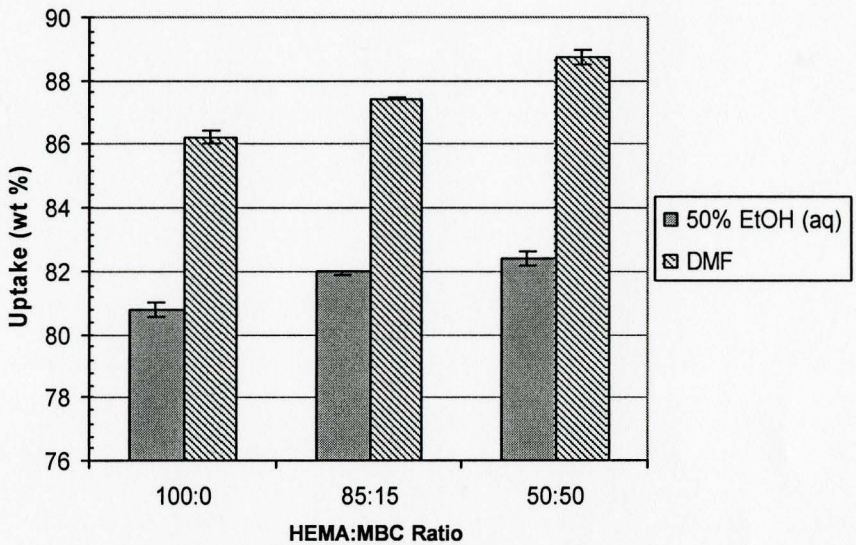


Figure 5-4 Equilibrium uptake of DMF and 50% ethanol (EtOH, aq) by various HEMA:MBC copolymers (mean ± SD, n = 3)

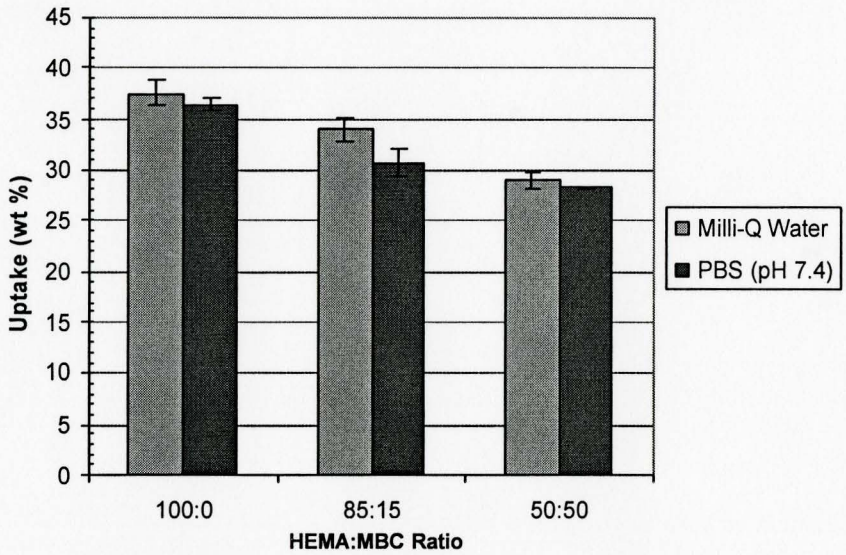


Figure 5-5 Equilibrium uptake of Milli-Q water and PBS (pH 7.4) by various HEMA:MBC copolymers (mean ± SD, n = 3)

Clearly, as shown in Figure 5-6, a general trend is apparent whereby an increase in solvent polarity results in a decrease in solvent uptake. However, if higher protein-drug loading into HEMA:MBC copolymers is to be achieved by using organic solvents with higher solvent uptake, care must be taken to ensure complete removal as these solvents are often toxic. Furthermore, the solubility and stability of the protein may limit the applicability of this method of drug loading.

Solvent Polarity

Solvent	Milli-Q Water	PBS (pH 7.4)	50% Ethanol (aq)	DMF
Average Uptake (%)	32.7 ± 5.6	31.0 ± 4.5	81.7 ± 0.8	87.5 ± 1.3

Equilibrium Solvent Uptake

Figure 5-6 Average solvent uptake by HEMA:MBC copolymers, ranging from 100:0 to 50:50 HEMA:MBC (mean ± SD, n = 3 for each HEMA:MBC composition)

5.3.4 Copolymer Swelling

Figure 5-7 summarizes the volume change observed when samples of various HEMA:MBC composition were swollen to equilibrium in DMF The diameters and thicknesses of the different copolymer samples were measured using a Vernier caliper (Mitutoyo Corp., Japan) when the samples were completely dry, and also after immersion in DMF These values were used to calculate the changes in sample volume. However, the accuracy of this method was poor due to the rubbery nature of the polymers which made them susceptible to deformation during the measurement. Nevertheless, from Figure 5-7 it is clear that the swelling of the HEMA:MBC copolymers in DMF increased as their MBC content increased. This is not surprising as increased MBC content of the polymers should result in increased polyurethane character, and DMF is a well-known polyurethane solvent (Lelah and Cooper, 1986). Similar data were obtained for samples that were immersed in 50% ethanol (aq). Such relationships between swelling and the HEMA content have been previously observed (Hermitte et al., 2004).

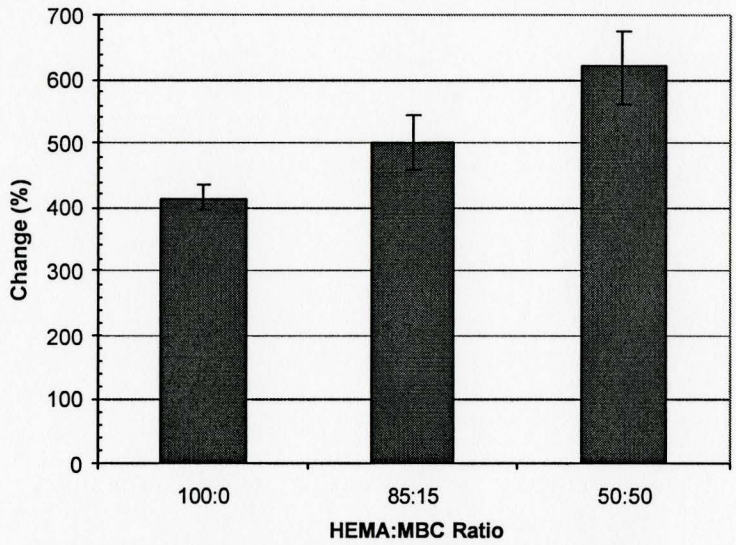


Figure 5-7 Volume change in various HEMA:MBC copolymers that had been immersed in DMF for three days (mean ± SD, n= 3)

Conversely, when samples were immersed in aqueous solvents such as Milli-Q water and PBS (pH 7.4), an increase in the MBC content was generally accompanied by a decrease in swelling as would be expected. The data for these solvents are shown in Table 5-2.

Table 5-2 Volume change in various HEMA:MBC copolymers that had been immersed in aqueous solvents for three days (mean ± SD, n= 3)

	Volume Change (%) in various HEMA:MBC Copolymers		
Solvent	**100:0**	**85:15**	**50:50**
PBS (pH 7.4)	44.6 ± 4.4	38.3 ± 8.5	28.1 ± 0.5
Milli-Q Water	52.2 ± 10.2	36.6 ± 4.0	23.6 ± 3.9

5.3.5 Copolymer Mass

An observation that supports the belief that different HEMA:MBC copolymers were being produced from the various feeds used (100:0, 85:15, 50:50, etc. HEMA:MBC), was based on measuring the dry masses of the disks (diameter = 3/16", thickness = 1.5 mm) that had been punched out of the products. From Figure 5-8, it can be seen that as MBC content increases in the feed, the dry mass of the final copolymer product increases. This is reasonable, considering that the copolymer products were originally swelled in Milli-Q water prior to punching out the discs. As was seen in Figure 5-5 and Table 5-2, copolymer products with lower MBC content swell more and take up larger amounts of aqueous solvent. When 3/16" diameter copolymer discs were punched out of the copolymer products and then dried, the samples with lower MBC content would consequently lose more aqueous solvent, and thus more mass, than samples with higher MBC content. These low MBC content samples would also have less polymeric HEMA:MBC content, since they have greater solvent content, again leading to reduced masses. This idea is confirmed by the results in Figure 5-8.

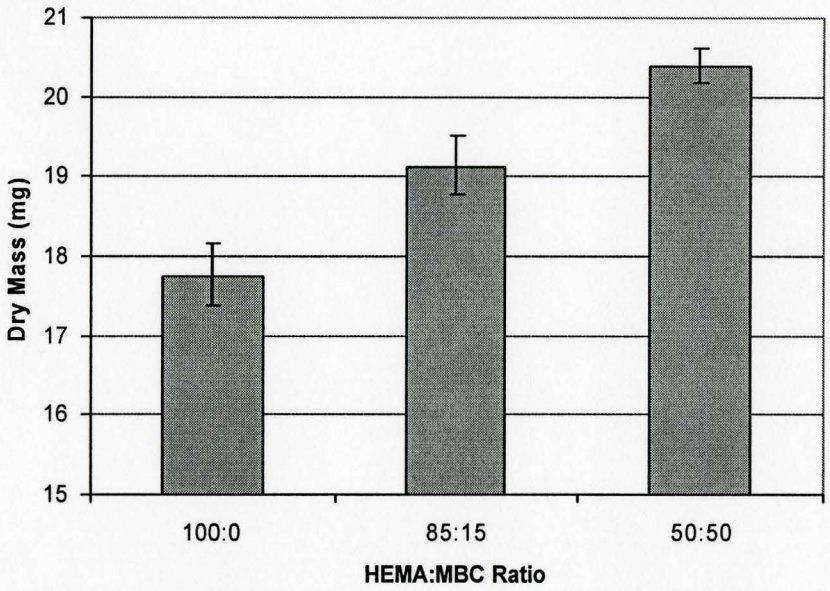


Figure 5-8 Average masses of various dried HEMA:MBC copolymer samples with identical swelled volumes (0.027 cm^3) (mean ± SD, n= 5)

5.3.6 Differential Scanning Calorimetry

Representative DSC scans for the 100:0 and 80:20 HEMA:MBC copolymers are shown in Figure 5-9. A single glass transition temperature (T_g) was observed for both the 100:0 and 80:20 HEMA:MBC compositions, indicating a single polymer phase consisting of a random copolymer with no significant homopolymer content. The T_g values obtained for the various HEMA:MBC samples are summarized in Table 5-3. The increase in T_g for the hydrated samples compared to the dry samples, and for the 80:20 sample compared to the 100:0 sample in the hydrated state, may be attributed to the water content increasing the plasticity of the copolymer (Roorda, 1994), and to the incorporation of the more rigid MBC monomer Based on the T_g data for the 100:0 and

80:20 HEMA:MBC materials, it may be reasonably assumed that the other HEMA:MBC compositions also possess a single T_g, and are thus homogeneous in nature.

The T_g values for pHEMA have been shown to be in the range of ~0°C to ~120°C, depending on such factors as water content, crosslinker content, initiator (Hill et al., 1999; Brahim et al., 2003; Roorda, 1994; Meakin et al., 2003). Values for the T_g of 100:0 pHEMA (Table 5-3) are similar to recently reported T_g values for pHEMA, which ranged from 98°C to 109°C (Hill et al., 1999; Hodge et al., 1998; Fambri et al., 1993). The similarity between the T_g of the 80:20 HEMA:MBC and the literature range of T_g values for pHEMA is expected since HEMA comprises the majority of the material in the 80:20 HEMA:MBC copolymer.

Table 5-3 Glass transition temperatures (T_g) for hydrated and dried 100:0 and 80:20 HEMA:MBC copolymers (n=1). Errors are typically ± 2%-3%

Sample	T_g for hydrated sample	T_g for dried sample
100:0 HEMA:MBC	107.3°C	102.8°C
8020 HEMA:MBC	113.3°C	101.6°C

5.3.7 Nuclear Magnetic Resonance (NMR) Spectroscopy

^{13}C NMR spectra were acquired for HEMA:MBC copolymers produced from 100:0, 95:5, 90:10, and 85:15 HEMA:MBC molar ratio solutions. Figure 5-10 and Figure 5-11 show the ^{13}C-NMR spectra, and peak assignments, for 100:0 and 85:15 HEMA:MBC copolymers, respectively. Peaks 1 to 6 can be assigned to the HEMA component of the copolymers, while peaks 7 to 16 can be assigned to the MBC

component. Comparing the ^{13}C-NMR spectra for 100:0, 95:5, 90:10, 85:15 in sequence, it is clear that with increasing amounts of MBC in the monomer feed, there are increasing amounts of MBC in the final copolymers since peaks 7 to 16 grow stronger and sharper as the MBC content increases (Figure 5-12).

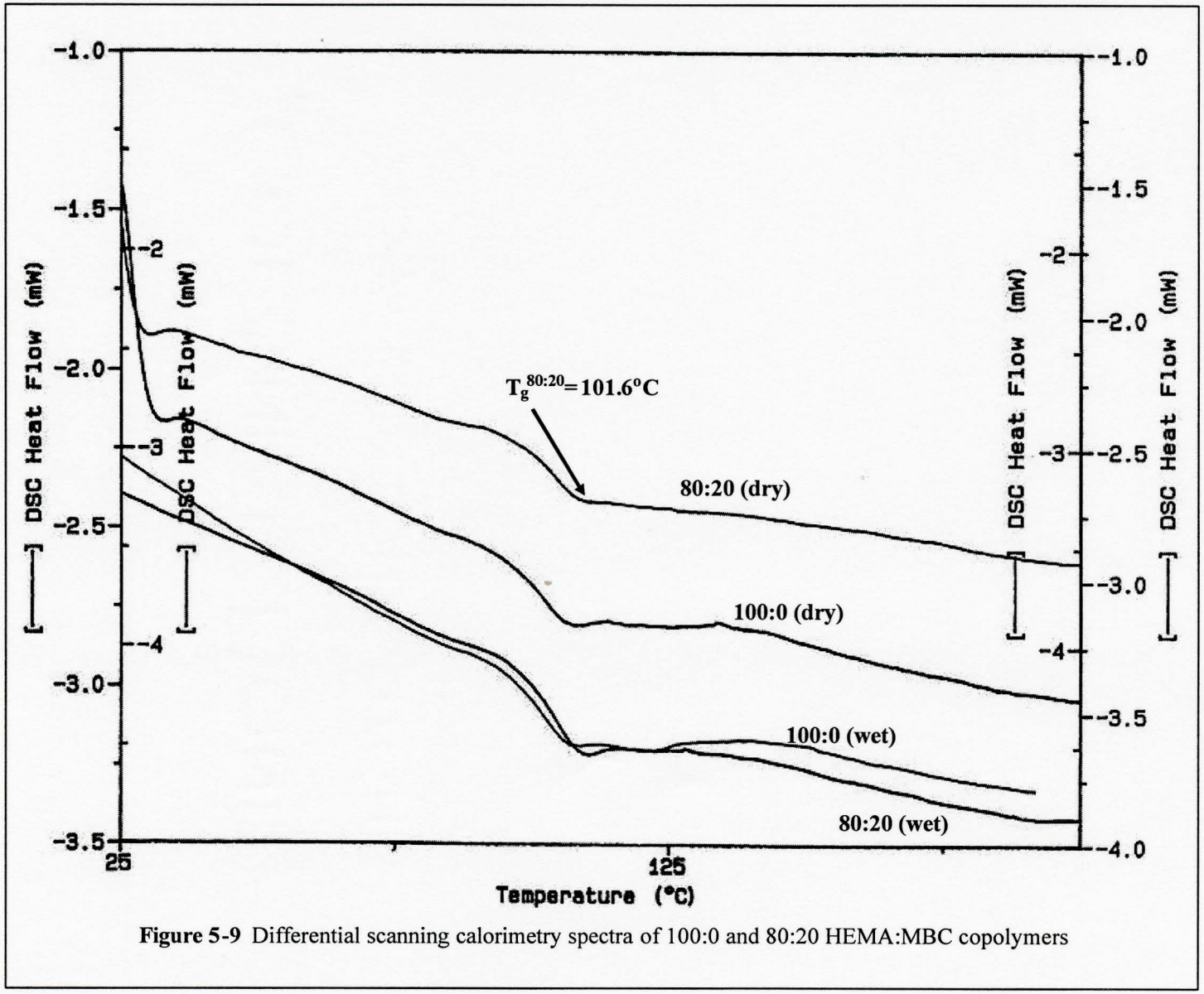


Figure 5-9 Differential scanning calorimetry spectra of 100:0 and 80:20 HEMA:MBC copolymers

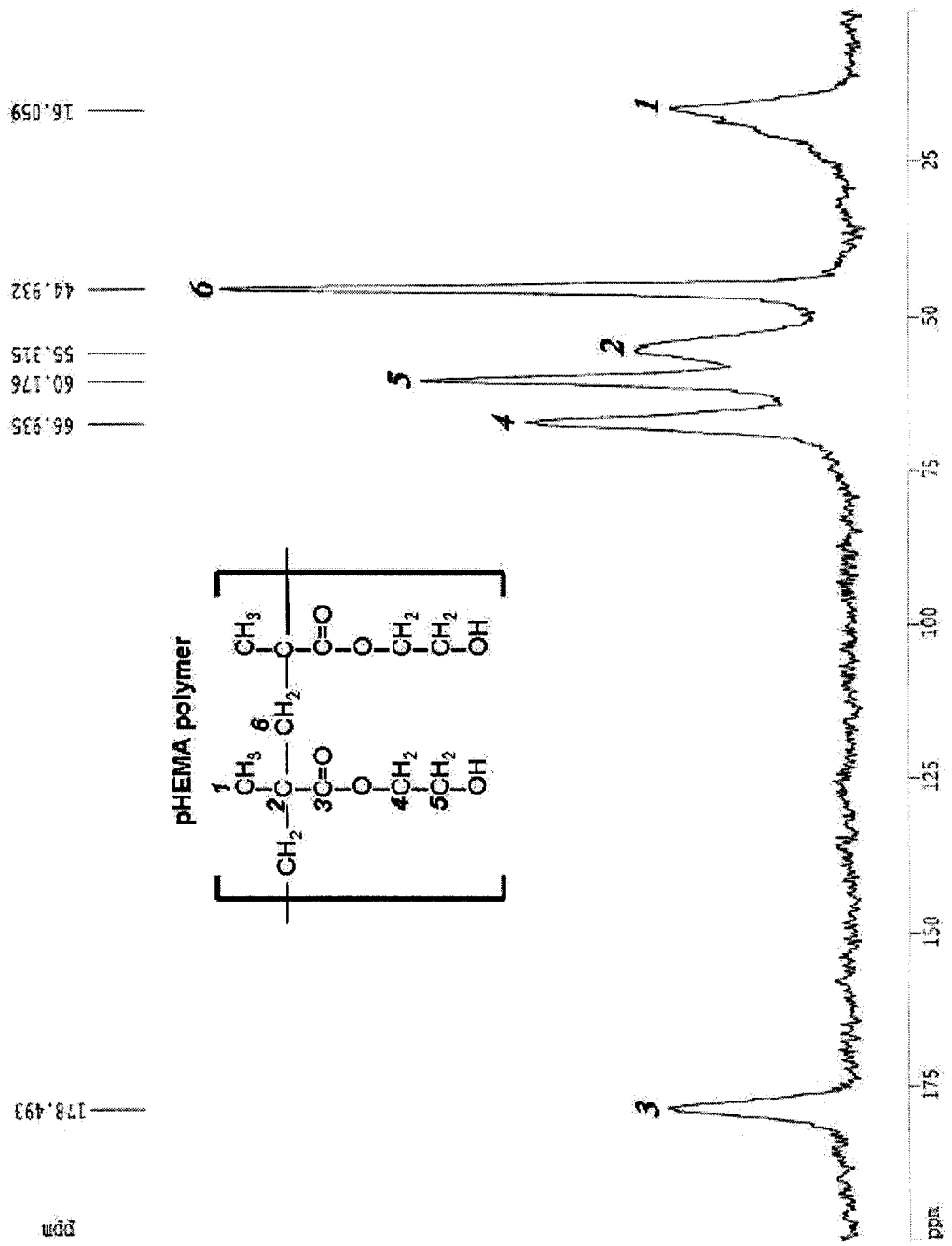


Figure 5-10 ^{13}C-Nuclear magnetic resonance spectrum of solid 100:0 HEMA:MBC copolymer

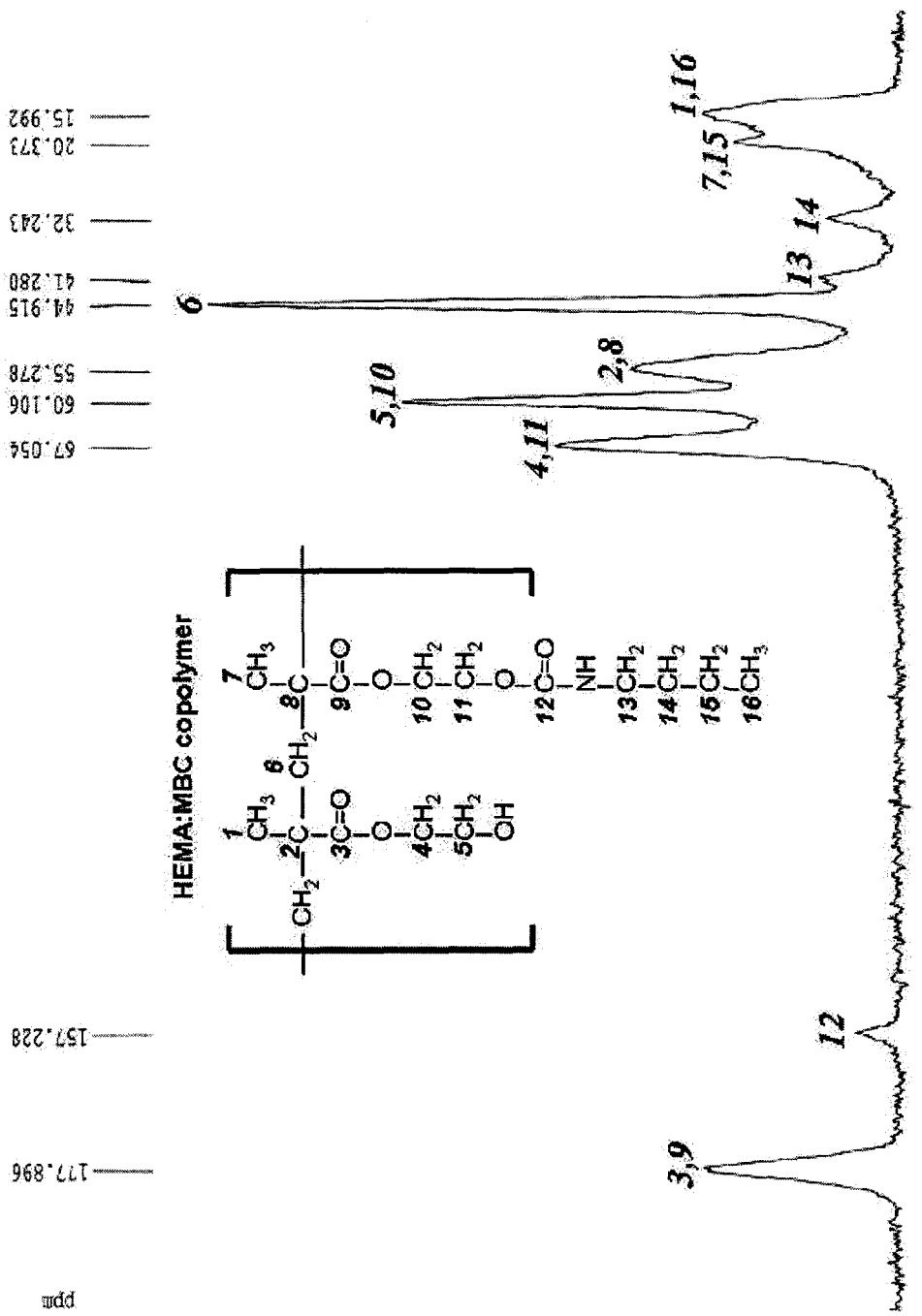


Figure 5-11 ^{13}C-Nuclear magnetic resonance spectrum of solid 85:15 HEMA:MBC copolymer

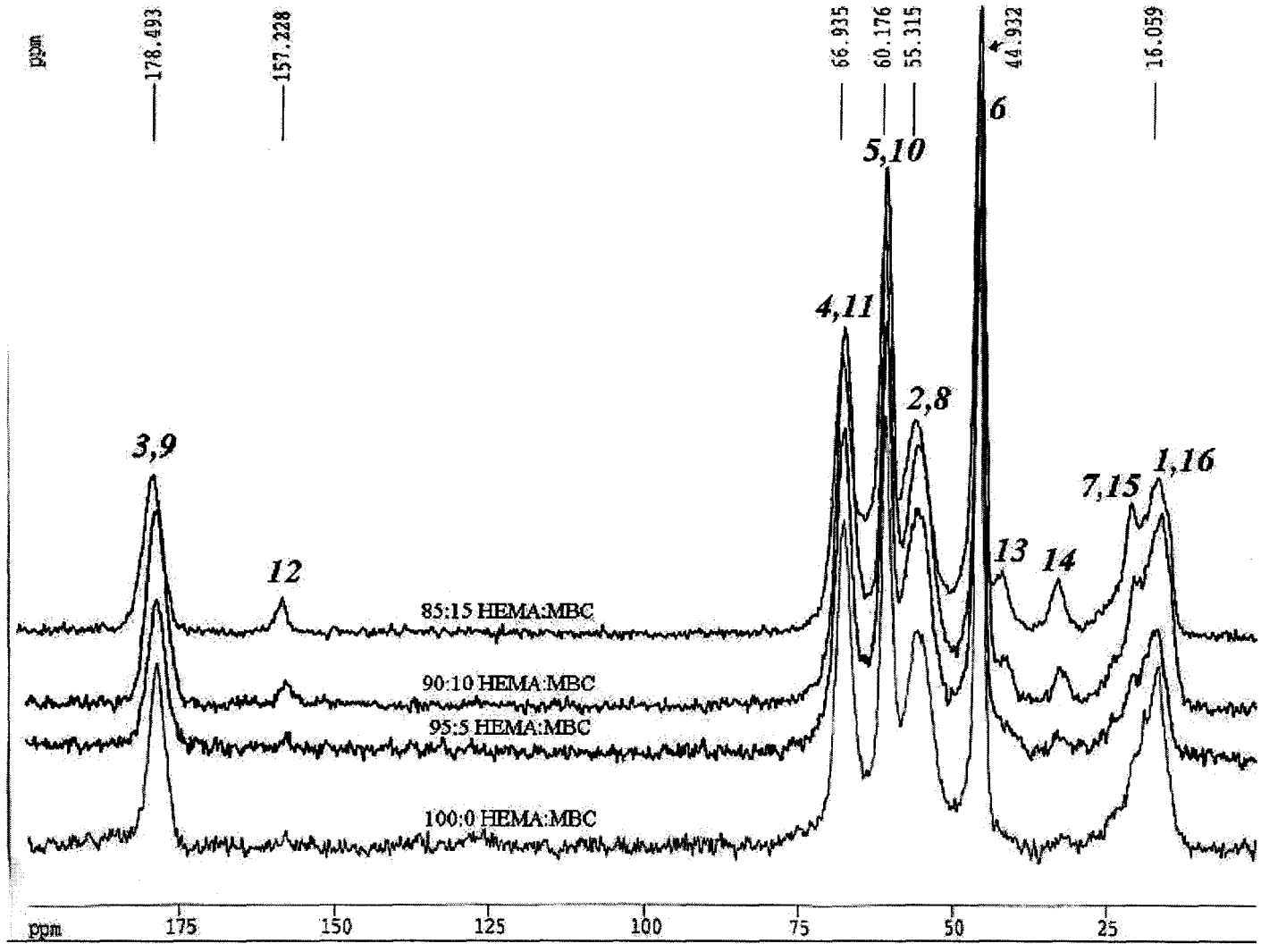


Figure 5-12 Superimposed ^{13}C-nuclear magnetic resonance spectra of HEMA:MBC copolymer (100:0, 95:5, 90:10, 85:15)

5.4 Labeling Proteins with 5-TAMRA SE

HSA was easily labeled with 5-TAMRA SE using an amine-reactive labeling procedure (Molecular Probes, 2003; Haugland, 1996), as shown by the absorption spectra for the reaction products in the UV-visible range (Figure 5-13). Two peaks of equal magnitude at 520 nm and 550 nm were seen consistently, indicative of the presence of protein-bound 5-TAMRA SE. However, reaction of rt-PA with 5-TAMRA SE under the same conditions gave a different result. While there was a noticeable peak at 550 nm, only a small shoulder peak was observed at 520 nm, indicative of a product that consisted mainly of unbound 5-TAMRA SE.

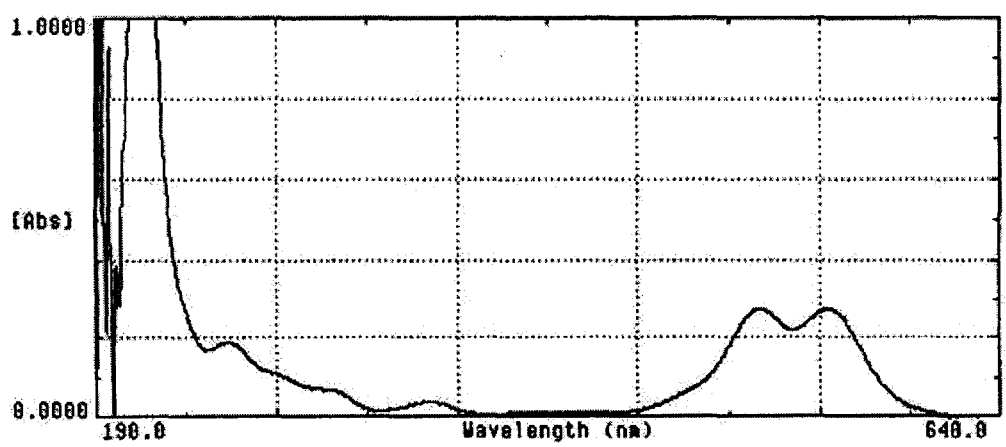


Figure 5-13 Absorbance spectra of human serum albumin conjugated to the fluorophor, 5-carboxytetramethylrhodamine succinimidyl ester, in PBS (pH 7.4)

5.5 Evaluation of t-PA Solubility

An obstacle to effective labeling of rt-PA with the 5-TAMRA SE fluorophor is the high L-arginine (L-Arg) content of the rt-PA material as received. This is because the large number of amine groups present in L-Arg inhibits the reaction of rt-PA with the fluorophor, through competitive inhibition. Following removal of the L-Arg by dialysis against Milli-Q water and freeze drying, various procedural modifications were attempted to label the rt-PA with 5-TAMRA SE. These included increasing the pH from 8.3 to 8.5 for the 0.1 M sodium bicarbonate buffer used, which increases the reaction efficiency of 5-TAMRA SE, and extending the reaction time to 2 h instead of 1 h (Molecular Probes, 2003). Under these conditions, a significant shoulder peak at 520 nm of approximately half of the magnitude of the peak at 550 nm was obtained. While there was considerably more protein-bound fluorophor, a significant amount of unbound dye remained which could not be removed by dialysis.

The solubility of the rt-PA in the $NaHCO_3$ buffer used in the labeling procedure was also a problem. The buffer components (phosphoric acid, polysorbate 80, and L-Arg) are apparently necessary to create the basic solution conditions necessary to solubilize the rt-PA. Without them, based on the fact that tPA has a pI of 6.5-8.8 (Moorhouse et al., 1996; Sueishi et al., 1982; Binder et al., 1979), reaction with the dye in $NaHCO_3$ buffer (pH of 8.3) is not feasible due to a lack of unprotonated amine groups. Solubility tests, the results of which are summarized in Table 5-4, were performed in an attempt to find appropriate conditions for the conjugation reaction.

Table 5-4 Descriptions of solubility tests for rt-PA, and their results

Description	Soluble?
~5 mg rt-PA per mL sterile water/$NaHCO_3$ buffer (pH 8.3)	YES
3.33 mg dialyzed rt-PA per mL $NaHCO_3$ buffer (pH 8.3)	No; rt-PA precipitate confirmed by peak at A_{280}
Heat ~ 1 mg dialyzed rt-PA per mL $NaHCO_3$ buffer (pH 8.3)	No
~4 mg dialyzed rt-PA per mL of rt-PA buffer (pH 1.61)**	YES, but only under acidic conditions
~4 mg dialyzed rt-PA per mL of rt-PA buffer (pH 1.61); added L-Arg substitute, e-amino-?-caproic acid	pH was increased to ~8 with increased solubility using e-amino-?-caproic acid, but large amounts of lysine-analog was needed
~4 mg dialyzed rt-PA per mL of rt-PA buffer (pH 1.61); added H-Lys(BOC)-OH	No; large amounts of H-Lys(BOC)-OH required
~4 mg dialyzed rt-PA per mL of rt-PA buffer (pH 1.61); added Nα-*t*-BOC-ω-Nitro-L-Arg	No; Nα-*t*-BOC-ω-Nitro-L-Arg is not soluble in aqueous solution

**rt-PA Buffer (pH = 1.61 instead of ~7.0 since L-Arg has been removed) = 0.1 g/mL phosphoric acid and 0.8 mg/mL polysorbate 80 in Milli-Q water (Genentech, 1999)

The results of the solubility tests indicate that rt-PA, when isolated from its buffering agents, is insoluble in aqueous solution. Heat does not visibly increase its solubility, although when an aqueous rt-PA buffer is used that is very acidic, the rt-PA will dissolve. Attempts to increase the pH to 8.3 as required for the amine-labeling reaction using substitutes for L-Arg, were unsuccessful since the quantity of these agents

was much more than the amount of L-Arg originally in solution. Additionally, the e-amino-?-caproic acid still has amine groups that can interfere with the 5-TAMRA SE-protein interactions, making the use of large amounts impractical. The Nα-*t*-BOC-ω-Nitro-L-Arg agent was also not soluble in aqueous solution, although the use of large amounts of glycerol or acetonitrile could resolve this problem. Based on these results, it seems that effective 5-TAMRA SE-labeling of rt-PA is not feasible and alternative methods were used.

5.6 HSA Loading into Polymer before Polymerization

As an alternative to loading the polymer with the proteins post polymerization via swelling of the polymer in a concentrated protein solution, loading of the protein during polymerization was investigated using HSA as the test molecule. Appropriate HEMA:MBC monomer solutions were prepared without Milli-Q water. Following this, various methods, summarized in Table 5-5, were used to try to solubilize the protein in the monomer solution.

Table 5-5 Descriptions of solubility tests for HSA and rt-PA, and their results

Description	Soluble?
Missing solvent, loaded with 5 wt% HSA, was mixed with 50:50 HEMA:MBC monomer solutions	No
Missing solvent, loaded with 1 wt% HSA, was mixed with 50:50 HEMA:MBC monomer solutions	Slightly, but precipitation observed after 1 day
Missing solvent, loaded with 1 wt% HSA, was mixed with 100:00 HEMA:MBC monomer solutions	Slightly, but precipitation observed after 3 days
Missing solvent, loaded with 2 wt% HSA, was mixed with 100:00 HEMA:MBC monomer solutions	No

Based on the observations in Table 5-5, 1 wt% HSA was dissolved in Milli-Q water and this solution was combined with various monomer feed mixtures. The samples were then polymerized. The resultant materials were not homogenous and had small bubble-like formations throughout. It is believed that these bubbles, which were not homogeneously dispersed in the products, were solid aggregated protein nodules. Loading prior to polymerization thus proved not to be feasible. Therefore, release studies were carried out on samples that were loaded post-polymerization.

5.7 Protein Adsorption – ^{125}I-Labeled HSA

The adsorption of plasma proteins onto biomaterials precedes a variety of other host responses such as intrinsic coagulation (Courtney et al., 1994). As such, it was considered important to evaluate the amounts of protein adsorbed on the HEMA:MBC

products. ^{125}I-labeled HSA was used as a model protein in this work. This information can be used to determine whether monolayers or multilayers of protein are being formed, which in turn may indicate whether protein-drug loading and release is primarily occurring on, and from, the surface, and/or into and from the interior of the biomaterial. Results obtained from various pre-swelled HEMA:MBC copolymers immersed for 2 h in a 1 mg/mL ^{125}I-labeled HSA solution are shown in Figure 5-14.

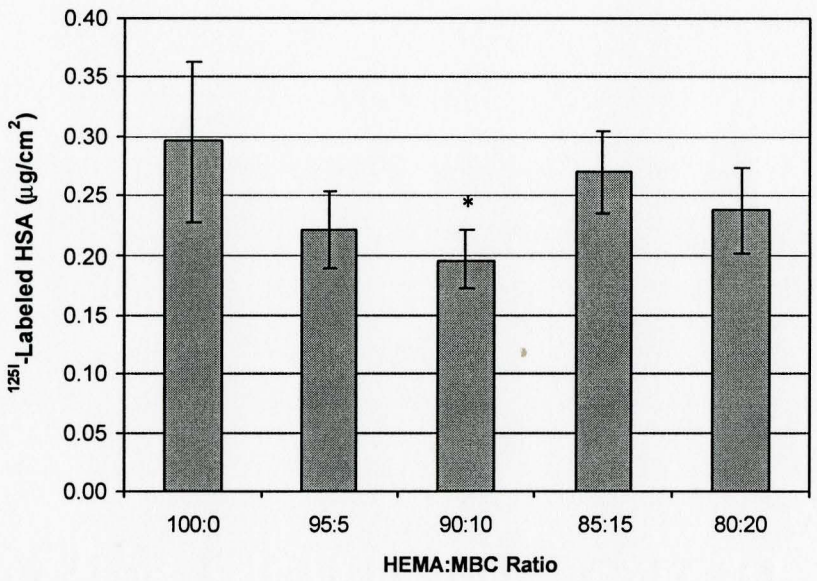


Figure 5-14 ^{125}I-labeled human serum albumin (HSA) adsorption to various HEMA:MBC copolymers. Pre-swelled samples were immersed for 2 h in a 1 mg/mL HSA solution (mean ± SD, n = 3). Statistical analysis was carried out using a one-way ANOVA test (P<0.05). Significant differences are indicated by * compared to the 100:0 HEMA:MBC copolymer

The amount of HSA adsorbed, ranging from 0.20 to 0.30 µg/cm^2, is slightly less than the theoretical amount predicted for monolayer adsorption of close-packed HSA --

0.40-0.46 μg/cm^2 (Dupont-Gillain et al., 2003). The value for 100% pHEMA (0.30 μg/cm^2) is also slightly less than the 0.34 μg/cm^2 value obtained by Pokidysheva et al. (2001) for fluorescein isothiocyanate-labeled HSA adsorbed onto pure pHEMA, although the difference is not likely significant. The data in Figure 5-14 suggest that the various HEMA:MBC products adsorb approximately a monolayer of HSA, and that the presence of MBC results in less HSA being adsorbed compared to pure pHEMA (100:0 HEMA:MBC). The data tend to suggest that increasing the MBC content and the surface hydrophobicity results in a decrease in protein adsorption, although this is not conclusive. While the reason for the increase in protein adsorption seen between the 90:10 and 85:15 HEMA:MBC copolymers is not known, it is possible that it is the result of a balance between diffusion of the protein into the swollen porous hydrogel copolymers that occurs over the two hour adsorption period, and the actual adsorption of protein to the polymer surface.

5.8 Protein Adsorption and Absorption – ^{125}I-Labeled HSA

The total amount of protein that can be loaded through imbibition into a protein-drug delivering biomaterial provides a quantitative assessment of the amount of drug that can be released. Coupled with adsorbed protein data, such as reported in the previous section, the efficiency of the loading process can be assessed. Therefore, a study of ^{125}I-labeled HSA uptake by initially dehydrated HEMA:MBC copolymers of varying composition was performed. Uptake of HSA was determined after 2 h and 48 h immersion in a 1 mg/mL HSA solution. The data are summarized in Figure 5-15.

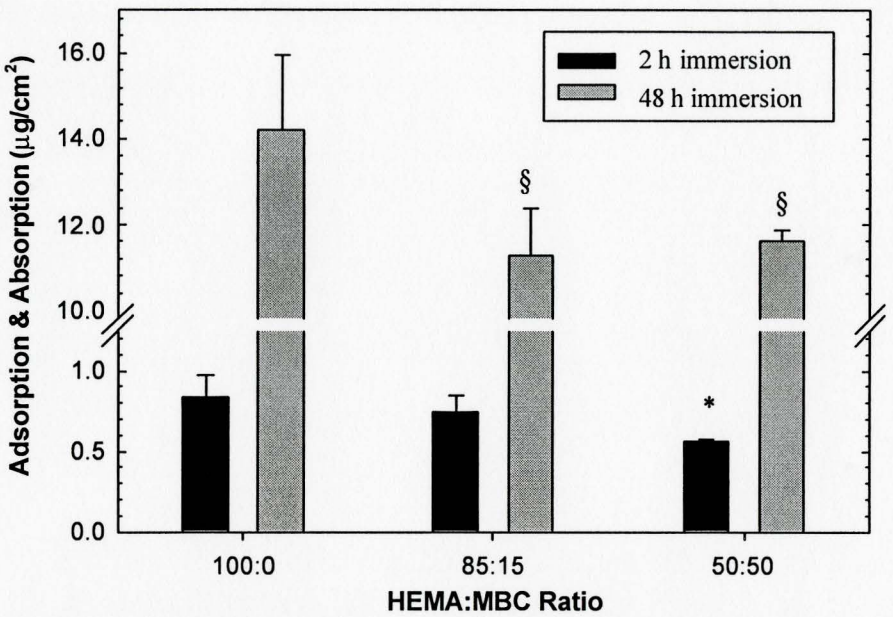


Figure 5-15 Amounts of ^{125}I-labeled human serum albumin (HSA) taken up by various compositions of HEMA:MBC copolymers after dried samples were immersed for different durations in a 1 mg/mL HSA solution Statistical analyses were carried out using one-way ANOVA tests (P<0.03). Significant differences are indicated by * and §. *compared to the 100:0 HEMA:MBC copolymer (for 2 h data). §compared to the 100:0 HEMA:MBC copolymer (for 48 h data)

There are differences in the amounts of HSA taken up by polymers of different composition both at 2 and 48 h, with increasing MBC content giving decreasing protein uptake as expected. This is consistent with previous data that showed that higher MBC content led to lower swelling and reduced imbibition of aqueous solvent, both of which would be expected to result in less protein uptake. The total quantity of HSA taken up at two h (0.57 to 0.84 μg/cm^2) is slightly above the theoretical amounts expected for a monolayer adsorption suggesting that the HSA is being both adsorbed onto and absorbed into the HEMA:MBC copolymers.

After 48 h of immersion, the difference between the two MBC-containing polymers was no longer significant, despite the fact that these compositions differ in their ability to take up PBS (pH 7.4) (Figure 5-5). This is likely due to the establishment of an equilibrium between the copolymers and the surrounding, relatively low concentration, solution rather than to differences in the copolymer samples. The use of a higher solution concentration of HSA would therefore be expected to result in greater differences in HSA uptake. Nevertheless, the quantities of HSA taken up after 48 h are much higher than at 2 h of immersion. Clearly, the finding of much larger uptake values (11.6 to 14.2 $\mu g/cm^2$) supports the notion that there is both adsorption and absorption, and that as solvent penetration increases over time, so does protein loading.

5.9 Adsorption and Absorption of ^{125}I-Labeled rt-PA

Similar uptake studies were performed using ^{125}I-labeled rt-PA. After imbibition, HEMA:MBC samples were removed from their rt-PA immersion solutions at various time points and then wiped with paper tissue to remove surface-adherent droplets. The total amount of rt-PA associated with the polymers was then determined using a gamma counter. The results for samples immersed over a period of 3 days are summarized in Table 5-6, and suggest that both adsorption and absorption occurred.

Table 5-6 ^{125}I-labeled rt-PA uptake by various HEMA:MBC copolymers immersed for three days at different rt-PA concentrations (mean ± SD, n = 3). Statistical analysis was carried out using a one-way ANOVA (P<0.05) test. Significant differences are indicated by * and †. *compared to the 100:0 HEMA:MBC copolymer. †compared to the 85:15 HEMA:MBC copolymer

	rt-PA Immersion Concentration	
Copolymer	**2 mg/mL**	**1 mg/mL**
100:0 HEMA:MBC	3.01 ± 0.02 μg/cm^2	2.15 ± 0.09 μg/cm^2
85:15 HEMA:MBC	2.87 ± 0.15 μg/cm^2	2.20 ± 0.05 μg/cm^2
50:50 HEMA:MBC	2.72 ± 0.14 μg/cm^2 *	2.01 ± 0.10 μg/cm^2 †

From the data, it appears that as the MBC content in the HEMA:MBC copolymers increases, the amount of rt-PA taken up decreases. Differences were, as expected, most evident for HEMA:MBC samples immersed in a 2 mg/mL rt-PA solution, since higher solution concentration should lead to a greater driving force for protein-drug loading. As with HSA, the protein-drug uptake trend is less evident for samples immersed in a 1 mg/mL rt-PA solution. This trend may be due to the samples not achieving their equilibrium protein uptake potential in the immersion time allotted, since lower protein-drug solution concentrations lead to slower protein-drug uptake driving forces. When the driving force is increased by immersing the copolymers in a 2 mg/mL solution, differences among the different copolymers are more clearly evident, perhaps because equilibrium protein-drug uptake is more likely to have been attained (Table 5-6). The small protein-drug loading by imbibition, at most 0.03 wt% in pHEMA (100:0 HEMA:MBC), is consistent with the work of Antonsen *et al.* (1993) who observed similar low levels (< 0.1 wt%) of α-amylase and lysozyme loading via imbibition into pHEMA.

5.10 HSA Release

5.10.1 Bradford Assay

The Bradford standard assay (100-1000 mg/mL sensitivity), microassay (10-100 mg/mL sensitivity), and modified microassay (1-10 mg/mL sensitivity) were considered as potentially useful methods to confirm HSA release from various HEMA:MBC copolymers. A preliminary step was to observe whether the prevention of protein adsorption to the surfaces of wells could be achieved, since unavoidable solution transfers between wells are required in this assay. Microassay tests using milk protein-coated wells confirmed that the addition of the milk protein resulted in slightly higher amounts of detected Coomassie brilliant blue G-250 (CBBG), as seen in Figure 5-16. However, the differences among the data points are quite small, and similar trends could not be reproduced using the standard and modified microprotein assay.

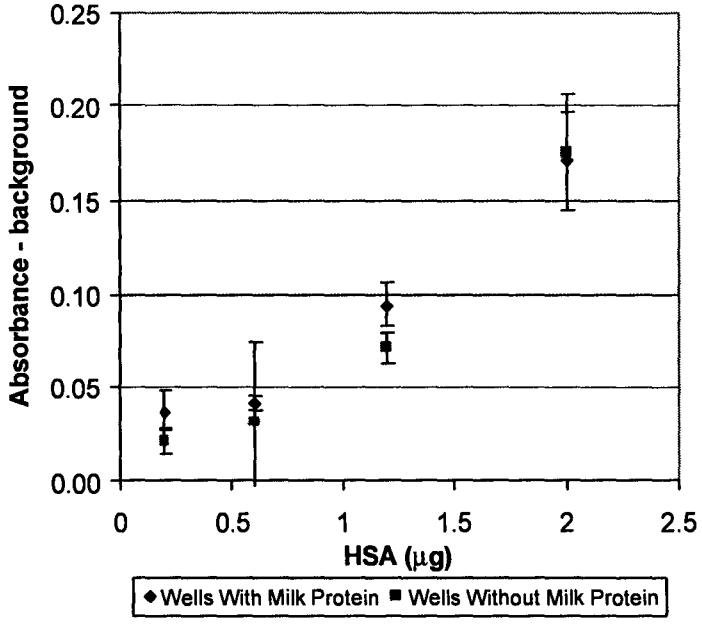


Figure 5-16 Comparison of the absorbance at 595 nm of human serum albumin (HSA) solutions in wells that were coated with milk protein, versus wells that were uncoated (mean ± SD, n= 3)

Another disadvantage of the Bradford assay is that although it avoids protein labelling, it requires that the approximate protein concentrations to be analyzed be known so that the appropriate Bradford assay can be selected. Because of the complexity and time consuming nature of this process (all solutions would need to be individually analyzed for concentration prior to the Bradford assay), this method was ruled out.

5.10.2 Fluorophor Labeling

Release of 5-TAMRA SE-labeled HSA into PBS (pH 7.4) at 37°C from the various copolymers was investigated. The results are summarized in Figure 5-17.

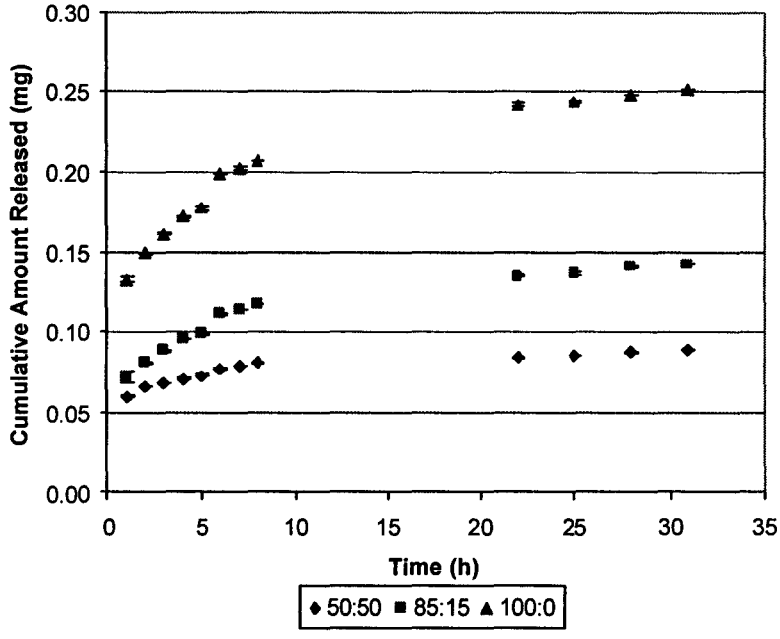


Figure 5-17 Release of 5-TAMRA SE-labeled HSA from various HEMA:MBC copolymers initially immersed in 5 mg/mL HSA solutions (mean ± SD, n = 3)

It is clear that as the MBC content in the copolymers increases, there is an associated decrease in the amount of HSA released. Similar trends were seen for protein release from dry HEMA:MBC copolymer immersed in 3 and 1 mg/mL 5-TAMRA SE-labeled HSA solutions. The results from these experiments as well as the data from Figure 5-17 can be seen in Figure 5-18. These results support the idea that the rate and pattern of drug release in a hydrogel can be regulated by copolymerization with appropriate monomers (Antonsen et al., 1993). It should be noted that there is an initial burst effect for all samples, followed by a gradually decreasing release rate. The release kinetics may be described as first order.

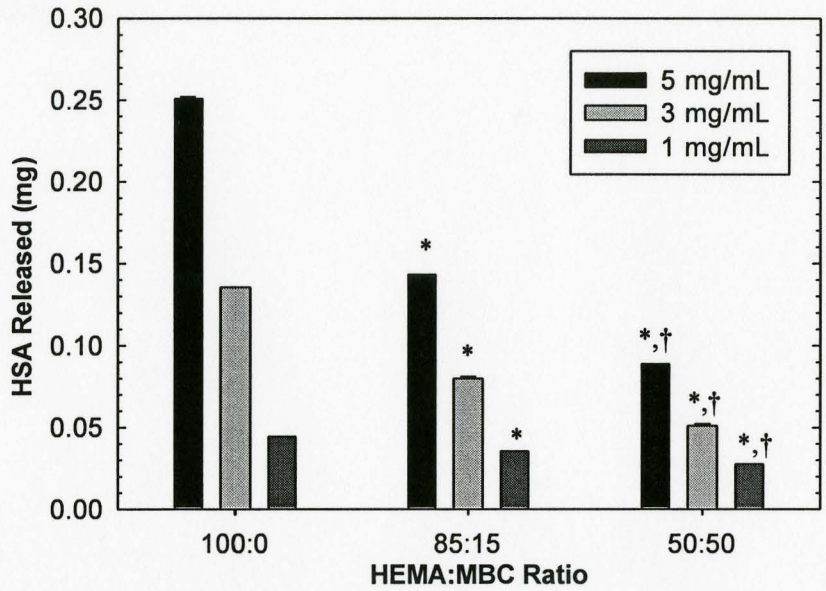


Figure 5-18 Cumulative 5-TAMRA SE-labeled human serum albumin released from HEMA:MBC copolymers initially immersed at various protein concentrations (see Figure 5-15 for HSA quantities loaded at 1 mg/mL). All samples were disk-shaped with a surface area of 0.581 cm^2 (duration of release: 31 h, data are mean ± SD, n= 3). Statistical analysis was carried out using a one-way ANOVA test ($P<0.01$). Significant differences are indicated by * and † *compared to the 100:0 HEMA:MBC copolymer †compared to the 85 15 HEMA:MBC copolymer

Figure 5-18 also shows information on the effect of varying the concentration of the soaking solution on the cumulative release from the different HEMA:MBC copolymers. From the data, partially depicted in Figure 5-19, it is apparent that higher protein concentrations lead to higher amounts of released protein (expected since based on Table 5-6, higher solution concentrations lead to higher protein uptake), again in agreement with the literature. As expected, there is a sharp drop in the rate of release from all samples after the initial burst. This relatively rapid first-order release profile is

somewhat longer in duration than that typically observed with pHEMA hydrogels (Saltzman, 2001).

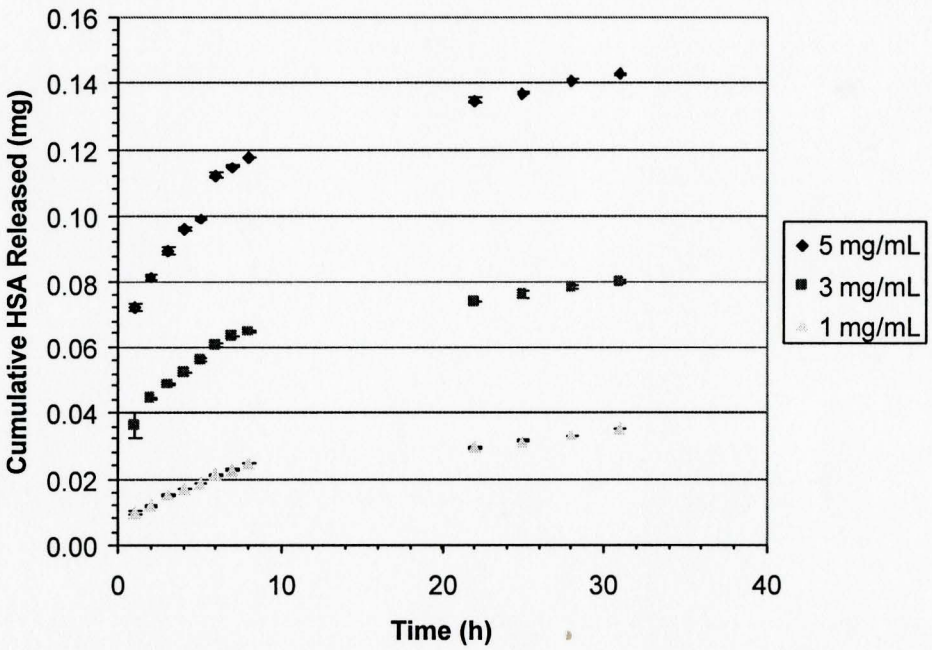


Figure 5-19 Release of 5-TAMRA SE-labeled human serum albumin from 85 15 HEMA:MBC samples initially immersed in solutions of varying HSA concentration (see Figure 5-15 for HSA quantities loaded at 1 mg/mL) (mean ± SD, n = 3)

5.11 rt-PA Release

Similar trends were observed for the release of rt-PA although there were some notable differences as seen in Figure 5-20. Most importantly, rt-PA release was lower than HSA release by approximately 2 orders of magnitude. This difference is probably due to the structural differences between the two proteins (e.g. amino acid composition, physical structure, and charge). Second, although Figure 5-20 again generally shows that

release decreases with increasing MBC content, this was not the case when the copolymers were initially immersed at 1 mg/mL rt-PA as seen in Figure 5-21 As previously discussed, this may be explained by the copolymers not having sufficient time to achieve distinguishable protein-drug uptake quantities. Similar to previous results with HSA, an initial burst release of rt-PA is followed by a gradual decrease in the release rate until a plateau is reached by ~60 h. This clearly shows that slow, but measurable, continuous release of protein-drug occurs for periods up to 3 days. This also suggests that the agents are being released primarily by diffusion, as is often seen in monolithic, amphiphilic hydrogels (Saltzman, 2001, Brahim et al., 2003).

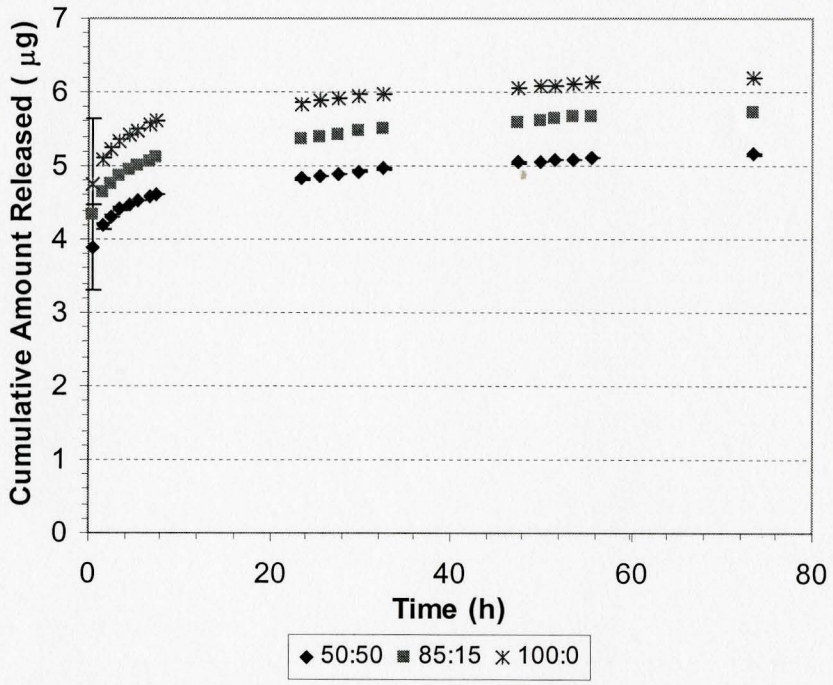


Figure 5-20 ^{125}I-labeled rt-PA release from HEMA.MBC copolymers initially immersed in 2 mg/mL rt-PA solutions; initial rt-PA loading quantities can be seen in Table 5-6. (mean ± SD, n = 3)

The large data scatter seen during the initial phase of release is common in drug release studies (Saltzman, 2001). It is believed that this is due to the fact that most of the initial release consists of loosely adsorbed drug, the amount of which can vary substantially on hydrogel surfaces. This variability in adsorption may be attributed to surface irregularities, or to clustering of hydrophobic crosslinking agents, causing localized high crosslink density and low swelling (Drumheller and Hubbell, 1995). Furthermore, free chain ends, chain loops, and molecular entanglements can also lead to network defects in hydrogels (Hoffman, 2002, Rosiak et al., 2002) which can also cause variable amounts of drug binding (and thus drug release).

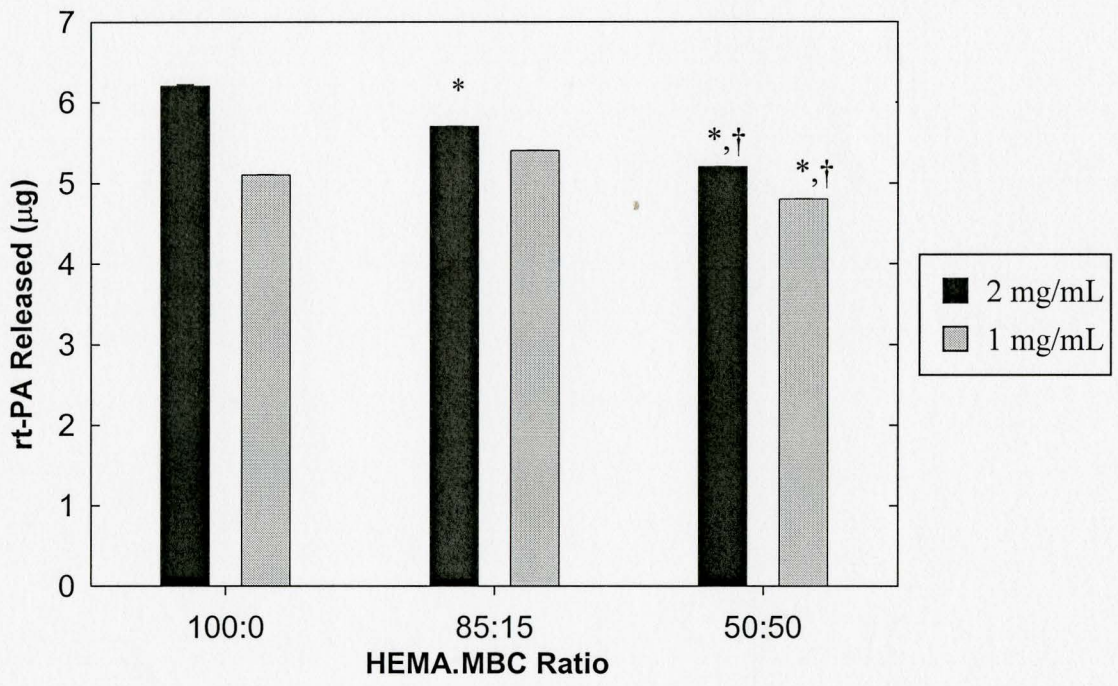


Figure 5-21 Cumulative ^{125}I-labeled rt-PA release from HEMA.MBC copolymers initially loaded at two protein concentrations (see Table 5-6 for rt-PA quantities loaded). All samples were disk-shaped with a surface area of 0.581 cm^2 (duration of release: 74 h, data are mean ± SD, n= 3). Statistical analysis was carried out using a one-way ANOVA test (P<0.01). Significant differences are indicated by * and † *compared to the 100:0 HEMA.MBC copolymer †compared to the 85 15 HEMA.MBC copolymer

Release durations of less than 100 h were consistently observed in these studies, and the release profiles indicate first-order release kinetics. Measurements beyond 176 h confirmed that no further release occurred. This limit may be important should a more prolonged release be desirable. For example, Dunn and Mohler (1993) suggest that four days is optimal for the administration of fibrinolytic agents via continuous catheter delivery to reduce postsurgical mesothelial or serosal tissue adhesion in rabbits. However, given that local therapy should be a more effective and more rapid method of dissolving clots, this duration of delivery may be unnecessary.

5.11.1 rt-PA Release vs. rt-PA Uptake

The amount of rt-PA released from the HEMA:MBC copolymers (Figure 5-21) is clearly greater than the amount taken up (Table 5-6). The differences in these values are thought to be due to an erroneously low measured rt-PA uptake. After incubation in the rt-PA solution, samples were rinsed thoroughly with TBS buffer (pH 7.35) and blotted (paper tissue) to remove surface droplets prior to analysis. It is likely that these steps remove significant amounts of the protein relative to the total amount present.

Another reason for the discrepancy in uptake versus released rt-PA may be the result of uptake and release measurements being performed on different copolymer samples.

5.11.2 rt-PA Release Summary

According to the AHFS ((American Hospital Formulary Service), 1996) and Majerus et al. (1995), the recommended therapeutic range to achieve t-PA efficacy *in vivo* is 0.45–1.00 μg t-PA/mL of body fluid at a thrombus site. The data in Figure 5-21 show that a total release of between 4.8 μg and 6.2 μg of rt-PA is achievable per 0.581 cm^2 of HEMA:MBC copolymer in the *in vitro* studies performed. However, what is actually ideal for a localized drug-delivery system remains unanswered. *In vivo*, fluctuations in the volume or replenishment of body fluid encompassing the material makes it difficult to predict exactly how much t-PA would be required locally, in a time-dependent manner. This also causes increased complexity in trying to correlate *in vivo* efficacy and release and *in vitro* experiments. Furthermore, it is not clear whether this recommended t-PA dose is appropriate for the dissolution of clots in small diameter vessels or for large vascular grafts. The duration for which t-PA release must be sustained in order to ensure the long term efficacy of the material also remains unknown, as it has been suggested that different areas of the body vary with respect to acute t-PA release (Schrauwen et al., 1994).

5.12 rt-PA Stability Assessment by SDS-PAGE

Although it was clear that the HEMA:MBC copolymers are able to take up and release both HSA and rt-PA, it was also important to demonstrate that the released HSA and t-PA remain structurally intact, and that t-PA is still enzymatically active.

5.12.1 SDS-PAGE

SDS-PAGE was used to evaluate the integrity of the rt-PA released during the first 30 min, and in the interval between 3.5 and 4.5 h, from the 100:0 and 50:50 HEMA:MBC copolymers. The results are shown in Figure 5-22 and Figure 5-23, respectively. A gel loading of 1 μg rt-PA was used for the control and 100:0 HEMA:MBC samples. For the 50:50 HEMA:MBC, the same sample volume used to deliver 1 μg rt-PA from the control and 100:0 HEMA:MBC was loaded, although it is likely that this contained less than 1 μg t-PA.

The band at ~70 kDa represents single-chain rt-PA, while the band at ~30 kDa probably represents a fragment of two-chain rt-PA. Fragments of molecular weight 32 and 39 kDa are generally present in any single-chain rt-PA preparation (American Diagnostica Inc., 2004). It can be seen that the standards resemble closely the t-PA released from the copolymers, indicating that no significant degradation has occurred in the released material. There appears to be less rt-PA in the 50:50 HEMA:MBC gel than in the pure HEMA, presumably due to lower protein release from the 50:50 material (Table 5-6, Figure 5-21).

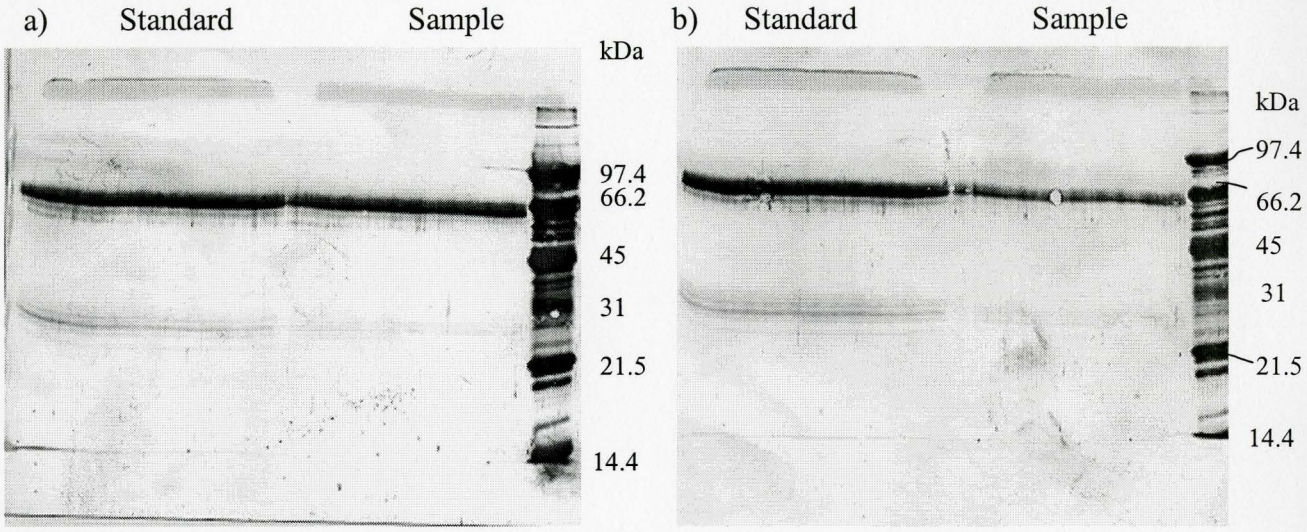


Figure 5-22 SDS-PAGE gel of ^{125}I-labeled rt-PA released during the first 30 mın from a) 100:0 and b) 50:50 HEMA.MBC copolymers. Standard ıs rt-PA stored at -70°C.

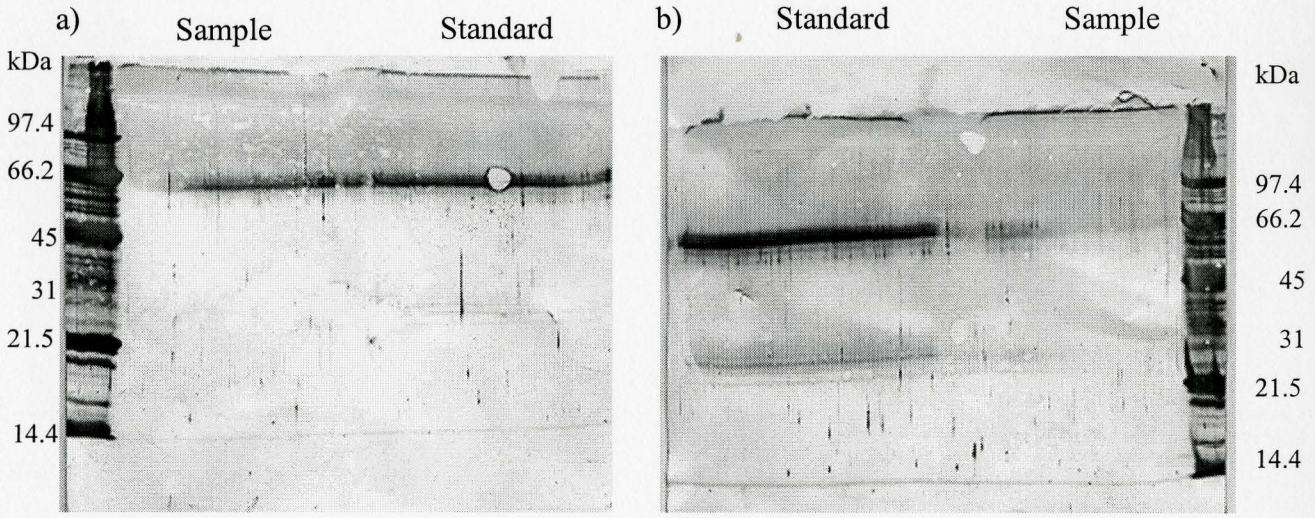


Figure 5-23 SDS-PAGE gel of ^{125}I-labeled rt-PA released in the interval between 3.5 to 4.5 h from a) 100:0 and b) 50:50 HEMA.MBC copolymers. Standard is rt-PA stored at -70°C.

5.12.2 S2251 Assay

It was found that rt-PA lost activity when stored at a temperature greater than -70°C (Figure 5-24). Differences in activity among rt-PA samples that had been incubated at temperatures of 37°C, 22°C, and 4°C were minimal, this trend was observed for samples that were analyzed repeatedly over an eight day period.

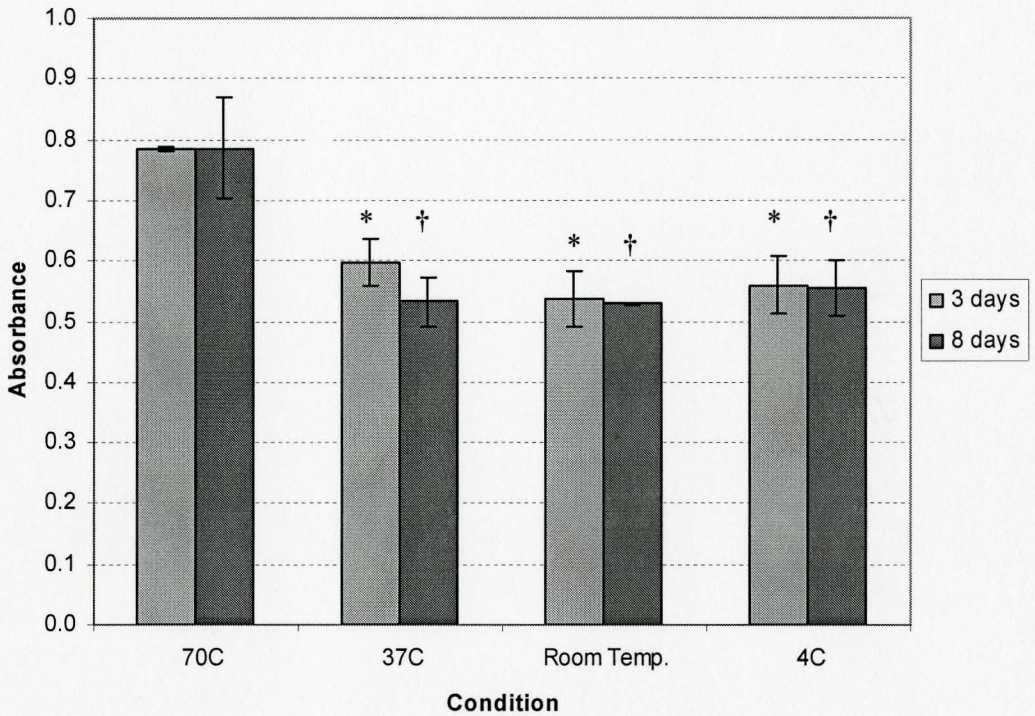


Figure 5-24 S2251 analysis of the enzymatic activity of rt-PA samples that had been incubated at various temperatures for different durations. Room temp. ~ 22°C (data are mean ± SD, n= 3). Statistical analyses were carried out using one-way ANOVA tests (3 days. $P<0.01$, 8 days: $P<0.03$). Significant differences are indicated by * and † *compared to -70°C data from 3 days. †compared to -70°C data from 8 days

Figure 5-25 demonstrates that a control rt-PA sample, which was kept at the same temperature as the rt-PA loaded into the HEMA.MBA copolymers, also lost activity

relative to rt-PA kept at -70°C. Furthermore, rt-PA released from the copolymers during the first 30 min was found to have decreased activity compared to the control sample. In contrast, t-PA released form the pure pHEMA (100:0) polymer had not lost activity. This "protective" effect of pHEMA has been previously shown for α-amylase, whose activity was not only preserved, but enhanced by 210% compared to controls, when it was imbibed into a pHEMA hydrogel and incubated under various conditions (Gehrke et al., 1998). Other studies have indicated preservation of t-PA activity after loading and release from a biomaterial, although these conclusions were drawn from qualitative data that did not measure the specific activity of released compared to native t-PA (Wiseman et al., 1992; Park et al., 2001; Hill-West et al., 1995). From the data in Figure 5.23, activity appears to decrease with increasing MBC content in the copolymers. This effect may be due to conformational change induced by the MBC component of the copolymers. It is well known that protein contact with surfaces can result in conformational change, with altered protein function (Roach et al., 2005; Wilson et al., 2005; Butler et al., 1992). In particular the hydrophobic nature of MBC relative to HEMA may result in significant rt-PA dehydration, denaturation, and loss of function (Gehrke et al., 1998).

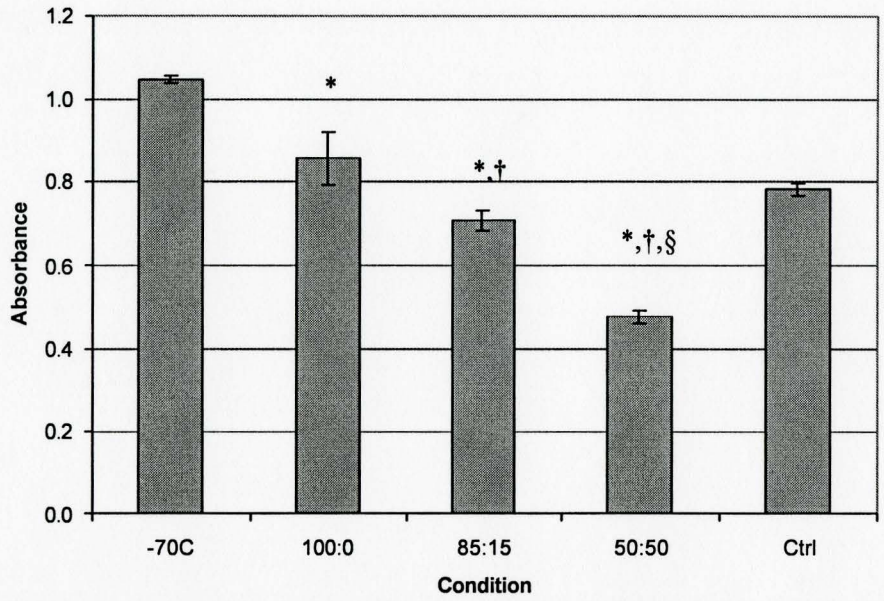


Figure 5-25 S2251 analysis of activity of ^{125}I-labeled rt-PA (1 μg) released during the first half hour from various HEMA:MBC copolymers initially immersed in 1 mg/mL ^{125}I-labeled rt-PA. The control rt-PA was kept under the same temperature conditions as the HEMA:MBC samples (data are mean ± SD, n= 3). Statistical analysis was carried out using a one-way ANOVA test (P<0.01, Ctrl data not included). Significant differences are indicated by *, †, and §. *compared to -70°C data. †compared to the 100:0 HEMA:MBC copolymer §compared to the 85:15 HEMA:MBC copolymer

6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In this work, novel HEMA:MBC copolymers with desirable chemical and mechanical properties were examined as prospective vehicles for extended protein-drug release. The polymers were successfully synthesized to yield homogeneous hydrogels as confirmed by FTIR, DSC, and ^{13}C NMR. Further characterization of these materials demonstrated that the incorporation of MBC into HEMA polymers affects their surface and bulk properties. Surface hydrophilicity, equilibrium aqueous solvent uptake, and swelling decreased with increasing MBC content. Conversely, the uptake of organic solvents was significantly increased with the incorporation of MBC into the copolymers. These effects can be used to control the amount of therapeutic agents that can be loaded into the copolymer materials.

It was demonstrated using fluorophor and radiolabeled proteins that by varying the drug concentration in the imbibition solutions, the amount of drug loaded and subsequently released could be varied. SDS-PAGE and a chromogenic assay demonstrated that rt-PA loaded into and released from the copolymers remained structurally intact and retained fibrinolytic activity. However, it appeared that as the MBC content increased, the enzymatic activity of the released rt-PA decreased.

While the trends for the release of HSA and rt-PA from the copolymers were similar, it was found that under similar conditions of loading, the total amounts of the two

proteins taken up and released differed significantly. The cumulative amounts of rt-PA released from HEMA:MBC samples (0.581 cm^2 surface area, loaded by immersion in a 2 mg/mL solution) ranged from 5.2 μg to 6.2 μg rt-PA in a 74 hour period.

The results of the current study support the possibility of the controlled delivery of t-PA as an approach to "thrombolytic" materials. Furthermore the HEMA:MBC copolymers investigated appear to be promising materials for this application.

6.2 Recommendations for Future Work

A number of additional studies and future research directions are suggested to more fully examine the potential of these materials as controlled delivery vehicles for thrombolytic drugs, and to elucidate the mechanisms by which the drugs are taken up and released.

- Further work in characterizing the surface morphology and composition of hydrated and dry HEMA:MBC copolymers using atomic force microscopy and X-ray photoelectron spectroscopy, respectively, is recommended. The internal and surface porosity of the different copolymer compositions should also be investigated. These studies would provide additional information about the structure-property relationships that could then be exploited to tailor the protein release profiles.
- The present work was focused on the release profiles of protein-drugs from samples that had been swollen in aqueous media. Release studies from samples swollen in organic solvents would also be of interest since it was demonstrated that solvent uptake increased with decreasing solvent polarity, thus possibly offering an

alternative method of drug uptake that would give higher loadings and altered release kinetics. Dimethyl sulfoxide, a well-known polyurethane solvent of relatively low polarity, is a likely candidate for such studies.

- It would be useful to evaluate the penetration of the protein into the polymer by fluorescent labeling and confocal microscopy, after loading and at various stages of the release process.
- Additional studies to evaluate the effect of the copolymer MBC content on protein adsorption would provide useful information about the balance between adsorption and absorption.
- Alternative drug loading methods should be considered, e.g. loading of the protein-drug during fabrication of the polymer. This would entail devising a system that would better allow the protein to remain dispersed in the polymer during the polymerization process. Protein release of longer duration would be expected from this method.
- Additional *in vitro* assays of t-PA activity should be performed to further assess the potential of these materials as clot lysing biomaterials. For example, a fibrin dissolution assay could provide additional information about the activity of the t-PA released from these materials.
- It would ultimately be of interest to examine the potential of these materials in combination with the ε-lysine presenting polyurethane developed by McClung et al. (2003). This PU has already been shown to "capture" plasminogen when in contact with blood and to have fibrinolytic activity in the presence of t-PA; such materials

would be considerably more effective if a local reservoir of t-PA were available. For example, it may be possible to graft the copolymers onto the polyurethane surface, or to coat the clot lysing polyurethane with these polyurethane-like t-PA releasing hydrogels. Coating methods for the HEMA:MBC copolymers would have to be developed. Additional studies to determine the optimal coating thickness, as well as the release rates of the t-PA from the coatings, would also need to be performed.

- Finally, it would be of interest to examine alternative fibrinolytic agents including t-PA derivatives such as reteplase, $K_1K_2P_u$, and tenecteplase, which show potential for quicker, safer, and more effective fibrinolysis than rt-PA (Verstraete, 1995; Genentech, 2005).

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Appendix A: t-PA and the Fibrinolytic System

Table A-1 Properties of members of the fibrinolytic system (Dobrovolsky and Titaeva, 2002)

Component	Molecular Mass (kDa)	Mean Plasma Concentration	Half-life	Function
Plasminogen	90	0.2 mg/mL	50 h	proenzyme
Tissue-type Plasminogen Activator (t-PA)	70	5-10 ng/mL	2-3 min.	plasminogen activator
Urokinase-type Plasminogen Activator (u-PA)	55, 31	1 ng/mL	3-5 min.	plasminogen activator
α_2-antiplasmin	70	0.07 mg/mL	50 h	plasmin inhibitor
α_2-macroglobulin	4 x 160	2.5 mg/mL		protease inhibitor
Plasminogen Activator Inhibitor type-1 (PAI1)	50	60 ng/mL	5-7 min.	inhibitor of u-PA and t-PA
Plasminogen Activator Inhibitor type-2 (PAI2)	70	< 5 ng/mL	--	inhibitor of u-PA and two-chain form of t-PA

Table A-2 Primary roles of the structural domains of tissue-type plasminogen activator (t-PA)

Domain	Residues	Function
Finger-like	4-50	-provides t-PA with strong fibrin-selectivity (i.e. a maximum molar binding ratio of 0.88 mole t-PA per mole of fibrin (Liu and Wallen, 1984))
Epidermal growth factor-like	50-87	-binds t-PA to endothelial cell receptors, which may mediate pericellular fibrinolysis through localized cell surface plasmin generation and by sheltering t-PA from PAI1's inhibitory effects (Hajjar, 1992)
K1	87-176	-possession of a high mannose-type glycosylation site makes it ideal for binding to mannose receptors on liver endothelial cells, which contributes to t-PA clearance (Otter et al., 1992)
K2	176-262	-restricts t-PA's activity by forming a 1:1 stochiometric reversible complex with t-PA's primary inhibitor, PAI1 (Dobrovolsky and Titaeva, 2002)
Trypsin-like serine protease	276-527	-stabilizes t-PA-PAI1 complexes -catalytic triad (His^{322}, Asp^{371}, Ser^{478}) associates with plasminogen to convert it into plasmin (Lijnen and Collen, 2001)

Table A-3 Properties of native tissue-type plasminogen activator (t-PA) (Collen, 1986; Dobrovolsky and Titaeva, 2002; Lijnen and Collen, 2001; Wun, 1988; Holvoet et al., 1986)

Molecular Mass (kD): Single-chain t-PA	~68-70
Molecular Mass (kD): Two-chain t-PA; heavy (A) chain, light (B) chain	A Chain = 39, B Chain = 32
Mean Plasma Concentration (ng/mL)	5-10
Half-life (min)	2-6
Specific Activity	580,000 IU/mg
Amino Acids (single-chain tissue-type plasminogen activator)	530[++]
Carbohydrate Content (%)	7

[++]originally incorrectly identified as 527 due to exclusion of 3 amino acid extension on NH_2-terminus of t-PA

Appendix B: Solutions and Reagents

Phosphate-Buffered Saline (PBS)

Disodium hydrogen phosphate	1.32 g
Sodium dihydrogen phosphate	0.345 g
Sodium Chloride	8.5 g

Fill to 1 L with Milli-Q water. Adjust pH to 7.4.

Tris-Buffered Saline (TBS)

Tris	6.05 g
Sodium Chloride	8.76 g

Fill to 1 L with Milli-Q water. Adjust pH to 7.35.

Iodine Monochloride (ICl) Reagent

ICl Stock solution
Dissolve 150 mg $Na^{125}I$ in 8 mL of 6N HCl.
Dissolve 108 mg $Na^{125}IO_3 \cdot H_2O$ in 2 mL of Milli-Q water.
Mix the above two solutions and bring the volume up to 40 mL with Milli-Q water.
Add 5 mL of CCl_4 and shake vigorously. Repeat until there is no longer any pink colour visible in the organic phase.
Remove residual CCl_4 by aerating the solution for 1 hour in the fumehood.
Bring solution volume to 45 mL with Milli-Q water.

For the labeling procedure, mix 1 part of the above ICl stock solution with 9 parts of 2 M NaCl to get 0.0033 M ICl in 1.8 M NaCl

Glycine Buffer (2 M Glycine in 2 M NaCl)

Glycine	75.0 g
Sodium Chloride	58.5 g

Fill to 500 mL with Milli-Q water. Adjust pH to 8.8 with 2 N NaOH.

Appendix C: Determination of Free Iodide Concentration by Trichloroacetic Acid Precipitation of Protein

a. Set up vials: 3 1.5 mL recovery eppendorf vials (pink)
   3 1.5 mL TCA (trichloroacetic acid) eppendorf vials (pink)
   3 1.5 mL control eppendorf vials (white)
b. 1/10 dilution of hot HSA = 100 μL hot HSA + 900 μL TBS
   Mix in a 1.5 mL eppendorf vial
c. Add 900 μL of 1% BSA (by wt. in H_2O) into first 6 vials (control, TCA)
   Use 1% BSA as carrier protein to pull down ^{125}I
d. Add 100 μL of 1/10 dilution hot HSA to first 6 vials (control, TCA)
e. Add 500 μL of 20 wt% TCA in H_2O (precipitates protein) to 3 pink TCA vials
f. Vortex the 3 pink TCA vials; let sit for 10 minutes
g. Place white control vials into tubes, with clasps cut off, into tube holder so that they are counted last (slots 4-6) in the gamma counter
h. Prepare last 3 vials:
   Centrifuge 3 pink TCA vials for 1 min (separate protein from centrifugate)
   Add 500 μL TBS to 3 pink recovery vials
   Take 500 μL of centrifugate in each TCA vial and transfer to recovery vials
   Cap all vials, cut clasps off, then place recovery vials in slots 1-3 of tube holder
   Count samples in gamma counter for 1 min
i. Free Iodide Calculation:

$$Free\ Iodide\ (\%) = \frac{3 * Average\ Radioactivity\ of\ A}{Average\ Radioactivity\ of\ B} \times 100$$

where A is the pink recovery vials and B is white control vials (less than 3% free iodide is acceptable)

*NOTE: when the TCA method is used to determine unbound fluorophor, all ^{125}I references refer to the fluorophor, and a fluorimeter is used in place of the gamma counter. As well, samples are analyzed in 96 well plates instead of vials.

Appendix D: Western Blot Procedure

Prepare Gels (12% separating gel, 4% stacking gel)
(ref: Mini Protean II Dual Slab Cell Instruction Manual and R.M. Cornelius)

Acrylamide/Bis:	Acrylamide/Bis (30% stock)	29.2 g
	N,N'-Methylenebisacrylamide	0.8 g

Dissolve above reagents in distilled water, then dilute to 100 mL and filter the final solution.

12% Separating Gel:	Distilled water	3.35 mL
	1.5M Tris-HCl, pH 8.8	2.5 mL
	10% (w/v) SDS stock (room temp)	100 µL
	30% stock (w/v) Acrylamide/Bis	4.0 mL

Mix above 4 reagents and degas for 15 min @ room temp

To initiate polymerization, add:	10 % ammonium persulfate (fresh)	50 µL
	TEMED	5 µL

Clean casting plates with distilled water and ethanol (95%), dry plates, then insert them into the casting assembly. Secure assembly to casting stand, then use syringe to fill gel plates with polymer solution. Ensure that enough space is left to add stacking gel later. After two minutes, layer a small amount of water over the gel. Allow the separating gel to polymerize for 1 h before adding stacking gel.

4% Stacking Gel:	Distilled water	3 mL
	0.5M Tris-HCl, pH 6.8	1.2 mL
	10% (w/v) SDS stock (room temp)	100 µL
	30% stock (w/v) Acrylamide/Bis	0.65 mL

Mix above 4 reagents and degas for 15 min @ room temp

To initiate polymerization, add:	10 % ammonium persulfate (fresh)	25 µL
	TEMED	5 µL

Fill remainder of gel plates with 4% stacking gel solution. Add comb. Allow to polymerize for 1 h before use.

Sample Preparation

Sample Buffer: (also referred to as tracking dye, TD)

Distilled water	4 mL
0.5M Tris HCl, pH 6.8	1.0 mL
Glycerol	0.8 mL
10 % (w/v) SDS	1.6 mL

Mix the above 4 reagents, aliquot into 225 µL volumes, and store in 4°C fridge until needed.

Add: (to 225 uL aliquot)

2-β-mercaptoethanol	30 µL
0.05% (w/v) Bromophenol blue	30 µL

Typical loading volumes for 3 well comb.

(Lane 1) 1 µL markers, 10 µL TD
(Lane 2) 150 µL sample, 80 µL TD
(Lane 3) 7.5 µL prestained markers

Typical loading volumes for 10 well comb.

(Lane 1) 1 µL markers, 10 µL TD
(Lanes 2-9) 1 to 20 µL sample, 10 µL TD
(Lane 10) 7.5 µL prestained markers

Once mixed, samples are placed in a 95°C water bath for 7.5 minutes.

Electrophoresis

Remove gels from casting stand, and place into clamp assembly. Place clamp assembly into buffer chamber. Fill upper buffer chamber to 3 mm below outer long glass plate with 1X electrophoresis buffer. Fill lower buffer chamber until 1 cm of gel is covered with 1X electrophoresis buffer. Add samples to gels. Operate power pack at 200 volts for ~45 minutes of electrophoresis. Layer a small quantity of Pyronin dye (in sample buffer) into wells just before the tracking dye (TD) reaches the bottom of the separating gel. Continue the electrophoresis until the Pyronin dye has just reached the top of the separating gel.

Electrophoresis Buffer: (5X stock solution, pH 8.3)

Tris Base	15 g
Glycine	72 g
SDS	5 g

Mix above 3 reagents, then fill to 1 L with distilled water. Ensure pH is 8.3+/- 0.3. Do not adjust with NaOH or HCl. Just before use dilute to 1X strength.

Gel Equilibration
Equilibrate gels in fresh, cold transfer buffer for 15-20 min upon removal from assembly.

Transfer Buffer:	3.03 g Tris
	14.4 g glycine
	200 mL methonal

Mix above 3 reagents, then fill to 1 L with distilled water. Ensure pH is 8.3+/- 0.3. Do not adjust with NaOH or HCl.

Electrophoretic Transfer
Immobilon PVDF transfer membrane was cut to size, pre-wetted with methanol (1-3 s) and water (1-2 min), and then immersed in transfer buffer (15 min). The gels and membranes were loaded in the transfer cassettes according to specifications and placed in the transfer chamber. The chamber was then filled with transfer buffer so that the entire gel surface was covered. A potential difference of 100V (200 mA) was applied for 1 h.

Gold Staining (>1 pg per band sensitivity)
The PVDF membranes were washed twice in PBS (pH 7.4). Unbound membrane sites were blocked by incubating the membrane in PBS (pH 7.4) containing 0.3% Tween 20 for 1 h at 20°C. This was followed by three washes of 5 min with this blocking solution, then three washes of 1 min with water.

Samples were then allowed to stain for 1 to 4 h, or overnight, in Protogold (BioCell Research Laboratories, Cardiff CF2 4AY, UK) solution.