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Biomedical Applications of Dendrimer Modified Polyurethanes with PEO (Polyethylene Oxide) Attached

by

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Abstract

A novel approach was used to synthesize bioactive polyurethanes by applying polypropylenimine octaamine dendrimers as the chain extenders, while another approach trying to incorporate star PEO (polyethylene oxide) directly into polyurethane failed to attain appropriate polymers. A protection/deprotection strategy was used to incorporate the dendrimers into the polyurethane chains, then PEO was chemically attached after deprotection to increase the biocompatibility of the material.

A generation 2.0 polypropylenimine octaamine dendrimer which has eight arms ending with amine groups, was used for the modification. The dendrimers were protected using the N-hydroxy-succinimide ester of a tert-butyloxycarbonyl (tBOC)-protected alanine or 9-Fluorenylmethyloxycarbonyl chlorocarbonate (Fmoc) in methylene chloride - triethylamine. A molar ratio of 6:1 (protecting group : dendrimer) was used to get a statistical distribution of protected dendrimers in which most of the dendrimers would have 6 arms protected. The partially protected dendrimers were used with ethylene diamine (ED) or butanediol (BDO) as a chain extender (molar ratio of dendrimer : ED/BDO = 1:9) to produce the dendrimer modified polyurethanes. After deprotection of the dendrimers, PEG-SPA (Polyethylene Glycol-Succinimidyl Propionate) was used to attach PEO to the polyurethane chains.

The NMR spectrum of the polyurethanes showed a significant increase of the peaks at 6.13 and 8.37 ppm after the incorporation of the dendrimers into the polyurethane. These two peaks were assigned to types of protons in the urea groups formed by the reaction of NH_2 of ED and the dendrimers with -NCO of the oligomer. In

the case of BDO as the chain extender, the reappearance of these two peaks after the dendrimer incorporation step further confirmed the incorporation of dendrimer into the polyurethane. The NMR spectra of polyurethanes after PEO attachment also showed a new peak at 3.51 ppm which was characteristic of PEO. The increase of the peaks at 3315 cm^{-1}, 1708 cm^{-1} and 1647 cm^{-1} in FTIR indicates the increase of hydrogen bonding of N-H and C=O of urethane and urea, due to the incorporation of the dendrimers into the polyurethane chains. The dendrimer-incorporated polyurethane had significantly lower molecular weight, possibly the result of the lower reactivity of the dendrimer due to steric hindrance of the branches. Water contact angle measurements revealed that the water contact angle of the polymer surfaces decreased significantly after PEO attachment indicating an increasing hydrophilicity of materials. No significant decrease in contact angle was observed after dendrimer incorporation when ED was used as chain extender. XPS (X-ray Photoelectron Spectroscopy) results not only further confirmed the incorporation of dendrimers and PEO into polyurethanes, but also indicated the presence of dendrimers on the polymer surfaces.

Fibrinogen adsorption on polyurethane surfaces was found to decrease significantly after incorporation of dendrimer and PEO. This decrease correlates with the PEO amount on polyurethanes estimated from GPC and NMR results. SDS-PAGE and immunoblotting experiment showed a decrease of several species of protein adsorptions from plasma on polyurethane surfaces after dendrimer and PEO modification, although the decrease of various protein adsorptions was not as significant as in the ED series after dendrimer and PEO incorporation because of the low amount of proteins adsorbed on the BDO polyurethane control.

Résumé

Une nouvelle approche a été utilisée pour synthétiser des polyuréthanes bioactifs en appliquant les dendrimers de polypropylenimine octaamine comme exoffres de chaîne, tandis qu'une autre approche essayant d'incorporer l'étoile OPE (l'oxyde de polyéthylène) directement dans le polyuréthane a échoué à atteindre des polymères appropriés. Une stratégie protection/déprotection a été utilisée pour incorporer le dendrimer dans les chaînes de polyuréthane, alors que l'OPE a été chimiquement attaché après la déprotection pour augmenter la biocompatibilité du matériel.

Une génération 2.0 du dendrimer polypropylenimine octaamine qui a huit bras finissant avec des groupes amine, a été utilisée pour la modification. Le dendrimer a été protégé utilisant l'ester N-hydroxy-succinimide d'un tert-butyloxycarbonyl (tBOC) protégé avec alanine ou chlorocarbonate 9-Fluorenylmethyloxycarbonyl (Fmoc) dans le chlorure de méthylène - triethylamine. Une proportion molaire de 6:1 (groupe protégeant : dendrimer) a été utilisé pour obtenir une distribution statistique du dendrimer protégé dans laquelle la plupart des dendrimers auraient 6 bras protégés. Le dendrimer partiellement protégé a été utilisé avec l'éthylène diamine (ED) ou butanediol (BDO) comme exoffre de chaîne (la proportion molaire de dendrimer:ED/BDO = 1:9) pour produire des polyuréthanes modifiés. Après la déprotection du dendrimer, SPA-PEG (le glycol-succinimidyl de polyéthylène propionate) a été utilisée pour attacher l'OPE aux chaînes de polyuréthane.

Le spectre NMR des polyuréthanes a montré une augmentation significative des sommets à 6.13 et 8.37 ppm après l'incorporation du dendrimer dans le polyuréthane. Ces deux sommets ont été assignés aux types de protons dans les groupes d'urée formés par la réaction du NH_2 du ED et du dendrimer avec le NCO de l'oligomer. Dans le cas de BDO comme l'exoffre de chaîne, la réapparition de ces deux sommets après l'incorporation du dendrimer a confirmé l'incorporation du dendrimer dans le polyuréthane. Les spectres NMR de polyuréthanes après l'attachement de l'OPE ont aussi montré un nouveau sommet à 3.51 ppm qui était caractéristique de l'OPE. L'augmentation des sommets à 3315 cm^{-1}, 1708 cm^{-1} et 1647 cm^{-1} dans le FTIR indique l'augmentation d'entreposage hydrogène de N-H et C=O d'uréthane et de l'urée, en raison de l'incorporation du dendrimer dans les chaînes de polyuréthane. Le polyuréthane dendrimer-incorporé avait le poids moléculaire significativement inférieur, probablement le résultat de la réactivité inférieure du dendrimer en raison de l'entrave stéric des branches. Des mesures d'angle de contact d'eau ont révélé que l'angle de contact d'eau des surfaces de polymère a diminué significativement après l'attachement de l'OPE indiquant une augmentation d'hydrophilicité des matériaux. Aucune diminution significative dans l'angle de contact n'a été observée après l'incorporation du dendrimer quand ED a été utilisé comme exoffre de chaîne. Les résultats de XPS (le Photoélectron de Radio Spectroscopy) non seulement ont plus loin confirmé le fait d'incorporation de dendrimer et d'OPE dans des polyuréthanes, mais ont aussi indiqué la présence de dendrimers sur les surfaces des polymères.

L'adsorption de fibrinogen sur des surfaces de polyuréthane a été trouvé pour diminuer significativement après l'incorporation du dendrimer et de l'OPE. Cette diminution corrèle avec la quantité d'OPE sur des polyuréthanes évalués de résultats de NMR et GPC. SDS-PAGE et l'expérience d'immunoblotting a montré une diminution de plusieurs espèces d'adsorptions de protéines du plasma sur des surfaces de polyuréthane après la modification du dendrimer et de l'OPE, bien que la diminution d'adsorptions de protéine diverses ne soit pas aussi significative que dans la série de ED après l'incorporation du dendrimer et de l'OPE à cause de la quantité basse de protéines adsorbées sur le contrôle de polyuréthane-BDO.

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Nomenclature

BDO	Butanediol
DBTDL	Dibutyl tin dilaurate
DMF	N, N-dimethyl formamide
DMSO	Dimethyl sulfoxide
ED	Ethylene diamine
FA	Formic acid
Fmoc	Fluorenylmethyloxycarbonyl
FTIR	Fourier transform infrared spectroscopy
G_2	Polypropyleneimine octaamine dendrimer generation 2.0
GPC	Gel permeation chromatography
HEP	Heparin
HMDI	Hydrogenated MDI
HSA	Human serum albumin
MDI	Methylene di-p-phenyl diisocyanate
NMR	Nuclear magnetic resonance spectroscopy
PBS	Phosphate buffered saline
PEO	Poly (ethylene oxide)
PFP	Polyfunctional polymers
PPO	Poly (propylene oxide)
PTMO	Poly (tetramethylene oxide)

PU	Polyurethane
PU-BDO	BDO chain extended polyurethane
PU-BDO-PEO	Dendrimer modified BDO PU with PEO attached
PU-ED	ED chain extended polyurethane
PU-ED-PEO	Dendrimer modified ED PU with PEO attached
PU-G2-BDO	Dendrimer modified BDO PU
PU-G2-ED	Dendrimer modified ED PU
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SPA-PEG 2000	Polyethylene glycol-succinimidyl propionate (2000 represents the PEG/PEO molecular weight)
SPR	Surface plasmon resonance
t-Boc	tert-Butyloxycarbonyl
TDI	Toluene diisocyanate
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TPU	Thermoplastic polyurethane
XPS	X-ray photoelectron spectroscopy

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Chapter 1: Introduction and Literature Review

1.1 Introduction

Biomaterials, defined as non-viable materials used in the fabrication of biomedical devices that interact with biological systems (Sefton, 1986), can be metals, glass, ceramics, or polymers. While such materials see widespread clinical use in the treatment of numerous diseases and conditions, there remains a need for materials with increased biocompatibility. It was previously believed that the "ideal" biomaterial would be completely "bioinert", however, the importance of appropriate interactions with the biological system of interest have been widely demonstrated and are considered to be a significant factor in the development of new biomaterials. Polymeric materials have seen widespread use in biomedical applications due to their excellent mechanical properties and the large variety of materials available. Polyurethanes in particular have been widely exploited in medical applications due to their outstanding physical-mechanical properties, the large number of available materials and their potential blood and tissue compatibility. The use of segmented polyurethane elastomers in clinical devices is well established. They have been widely used in medical applications, particularly blood contacting applications in such devices as pacemaker leads, blood bags and stents.

It is generally accepted that when polymeric biomaterials come in contact with blood, the initial and fate-determining step is the adsorption of plasma proteins. This

adsorption can lead to a variety of reactions including activation of the intrinsic coagulation pathway or the complement cascade and activation of platelets as well as interactions with other cellular components present in the blood (Colman et al., 1994). Therefore, it is desirable to control or eliminate the adsorption of proteins to biomaterial surfaces following exposure to protein-containing solutions. There is considerable research aimed at improving the bulk and surface properties of polymers, including polyurethanes, to achieve this goal. The most effective polymer for protein resistant surfaces appears to be polyethylene oxide (PEO). PEO is a very biocompatible synthetic polymer with a wide range of useful properties, including solubility in water and organic solvents, non-degradability, a lack of toxicity and low immunogenicity (Lee et al., 1998). Surface modification of biomaterials with PEO has been shown to impart protein repellant properties to the surfaces, presumably by a mechanism of steric repulsion (Desai et al., 1991; Fujimoto et al., 1993; Silver et al., 1994; Lee et al., 1998). PEO graft density and PEO molecular weight have been suggested as important parameters in the effectiveness of this technique. Furthermore, coupling of bioactive ligands via a PEO tether has been shown to increase the bioactivity of these ligands (Piao et al., 1992; Han et al., 1996, 1998; Bentz et al., 1998). Star PEO has been used in place of linear PEO to increase the surface density of the polymer with good results (Merrill, 1993; Du et al., 1999; Banerjee et al., 2000).

Dendrimers have also been shown to have interesting biological properties and have been used for the delivery of drugs and in other biomedical applications (Martin et al., 1995; Kojima et al., 2000; Kawase et al., 2001). These three-dimensional, highly ordered oligomeric and polymeric compounds formed by reiteration reaction sequences

starting from smaller molecules or "initiator cores" have precise geometry and can be considered to be virtually monodisperse. The shells of monomers are referred to as generations with the number of terminal functional groups doubling with each generation, resulting in a molecule with a potentially high number of terminal functional groups and a star-like geometry.

In the current work, dendrimers were used as a means of increasing PEO density. PEO functionalized dendrimers were incorporated into a polyurethane backbone. Incorporation of both the dendrimer and the PEO were evaluated using NMR and FTIR; molecular weights of the functionalized polymers were determined by GPC. Surface properties were examined by XPS and water contact angles. The effects of these modifications to the polymer structure on its biological properties were examined by measuring the adsorption of plasma proteins from buffer and from plasma. It is hoped that the modification of polyurethane structure through the incorporation of dendrimers will allow more biological function to be attributed to the polymers and that these polymers can be subsequently modified with other biologically relevant molecules in addition to PEO.

1.2 Literature Review

1.2.1 Blood Coagulation on Artificial Surfaces

The hemostatic mechanism is the naturally designed physiologic response to arrest bleeding from injured blood vessels involving a complex set of interdependent reactions between the surface, at least 12 plasma proteins and platelets resulting in the

formation of a clot or thrombus (Colman et al., 1994). In the cascade, inactive factors become enzymatically active following surface contact or exposure to the damaged blood vessels. While amplification is rapid, the process is localized at the surface by complicated activation and inhibition mechanisms to maintain the fluidity of blood in the circulation. There are two independent pathways for the initiation of the coagulation cascade as shown in Figure 1.1.

The extrinsic pathway, of particular importance following damage to a blood vessel, is initiated when blood comes in contact with a traumatized vascular wall or extravascular tissues. The intrinsic pathway is of more importance in the study of blood contacting surfaces because this cascade is initiated when blood contacts any material that is not the naturally occurring intact vascular endothelial layer. In both pathways, inactive factors, or zymogens, become enzymatically active following contact with a foreign surface. Active zymogens subsequently activate the next factor in the cascade, with the final result of both pathways being the activation of thrombin, which catalyzes the conversion of fibrinogen to fibrin and the polymerization of the fibrin to form a polymeric plug. Many of the activated zymogens can both feed forward to activate the next enzyme in the cascade and feed back to amplify the system. This amplification system allows for the rapid formation of a fibrin plug, limiting the loss of blood following injury.

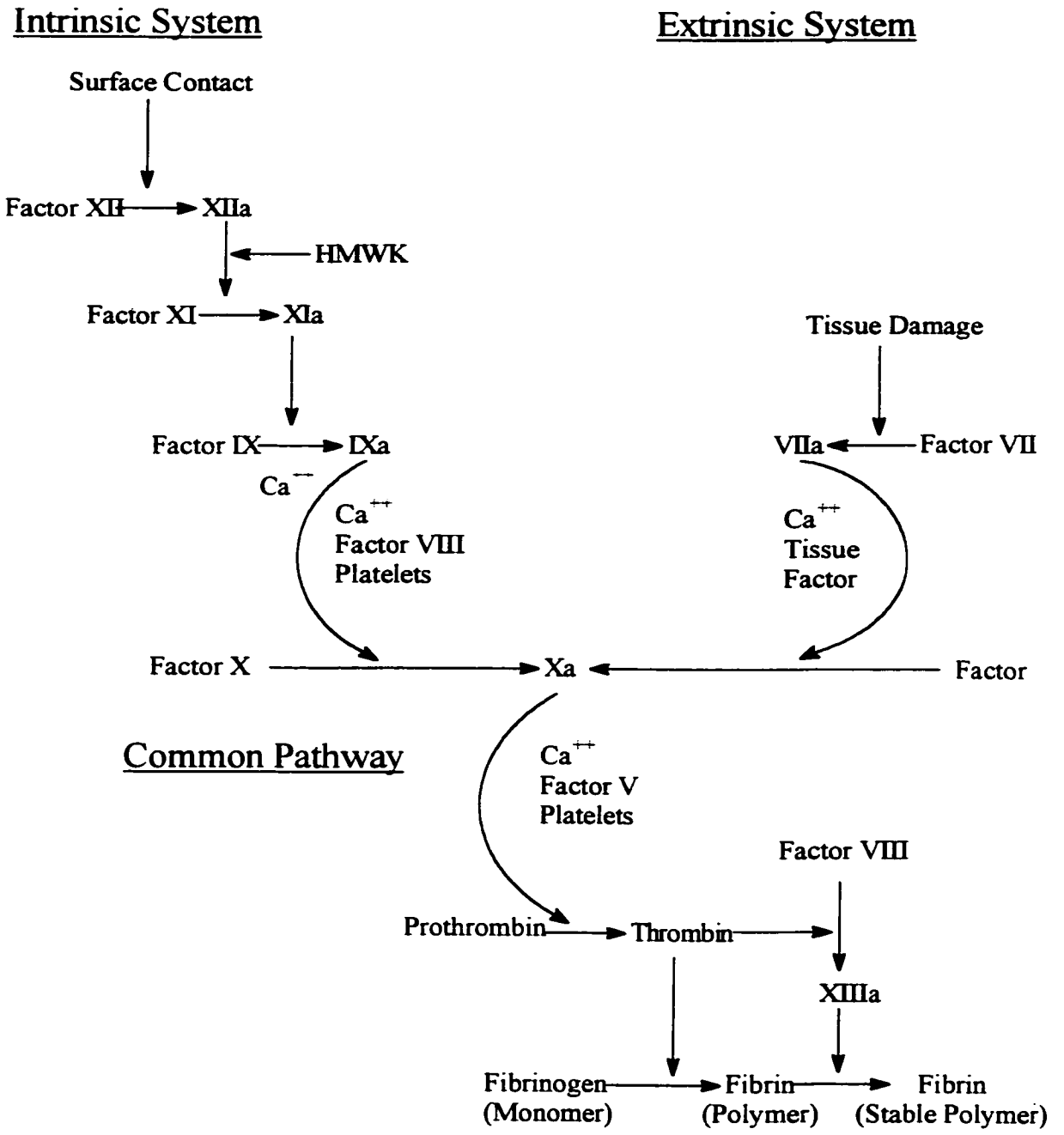

Figure 1.1 Schematic diagram of the blood coagulation pathways. Roman numerals represent enzyme or zymogen factors in coagulation cascade. Arrows indicate direct action of enzyme on zymogen or protein. HMWK means High-Molecular-Weight Kininogen. Adapted from (Colman et al., 1994)

In addition to converting fibrinogen to fibrin, the conversion of prothrombin to

thrombin also leads to the activation of platelets in the blood, making them adhesive. These cells become incorporated into the growing fibrin polymer plug as well as releasing additional activation factors that also amplify the system.

Blood coagulation on biomaterials can result in a catastrophic outcome for the patient. An occlusion can limit the blood flow through a vessel, resulting in a failure to deliver an adequate amount of oxygen and nutrients to the tissues, and subsequently to tissue necrosis. Furthermore, the surface bound thrombus may embolize and become lodged in the smaller vessels of either the heart or the brain leading to either heart attack or stroke.

1.2.2 Properties of Polyurethanes

Thermoplastic polyurethanes are a versatile class of thermoplastic elastomers formed by incorporating two chemically dissimilar blocks in a linear polymer backbone. These copolymers have an $[H\text{-}S]_n$ type structure. At service temperature, the soft segment (S) is viscous or rubbery, while the hard segment component (H) is in a rigid (glassy or semicrystalline) state. The hard segment is diisocyanate and is chain extended with a low molecular weight diol or diamine to form a polyurethane urea. The soft segment is usually a polyol, polyether, polyester, polyalkyl or polydimethylsiloxane. The three building blocks of the polyurethane combine to form the polyurethane shown in Figure 1.2.

Due to incompatibility between the two types of structural units, the soft and hard segments, the polymers undergo microphase separation, resulting in a hard segment rich

hard domain, a soft segment rich soft matrix and a poorly characterized interphase. It is generally accepted that the high strength of polyurethanes is due to the hard domains, which are stabilized by hydrogen bonding between hard groups. Hydrogen bonding may occur between the N-H and C=O groups in the hard segments. In polyether-based polyurethanes, hydrogen bonding may occur between the N-H groups of the hard segment dispersed in the soft matrix and the C-O-C groups of the soft segment. The morphology and resulting physical properties can then be rationalized in the following manner. The continuous matrix, which consists of the flexible soft segments, gives rise to the high deformability of the material. The hard segment domains, in which the molecules are relatively immobile due to hydrogen bonding, perform the same function as chemical crosslinks in conventional elastomers.

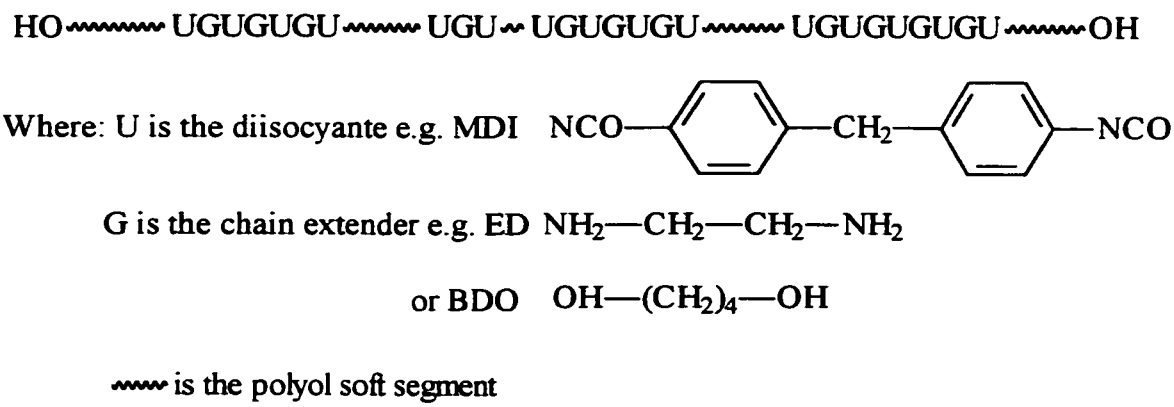

Figure 1.2. Segmented Polyurethane Structure

The physical properties of the polyurethane can therefore be manipulated by changing the molecular weights, chemical compositions and molar ratios of the segments. The most commonly used diisocyanates for polyurethane synthesis are toluene

diisocyanate (TDI) and methylene di-p-phenyl diisocyanate (MDI), although other alternatives have been used for creating polymers with different physical properties (Klees and Gerald, 1999). The isocyanates react with the chain extenders to create the hard segment. A wide variety of chain extenders can be used, each providing the polymer with different characteristics. Generally, aliphatic chain extenders yield softer polyurethanes than aromatic chain extenders.

The polymerization procedures can be carried out in a single or two step process, with the two step process being more common. In the first step, an intermediate oligomer, the prepolymer is formed by reacting the diisocyanate and the polyol, as shown in Figure 1.3.

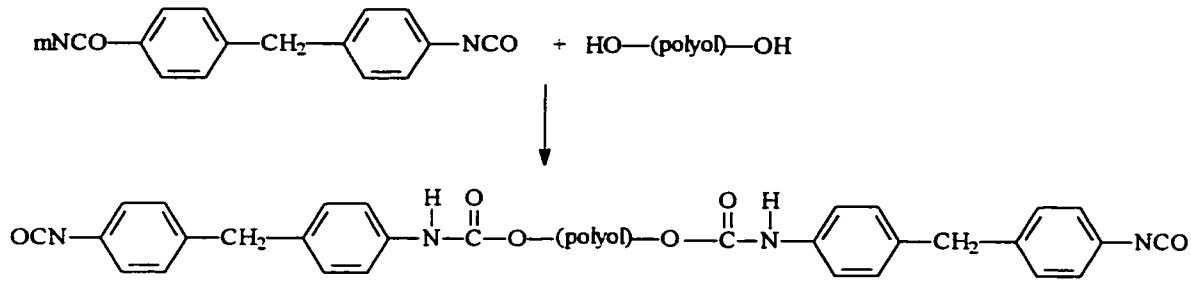

Figure 1.3 Polyurethane prepolymer synthesis. The polyol reacts with diisocyanate (e.g. MDI) to form the prepolymer with -NCO groups on both ends for further reaction

After the prepolymer is formed, the second step is the reaction of the prepolymer with the chain extender in the chain extension step. The reaction is shown in Figure 1.4.

All polyurethanes are based on the exothermic reaction of polyisocyanates with polyol molecules, containing hydroxyl groups. Relatively few basic isocyanates and a range of polyols of different molecular weights and functionalities are used to produce

the whole spectrum of polyurethane materials with a wide spectrum of properties, many of which make them attractive for use in biomedical applications.

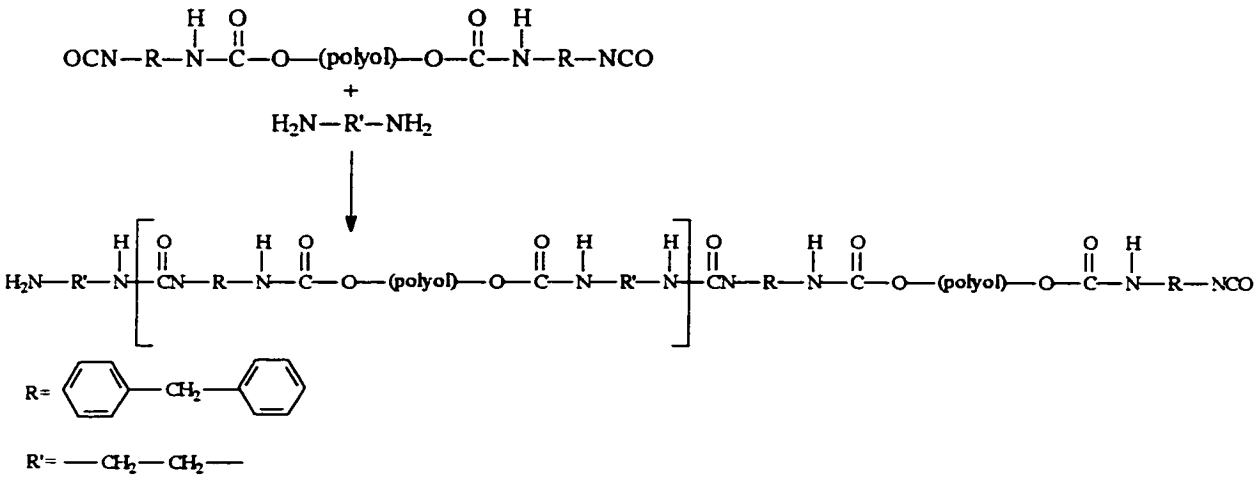

Figure 1.4 Polyurethane chain extension reaction. The chain extender (e.g. ethylene diamine or butanediol) reacts with the -NCO groups of the prepolymer to form polyurethaneurea or polyurethane

1.2.3 Polyurethanes in Biomedical Applications

A biomaterial is defined as a non-viable material used in the fabrication of a biomedical device that interacts with biological systems (Sefton, 1986). Therefore, metals, glass, ceramics, polymers, etc., when used in connection with the living body, are considered biomaterials. The material could become a part of the body as an implant supporting, augmenting, or restoring the function of an organ such as a total artificial heart (TAH) or an artificial pancreas or could have as minimal an interaction as an intravenous catheter (IV) used over one million times per month, worldwide. Until 10 years ago, it was generally believed that the "ideal" biomaterial would be completely

"bioinert", i.e. would not have any interaction with the human body under the biological conditions of application (Zdrahala, 1999). However, the concept of "biocompatibility" has been reevaluated and it is currently accepted that the biocompatibility of a material is specific to its application and therefore can only be assessed in conjunction with the host response evoked by a material intended for a specific end use (Hunt et al., 1996). Inherent in this definition is the "biological safety" of the material. Materials generating deleterious effects including mutagenicity, carcinogenicity, hypersensitivity, thrombogenicity, haemolysis, and tissue necrosis in specific applications should obviously be excluded from use as biomaterials.

While a wide spectrum of polymeric materials including natural rubber, polyethylene, poly vinyl chloride (PVC), fluoropolymers, hydrogels, and silicones have been used as biomaterials, the broad palette of available polyurethanes with outstanding physical-mechanical properties and potential blood and tissue compatibility has given these materials significant status in biomedical applications. Biomer®, one of the earliest polyurethanes for biomedical applications, gained wide acceptance due to its high modulus of elasticity and tensile strength and high resistance to flex fatigue (Boretos et al., 1975), physiological acceptability (Boretos, 1980), and high blood "tolerability" (Ihlenfeld et al., 1979). The Biomer® formulation contained MDI, a soft segment of PTMO (Polytetramethylene oxide, molecular weight 2000) and 1,2-ethylene diamine (ED) as the chain extender, generating both urethane and urea linkages. The urea linkages significantly hinder thermoplasticity of the material for any practical polymer melt processing, thus limiting its utilization for solution coating, dipping, or casting applications. This material was withdrawn from the market in 1991 because of its

difficulty in processing and lack of long term resistance to hydrolysis in the human body . A similar system, suitable only for solution processing, was developed by using polydimethyl siloxane as a modifying, reacted-in additive. Originally named Avcothane®, it was renamed Cardiothane® and has been widely used in various medical applications. In the late 1970s, a series of polyether-based thermoplastic polyurethanes (TPU), trade-named PELLETHANE-2363®, was introduced by the Upjohn Co. as a series of different hardness products designed for the medical field. Vascular catheters, blood bags, implants targeting both soft and hard tissues, and a number of other medical devices, components and/or parts of those were prospective targets. In the early 1980s, the VIALON® family of specially designed, medical grade TPUs was developed for use in vascular catheters. As reported by Zdrahala and McGary (1986), the unique feature of those materials was a controlled degree of softening after insertion into the vascular system. This softening, related to the phase separation between the hard and soft segment domains and the formation of mixed-phases or an "interphase" connecting hard and soft segment domains (Zdrahala et al., 1988), was extremely important for reduction of abrasion and injury to the vascular vessel wall, resulting in a significant lowering of mural thrombosis, thus extending the service time of the inserted catheter (Solomon et al., 1986).

Both the Pellethane® and Vialon® families of biomedical grade TPU are based on the aromatic diisocyanate MDI. Inherent to these types of TPU is yellowing following prolonged exposure to the surrounding environment, especially following exposure to ultraviolet (UV) light. The formation of chromophore groups in the hard segment is responsible for this exclusively cosmetic change. However, as many applications require

a clear, transparent appearance, Szycher and collaborators (Szycher et al., 1977) introduced an aliphatic diisocyanate based family of biomedical TPUs in 1979 under the name of Tecoflex®. These materials, based on cycloaliphatic, hydrogenated MDI (HMDI) became recognized as alternative biomedical TPUs for applications requiring a non-yellowing, clear appearance (Szycher et al., 1983, 1984). Some of the commercially available biomedical grade polyurethanes are listed in Table 1.1.

Table 1.1. Commercially available biomedical grade polyurethanes

Material	Composition	Source
Pellethane 2363®	MDI/BDO(Butane Diol)/PTMO	Dow Chemical, Inc.
Tecoflex®	HMDI/BDO/PTMO	Thermodics, Inc.
Bionate®(formerly Corethane®)	MDI/BDO or ED/polycarbonate	Polymer Technology Group, Inc.
Biospan®	MDI/BDO or ED/PTMO/Chain end modifiers	Polymer Technology Group, Inc.
Chronoflex® AL, AR	HMDI(MDI)/BDO(ED)/ Polycarbonate	Cardiotech International, Inc.
Estane	MDI/BDO/polyethers	B.F. Goodrich, Inc.

To further expand the bioperformance and biodurability of both aromatic and aliphatic polyurethanes, additional intermediates were utilized, usually as the second soft segment. Utilization of PTMO and PEO as co-soft-segments has led to materials having specific hydrophilic/hydrophobic balance which, in turn, produced materials with enhanced hemocompatibility and internally "built-in" surface lubricity (Zdrahala et al., 1987). Modification of segmented polyurethane surfaces by PEO- and sulfonated PEO-

containing block copolymer additives led to reduced platelet adhesion with increasing PEO chain length (Lee et al., 1998). PEO chains of length 80 units were particularly effective in preventing platelet adhesion. The surfaces of these PEO additive polymers were in a gel-like state and some extraction of the PEO additives was noted.

Surface immobilization of heparin has also been used to improve the biocompatibility of polyurethanes (Piao et al., 1992). The surface of polyurethane-urea (PU) coated glass beads was first modified with diisocyanates, followed by surface grafting of polyfunctional polymers (PFP), including: poly (vinyl alcohol), poly (ethyleneimine), and poly (allylamine). The functional groups of the surface grafted PFP (i.e. -OH, -NH, or $-NH_2$) were modified with diisocynates (TDI) to amplify the surface concentration of isocyanate groups. Bioactivity of the heparin increased when immobilized through PFP's and PEO compared with PFP and PU alone. Heparin like groups were used in the research of Santerre et al. (1992) who studied the effect of sulfonation of segmented polyurethanes on the adsorption of fibrinogen from plasma. It was found that thrombin times of human plasma were prolonged with increasing sulfonate content of the polymers.

Polyurethanes have played a dominant role in a myriad of unique medical devices with excellent performance in life improvement and life saving areas. Despite the problems associated with material degradation (Santerre et al., 1993) and calcification (Bernacca et al., 1998) when implanted in the human body, the physical and mechanical properties associated with these materials, in conjunction with the wide range of chemical functionalities that can be incorporated into the structure, make polyurethanes an

excellent choice of elastomeric polymer for biomedical applications as well as for experimental study.

Today, PUs are used in cardiovascular applications, artificial organs, tissue replacement and augmentation and other miscellaneous applications, as listed in Table 1.2.

Table 1.2 Polyurethanes in Medicine and Medical Devices (derived from Zdrahala et al., 1999)

Cardiovascular Applications
Catheters
Intravenous (IV)
Central Venous (CV)
Vascular Access
Balloon, Pulmonary/Thermodilution
Balloon, Angiography, Angioplasty
Urological
Specialty Conduits
Pacemaker Leads Insulation
Vascular Prostheses
Heart Valves
Cardiac Assist Devices
Artificial Organs:
Total Artificial Heart (TAH)
Artificial Kidneys-Hemodialysis
Artificial Lungs-Blood Oxygenators
Artificial Pancreas
Tissue Replacement and Augmentation:
Breast Implants
Wound Dressing
Facial Reconstruction
Surgical Adhesives
Others:
Controlled Drug Delivery

1.2.4 Development of Bioactive Biomaterials

While biomaterials have had an enormous health impact, saving countless lives and

improving the quality of life, the need still exists for better polymers and surfaces as well as improved methods of characterizing and testing them. Considerable research has been done to improve the biocompatibility of the biomaterials in recent years. One of the most important advances in the field of biomaterials over the past few years has been in the development of bioactive biomaterials based on the principles of biological recognition. By incorporating biologically motivated, adhesion promoting sites, growth factors or other biologically based moieties, biomaterials have been developed to inhibit receptors or enzymes, block binding sites, regulate certain biological pathways or improve cell adhesion. In addition to the highly biospecific biological recognition, it is also possible to incorporate moieties to mimic the natural biological recognition phenomena that proceed by less specific physicochemical interactions, such as binding of a charged polysaccharide to a protein.

1.2.5 PEO Surface Modification for Reducing Protein Adsorption and Cell Interactions

Polyethylene oxide (PEO), an uncharged polyether with the chemical formula $H\text{-}(O\text{-}CH_2\text{-}CH_2)_n\text{-}OH$, has been widely used to make non-interactive or non-fouling surfaces by limiting non-specific interactions between cells, proteins and surfaces. At room temperature, PEO is completely miscible with water in all proportions for all degrees of polymerization. However, the solubility of PEO in water decreases with increasing temperature (Claesson, 1993). PEO in water has a lower consolute

temperature, or cloud point; raising the temperature above the cloud point will result in insolubility and formation of two phases. The flexibility of the polymer depends on the number of possible chain conformations and transition probabilities among conformations. The presence of bulky side groups in the polymer backbone introduces steric hindrance, reducing the probability of conformational transitions by limiting the mobility of the polymer segments. PEO does not have bulky side groups. Moreover, the rotation of the chain around the C-O bond of the ether oxygens is relatively facile compared even to an unsubstituted C-C bond. The result is a polymer of very high chain flexibility.

It has been widely demonstrated that PEO modified surfaces resist protein and cell uptake (Desai et al. 1991; Fujimoto et al., 1993; Silver et al., 1994; Lee et al., 1998;). The reasons for these properties are not fully understood, although several explanations have been proposed. A number of approaches can be used to immobilize PEO to surfaces including covalent attachment of the polymer (Ratner, 1996), adsorption of pluronics including PEO-PPO-PEO (Lee et al., 1998; Pavey et al., 1999,) and PEO-PU-PEO (Tan, 2000), surface interpenetration (Desai et al., 1992), chemisorption (Du and Brash, 1999) and crosslinked network formation (Ratner, 1996). In Fujimoto et al.'s research (1993), the surface of a PEO grafted PU film was characterized by dye staining, x-ray photoelectron spectroscopy, water contact angles and zeta potential. All measurements indicated that PEO chains were immobilized on the PU surface. The PU surface showed reduced protein adsorption *in vitro* and reduced platelet adhesion *in vitro* and *ex vivo*. The optimum graft density for suppressing the protein adsorption was as low as 5 micrograms per cm^2. The extent of reduction in serum albumin adsorption was always less than that

of gamma-globulin. Silver et al. (1994) studied the effect of polyol molecular weight on the physical properties and hemocompatibility of polyurethanes containing PEO chains. It was found that the hard segment content at the surface increased as the polyol block length decreased. Three PEO molecular weights 600, 1450, 8000 were studied. Only for the shortest block length studied, PEO-600, were differences in blood compatibility observed. This material was the most thrombogenic.

Possible explanations for protein repelling properties of longer PEO chains include its minimum interfacial free energy with water, hydrophilicity, high surface mobility and steric stabilization effects and unique solution properties (Lee et al., 1998). Since PEO is not hindered sterically in water, it appears to have a large excluded volume in water (Lee et al., 1995). The rapidly moving hydrated PEO chains and a large excluded volume tend to repel protein or platelet molecules that approach the surface as shown in Figure 1.5.

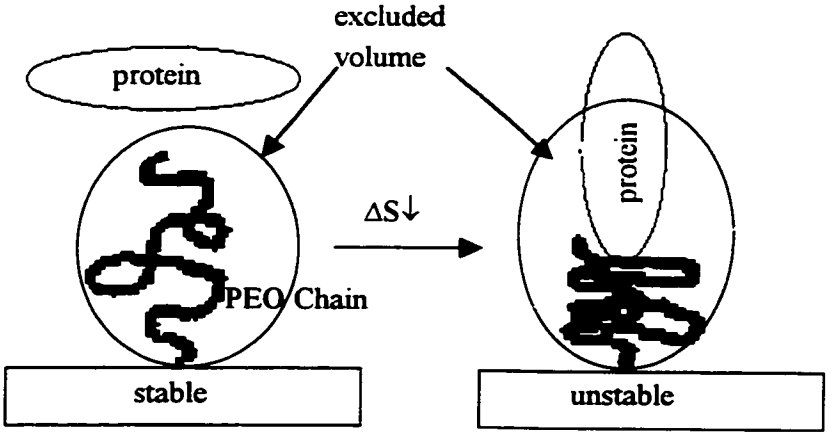

Figure 1.5 Proposed repulsion mechanism of PEO chains. An excluded volume resulting from mobile PEO chains tend to repel protein or platelet molecules from the surface. Adapted from (Han et al., 1991)

The effect of PEO molecular weight has not been clearly defined. While it has been suggested that longer PEO chains suppress protein adsorption or platelet adhesion more effectively than shorter chains, Pavey et al. (1999) found the mixture of long and short PEO chain copolymers showed significant reduction of binding of BSA (bovine serum albumin) to the surfaces, compared to the more commonly used long chain PEO copolymers. Furthermore, recent results of Unsworth (2001) demonstrate that low molecular weight PEO inhibits protein adsorption to a greater extent than higher molecular weight PEO. Therefore, it is apparent that that a complex interplay of factors is important in determining protein adsorption to PEO modified surfaces.

Increased densities of PEO compared to linear PEO polymers have been achieved with a divinyl benzene core from which 10 to 50 PEO arms extend (Merrill, 1993). According to Merrill, this approach potentially enhances biocompatibility because the high PEO density is more effective in sterically repelling proteins or cells, making it difficult for them to "see" a non-PEO surface. Du and Brash (1999) demonstrated that HSA adsorption was significantly decreased on star PEO modified surfaces and that protein adsorption appeared to decrease as the number of arms increased. Furthermore, star PEO was found to decrease protein adsorption more than linear PEO. Others have used comb PEO polymers to mimic this effect (Banerjee et al., 2000). In these studies, acrylic latexes with glass transitions ranging from -30 to 100°C were synthesized by dispersion polymerization in a water and alcohol solution using an amphiphilic comb copolymer as a stabilizing agent. The comb had a poly(methyl methacrylate) backbone and hydrophilic poly(ethylene glycol) (PEG) side chains, which served to stabilize the dispersion and create a robust hydrophilic coating on the final latex particles. The end

groups of the comb stabilizer can be selectively functionalized to obtain latex particles with a controlled density of ligands tethered to their surfaces. Latexes were prepared with adhesion peptides (RGD) linked to the surface of the acrylic beads to induce attachment and spreading of cells. It was found that cell attachment and morphology varied with the surface density of the RGD-bearing latex beads.

Materials can also be synthesized directly so that desirable chain segments or functional groups are built into the material. PEO-containing polyurethanes for biomedical applications have been synthesized and characterized by some biomaterial researchers (Corneillie et al., 1998). Surface plasmon resonance (SPR) experiments indicated a significant reduction in the adsorption of human serum albumin (HSA) on these PEO-containing polymers. However, the incorporation of PEO has an adverse effect on the polyurethane mechanical strength (Silver et al., 1994).

Coupling of bioactive ligands via a PEO tether has been shown to increase the bioactivity of these ligands. PEO spacers have been used, for example, to immobilize heparin (Piao et al., 1992), sulfonate groups (Han et al., 1998), cell adhesion peptides (Banerjee et al., 2000) or growth factors (Kuhl and Griffith-Cima, 1996; Bentz et al., 1998). Bae et al. (1999) synthesized heparinized polyurethanes with PEO spacer using a plasma glow discharge technique and observed increased hydrophilicity and improved blood compatibility. In the study of Han et al. (1991), a negative cilia concept for thromboresistance was presented. Polyurethanes surface-grafted with PEO chains that were end-terminated with sulfonate groups significantly prolonged occlusion time in a

rabbit arterio-arterial shunt model. They explained this phenomenon with a molecular negative cilia model where the hydrated flexible long PEO chains prevented stagnation and adhesion of proteins and platelets by a volume restriction effect, and by their high chain mobility. They also felt that the PEO chain motion might be increased by electrical repulsion of negative sulfonate end groups to each other, further repelling the blood components. In the case of surface immobilized heparin using PEO as a hydrophilic spacer (Park et al., 1991; Piao et al., 1992), blood compatibility was further enhanced by the increased biological activity of heparin due to a possible synergistic effect of PEO and heparin (Park et al., 1991).

Veronese (2001) discussed the general problems in using PEO for conjugation to high or low molecular weight molecules such as proteins or polypeptides in a recent review. Problems encountered in conjugation, such as the evaluation of the number of PEG chains bound, the localisation of the site of conjugation in polypeptides and the procedure to direct PEGylation to the desired site in the molecule are discussed. More specific methods regarding reversible PEGylation, cross-linking reagents with PEG arms, and PEG for enzyme solubilization in organic solvent were reported.

1.2.6 Dendrimers

Dendrimers are three-dimensional, highly ordered oligomeric and polymeric compounds formed by reiteration reaction sequences starting from smaller molecules or "initiator cores" (Tomalia et al., 1990). These branched or even hyperbranched molecules,

also called arboroles, cascade molecules, dendritic molecules or starburst-dendrimers (Tomalia et. al., 1990), are constructed from identical monomeric building blocks carrying branching sites which are located in a spherical manner around a core. The shells of monomers are called generations, with the numbers of functional groups doubling or tripling with the addition of an additional monomer layer. Therefore, at the periphery, dendrimers can carry numerous functional groups that can eventually lead to a surface congestion due to the steric interactions and thus render the molecules towards further reactions (de Gennes et al., 1983). Polypropyleneimine octaimine dendrimers of generations 1, 2, 3, and 4 (G_1-G_4) are depicted in Figure 1.6.

1.2.6.1 Properties of Dendrimers

Dendrimers offer an important example in the rapidly growing field of supramolecular chemistry. Supramolecular chemistry is based on molecular recognition, i.e., the study of an ensemble of polymolecular species and their assemblies in which non-covalent intermolecular forces including aromatic stacking, H-bonds, polar and van der Waal's forces bring molecules together as structurally interrelated species. Unlike linear polymers, which possess random-coil structures, dendrimers express unique structural features in three dimensions. At higher generations, they tend to adopt a spherical surface with pockets of spaces in the interior, acting as "unimolecular" micelles capable of guest inclusion. These empty spaces within the dendrimers may be termed as "dendritic voids".

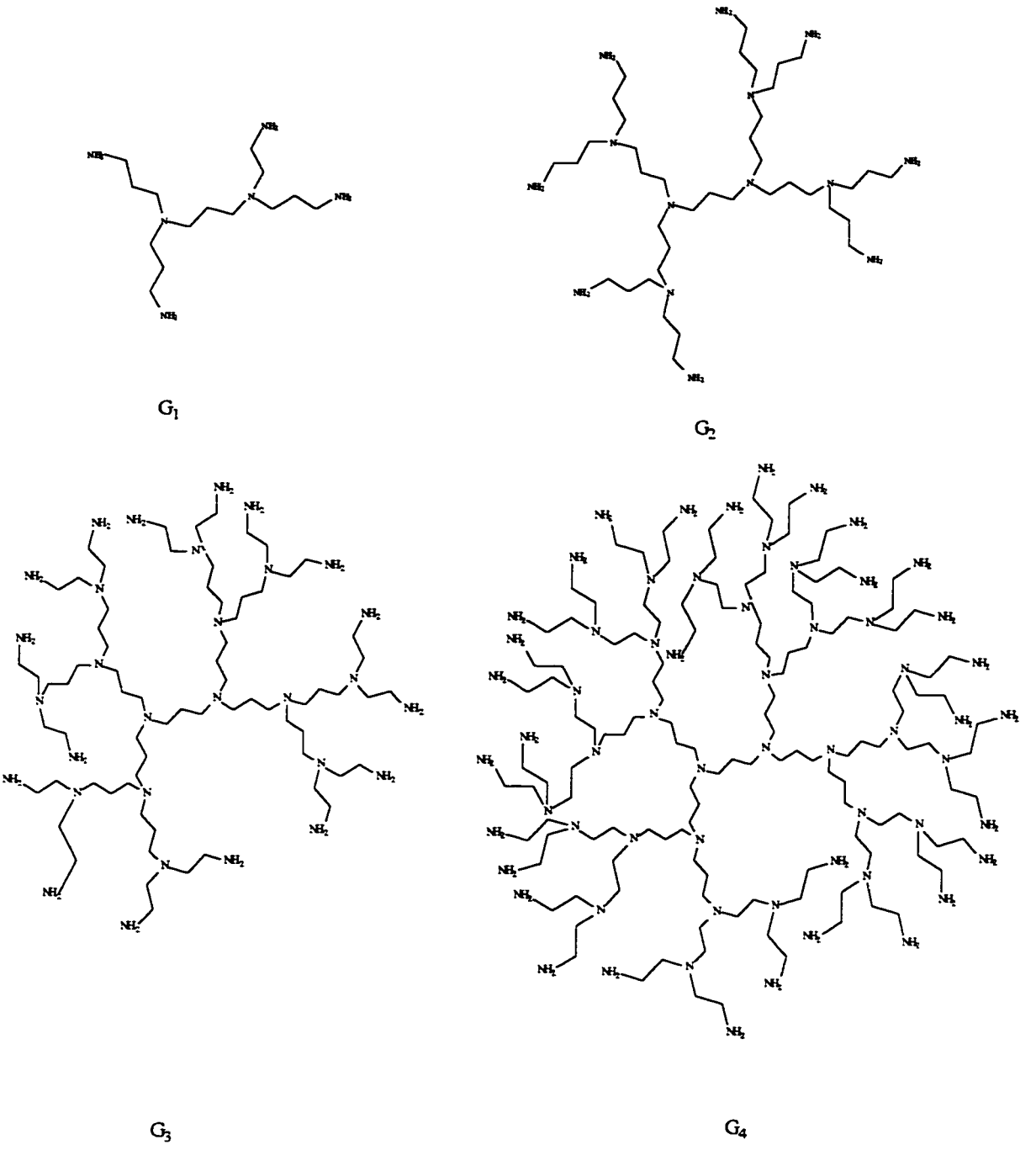

Figure 1.6 A two-dimensional picture of different generations of poly(propyleneimine) dendrimers. The number of arms of dendrimers doubles after each generation. Adapted from (Jansen et al., 1994).

It has been hypothesized that these synthetic models that can mimic functions of natural proteins such as molecular recognition of substrates either in the interior or on the surface. Molecular recognition could be achieved by encapsulation of non-specific guest(s) (Jansen et al., 1994), site-specific interior complexation and site-specific H-bonding (Newkome et al., 1996).

Another important characteristic of dendrimers is the ability for self-assembly. These self-assembly/aggregation processes can be assisted by non-directional forces such as dendritic amphiphiles, π-π interactions, liquid crystals etc. or via intermolecular H-bonding, and with metal centers as linkers and branching points in metallo-dendrimers.

Dendrimers, in a unique way, combine characteristics, such as monodispersity and definite composition, typical of small organic molecules, with attributes unique to traditional polymers, such as high molecular weight. Surface properties such as shape, reactivity, stoichiometry, congestion, special kinetic features, and flexibility govern divergent recognition and as such dendrimers may be expected to exhibit exo-receptor properties. Significant biological analogies include antibody-antigen interactions in immunological recognition processes as well as most protein-protein interactions and recognition at membranes or cell walls.

1.2.6.2 Dendrimers in Biomedical Applications

Dendrimers are being widely examined for their potential use in biological and biomedical applications. Amine terminated PAMAM dendrimers (Haensler et al., 1993; Tang et al., 1996; Kukowska-Latallo et al., 1996; Bielinska et al., 1996) possessing positively charged surfaces at physiological pH can be bound with polyanionic DNA and

function as in vitro gene-transfer agents. Attachment of multiple copies of peptide sequences to small dendritic scaffolds has afforded novel peptide dendrimers (Tam, 1988; Tam et al., 1989) with increased immunogenic effects. These molecules have shown promise as a synthetic AIDS vaccine (Defoort et al., 1992). A sialic acid dendrimer, which inhibits the influenza A virus haemaglutinin, is a good example of the rapidly growing field of glycodendrimers and glycopolymers (Roy et al., 1993). Surface modification of dendrimers with varied functional units has generated dendrimers having potential applications as sugar-binding receptors (James et al., 1995), novel catalysts (Issberner et al., 1996) and radiotherapeutic agents (Martin et al., 1995). Fructose modified dendrimers were found to be very suitable scaffolds for use in a high-performance bioartificial liver support system (Kawase et al., 2001). When fructose-modified dendrimers were immobilized on a polystyrene culture plate, the number of initially adhered hepatocytes increased. Moreover, increasing the number of generations of fructose-modified dendrimer also increased the number of adherent hepatocytes. Polyamidoamine dendrimers having poly(ethylene glycol) grafts were designed as a novel drug carrier with an interior suitable for the encapsulation of drugs and a biocompatible surface (Kojima et al., 2000). New methods have been developed for the modification of PAMAM dendrimers that allow the covergent synthesis of either peptide or carbohydrate-bearing dendrimer molecules for biomedical applications (Mitchell et al., 1999). Dendrimers have also been shown to be useful in providing strong signal amplification in diagnostic applications (Capaldi et al., 2000).

1.3 Objectives of thesis

In this work, the objectives are to incorporate dendrimers into a polyurethane backbone and to chemically attach PEO chains to polyurethane. The effect of incorporation of polypropyleneimine octaamine generation 2.0 dendrimers into a polyurethane and subsequent attachment of PEO chains to polyurethane on the biocompatibility of the material, specifically protein adsorption characteristics, was studied. Through the incorporation of the dendrimers, the potential density of reaction sites for further modification with PEO, cell adhesion peptides or other biologically relevant molecules is increased. Furthermore, the dendrimers afford the possibility for modification with multiple functional groups. In the current work, the focus was on modification of the dendrimer incorporated polyurethanes with PEO for reducing adsorption of plasma proteins.

Different approaches for incorporating the dendrimers into the polyurethane structure and two different protecting groups were used in this study. The modified polyurethanes were evaluated chemically as well as biologically. The bulk structure characterization techniques, ^{1}H-NMR and FTIR, were used to confirm the structural information of the modified polyurethanes. Surface analysis techniques, including water contact angles and x-ray photoelectron spectroscopy, were used to obtain surface information. Protein adsorption studies and SDS-PAGE were used to obtain information about the protein adsorption on various surfaces.

Chapter 2 Experimental Methods and Materials

2.1 Materials

The materials used in the synthesis and characterization of the polyurethanes and their sources are summarized in Table 2.1.

Table 2.1 Materials used in the synthesis and characterization of the polyurethanes

Chemical	Source
Polytetramethylene oxide (PTMO) MW 650	Sigma Aldrich®
Ethylene diamine (ED)	Sigma Aldrich®
Butanediol (BDO)	Sigma Aldrich®
Dibutyl tin dilaurate (DBTDL)	Sigma Aldrich®
Anhydrous dimethyl sulfoxide (DMSO)	Sigma Aldrich®
N,N-dimethyl formamide (DMF)	Sigma Aldrich®
Tetrahydrofuran (THF)	Sigma Aldrich®
Polypropyleneimine octaamine dendrimer generation 2.0	Sigma Aldrich®
N-hydroxy-succinimide ester of a tert-butyloxycarbonyl (tBOC)-protected L-Alanine	Sigma Aldrich®
9-Fluorenylmethyloxycarbonyl Chlorocarbonate (Fmoc)	Sigma Aldrich®
Methylene chloride	Sigma Aldrich®
Triethylamine	Sigma Aldrich®
Formic acid	Sigma Aldrich®
Trifluoroacetic acid	Sigma Aldrich®
Diethylamine	Sigma Aldrich®
Methylene-di-p-phenyl diisocyanate (MDI) (98%)	Acros®
SPA-PEG (PEG-Succinimidyl Propionate) 2000	Shearwater® Polymers Inc.
SPA-PEG (PEG-Succinimidyl Propionate) 5000	Shearwater® Polymers Inc.
Fibrinogen	ERL
Iodogen®	Pierce
Radioactive sodium iodide ($Na^{125}I$)	Amersham

Chemical structures of the above chemicals are shown in Appendix A.

Antibodies used for immunoblotting protein adsorption characterization of polyurethanes are listed in Table 2.2.

Table 2.2 Polyclonal antibodies to human proteins in the form of fractionated antisera (IgG) fractions developed in goat, sheep or rabbit

Protein	Host	Supplier
Factor XI	Goat	Cedarlane Lab Ltd. Hornby ON
Factor XII	Goat	Cedarlane Lab Ltd. Hornby ON
Prekallikrein	Goat	Cedarlane Lab Ltd. Hornby ON
HMWK	Goat	Cedarlane Lab Ltd. Hornby ON
Fibrinogen	Goat	Cedarlane Lab Ltd. Hornby ON
Plasminogen	Goat	Sigma, St. Louis MO
ATIII	Sheep	Cedarlane Lab Ltd. Hornby ON
C3	Goat	Cedarlane Lab Ltd. Hornby ON
Transferrin	Goat	Sigma, St. Louis MO
Alpha-1-antitrypsin	Goat	Enzyme Research Labs, South Bend IN
Fibronectin	Rabbit	Calbiochem, Bering Diagnostic, La Jolla CA
Albumin	Goat	Cedarlane Lab Ltd. Hornby ON
IgG	Goat	Sigma, St. Louis MO
Beta-lipoprotein	Goat	Sigma, St. Louis MO
Alpha-2-macroglobulin	Rabbit	Sigma, St. Louis MO
Vitronectin	Sheep	Cedarlane Lab Ltd. Hornby ON
Protein C	Goat	Cedarlane Lab Ltd. Hornby ON
Prothrombin	Sheep	Cedarlane Lab Ltd. Hornby ON
Haemoglobin	Rabbit	Sigma, St. Louis MO
Factor B	Goat	Calbiochem, Bering Diagnostic, La Jolla CA
Factor H	Goat	Calbiochem, Bering Diagnostic, La Jolla CA
Factor I	Goat	Calbiochem, Bering Diagnostic, La Jolla CA
Protein S	Sheep	Cedarlane Lab Ltd. Hornby ON
Apolipoprotein A1	Goat	Chemicon International Inc., Temecula CA

PTMO, BDO, ED, DBTDL, DMSO, DMF, THF, methylene chloride, triethylamine, formic acid, trifluoroacetic acid and diethylamine were stored at room temperature. Polypropyleneimine octaamine dendrimer G_2, N-t-Boc-L-Ala-NHS, 9-Fmoc

Chlorocarbonate and MDI were store at 4°C until used. SPA-PEG 2000 was stored in a dessicator at -20°C because of their high sensitivity to water at room temperature.

The PTMO was degassed under vacuum at 80°C for 6 hours prior to use. All other reagents were used as received.

2.2 Synthesis of control polyurethanes

A conventional two-step polymerization method was used to synthesize all polyurethane samples. The synthesis is highly water sensitive because of the reaction between water and the -NCO groups of diisocyanate (MDI). Therefore, all glassware used in the synthesis was dried thoroughly in a 60°C oven overnight prior to the polymerization reaction. PTMO was degassed under vacuum at 80°C for 6 hours prior to use in order to remove trace water absorbed from the air and predried high purity N_2 was purged into the reactor to produce a water free, inert environment for the reaction. In the first step, prepolymers were formed by reacting MDI with PTMO dissolved in DMSO (10% wt./vol.) at a 2:1 molar ratio at 50°C for 90 minutes. For the control polymers, the chain extension step involved reaction with either ED or BDO. The chain extender was dissolved in DMSO (2% wt./vol.) and added to the reaction flask dropwise so that the molar ratio of MDI:PTMO:ED (or BDO) was 2:1:1. The reaction was allowed to proceed at room temperature (~25°C) when using ED as the chain extender, or at 40°C when using BDO. Two to 3 drops of DBTDL in THF were added as catalyst when using BDO as the chain extender. The reactions were carried out in a 500 mL three necked round

bottom flask equipped with a mechanical stirrer and a dry nitrogen purge as shown in Figure 2.1.

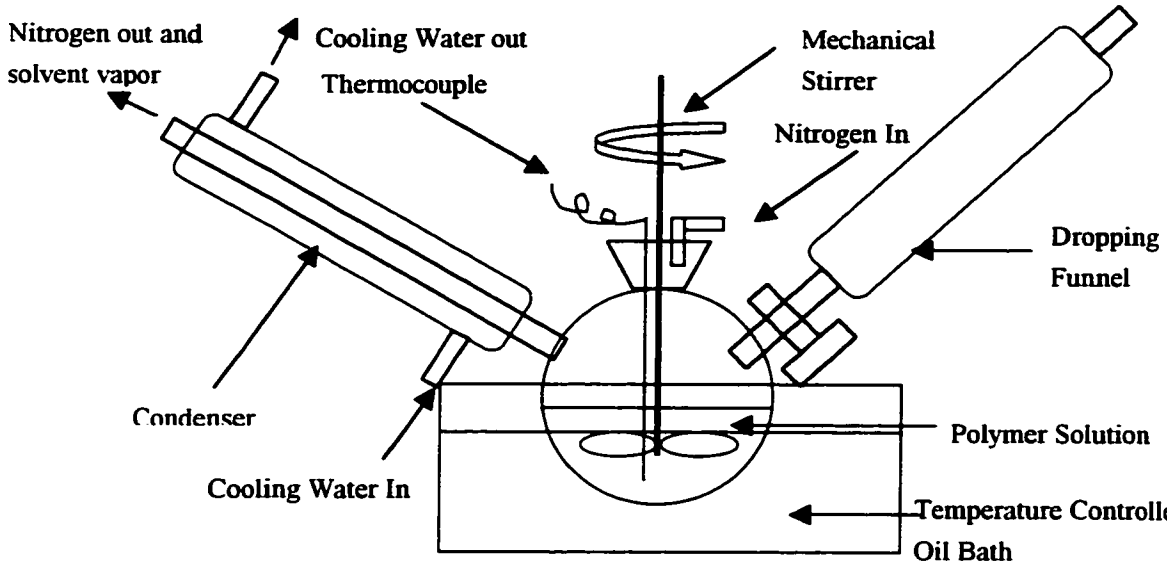

Figure 2.1 Schematic diagram of polyurethane synthesis apparatus. Nitrogen is passed in the reactor to maintain an inert and dry environment

The final reaction mixture was poured into a large quantity of distilled water in small portions for precipitation. After precipitating and rinsing with water 3 times, the precipitate was filtered and dried in an oven at 60°C. The polyurethane samples were then dried under vacuum at 60°C and extracted with methanol for 24 hours. The purified polyurethanes were vacuum dried prior to further characterization.

2.3 Synthesis of dendrimer modified polyurethanes

2.3.1 Approach 1: Incorporation of PEO into the dendrimer prior to polyurethane synthesis

The experimental design for approach 1 is shown in Figure 2.2.

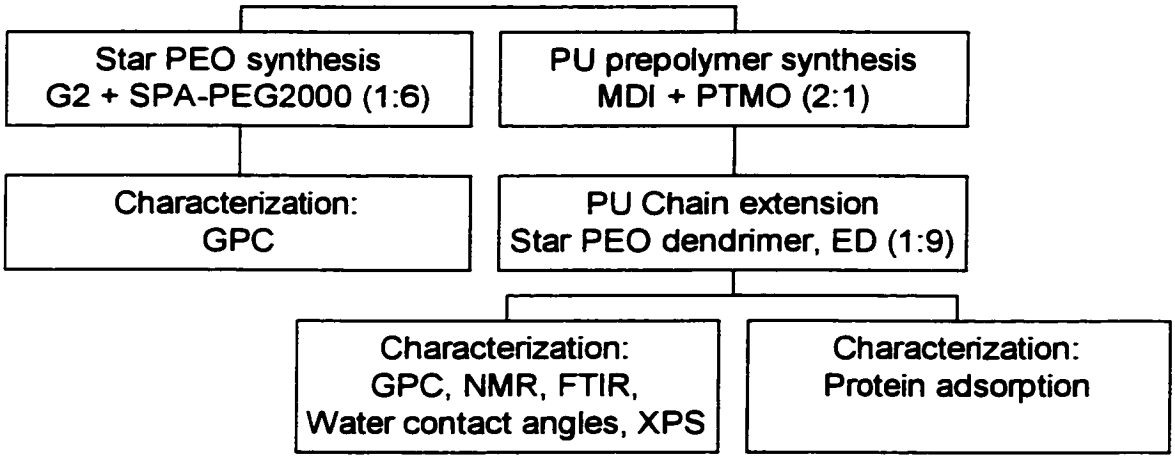

Figure 2.2 Schematic of approach 1 used to synthesize dendrimer modified polyurethanes with PEO attached. In this approach, PEO was incorporated into the dendrimer prior to polyurethane synthesis.

2.3.1.1 Synthesis of star PEO dendrimers

A solution of polypropyleneimine octaamine dendrimer generation 2.0 (G_2) and SPA-PEG 2000 in CH_2Cl_2 was stirred at room temperature in a 50 mL beaker covered with laboratory film to inhibit CH_2Cl_2 vaporization. In this case, 1.01 equivalent per NH_2 end group, and a molar ratio of 8:1 (SPA-PEG2000:G_2) was used to determine the duration of the reaction. Every 15 to 30 minutes, the reaction was monitored by the addition of 50 μL of the reaction mixture to 0.5 mL ninhydrin solution. When the ninhydrin test became negative (i.e. the ninhydrin solution did not turn blue), the reaction was considered complete. GPC analysis was performed on this product to confirm the formation of the star PEO. In all subsequent reactions, a molar ratio of 6:1 (SPA-PEG

2000:G_2) was used to yield a statistical distribution of star PEO molecules in which the star PEO molecules with 6 PEO arms would be the maximum.

2.3.1.2 Synthesis of dendrimer modified polyurethanes with star PEO

The polyurethane prepolymers were synthesized using the same method as the PU control (see section 2.2). The star PEO dendrimers with 6 PEO arms were incorporated during the chain extension step with ED using a ratio of 9 moles of standard chain extender to one mole of dendrimer. The final reaction mixture was slowly poured into 1L distilled water for precipitation.

2.3.1.3 Derived approach 1: Incorporation of PEO and dendrimers into polymer structure simultaneously

Using the same molar ratio of SPA-PEG2000 and dendrimers as was applied in approach 1, SPA-PEG2000 and dendrimers were introduced in the following sequence in the chain extension step: standard chain extender (i.e. ED or BDO), SPA-PEG2000 and dendrimer G_2. This sequence was chosen in order to minimize the crosslinking caused by the multifunctional dendrimers. The final reaction mixture was poured slowly into 1 L distilled water for precipitation and then washed with copious quantities of distilled water.

2.3.2 Approach 2: Incorporation of dendrimers into polymer structure and subsequent PEO modification

Approach 2 was developed and used to produce a crosslink-free (or low crosslink density) polyurethane with incorporated dendrimer and PEO attached (as shown in Figure 2.3).

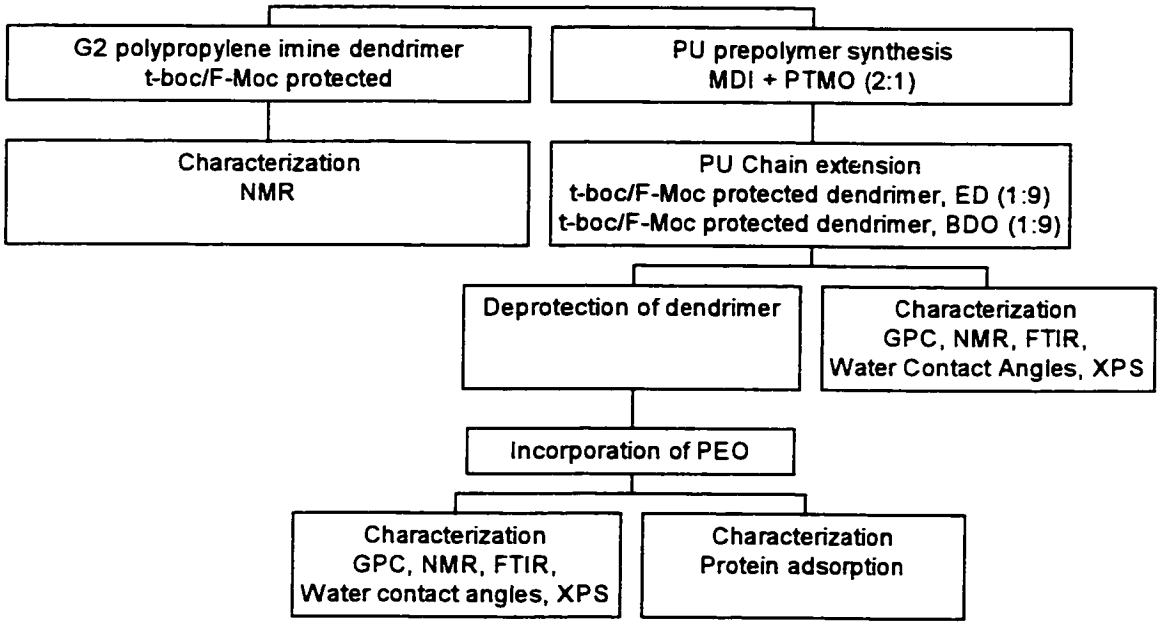

Figure 2.3 Schematic of approach 2 used to synthesize dendrimer modified polyurethanes with PEO attached. In this approach, dendrimer was incorporated into the polymer structure and PEO was attached to dendrimers subsequently.

2.3.2.1 Protection of dendrimers with t-Boc and Fmoc groups

t-Boc protected dendrimers were prepared following the procedure described by Jansen et al. (1994). To a stirred solution of polypropyleneimine octaamine dendrimer generation 2.0 (0.193 g) in 20 mL CH_2Cl_2 with triethylamine (0.2 mL), 0.434 g N-t-Boc-

L-Ala hydroxy succinimide ester (1.01 equivalent per NH_2 end group at a molar ratio of 6:1) was added. After stirring overnight, the reaction mixture was diluted with 50 mL of CH_2Cl_2 and subsequently washed with distilled water (three times with 30 mL) and saturated Na_2CO_3 (three times with 30 mL). Drying of the resulting product with Na_2SO_4 was followed by evaporation of the solvent in a rotary evaporator to yield t-Boc protected dendrimers.

Fmoc protection of the NH_2 groups of dendrimer was achieved using the following procedure. Polypropyleneimine octaamine dendrimer generation 2.0 was dissolved in CH_2Cl_2. 9-Fluorenylmethyloxycarbonyl Chlorocarbonate (9-Fmoc) at a molar ratio of 6:1 was then added in small portions (1~2 mL aliquots every 30 seconds). Stirring was continued in an ice water bath for 4 hours. After another 8 hours of reaction at room temperature, CH_2Cl_2 was evaporated in the rotary evaporator and the product was dried at 60°C in a vacuum oven to yield Fmoc protected dendrimers.

2.3.2.2 Synthesis of polyurethanes with dendrimers as chain extenders

Polyurethane prepolymers were synthesized as previously described (see section 2.2). The t-Boc or Fmoc protected dendrimers were incorporated during the chain extension step with either the ED or BDO at a ratio of 9 moles of standard chain extender to one mole dendrimer and the mixture reacted as in the case of the controls. The final reaction mixture was poured slowly into 1 L of distilled water for precipitation. After precipitation and extensive water washing (three times in 1 L of distilled water each time), the precipitate was filtered and dried in an oven for 3 days at 60°C. The polyurethane samples were then dried under vacuum at 60°C overnight and extracted

with methanol for 24 hours. The purified polyurethanes were vacuum dried overnight for further modification and characterization.

2.3.2.3 Deprotection of dendrimer modified polyurethane and PEO attachment

2.3.2.3.1 t-Boc protected dendrimer

0.5 g of the dendrimer incorporated polyurethane was stirred overnight in 100 mL of 90% formic acid. This reaction mixture was diluted with 1000 mL of distilled water to recover the deprotected polyurethane. The polyurethane was washed thoroughly with distilled water, dried in a 60°C oven for 2 days, and then dried under vacuum at 60°C overnight. The deprotected dendrimer polyurethane was redissolved in DMF (0.5 g in 10 mL), and the theoretically calculated amount of SPA-PEG2000 in DMF was added with stirring, at room temperature. The reaction was allowed to proceed overnight. The solvent was subsequently removed from the reaction mixture by vacuum distillation and the remaining solid was washed with distilled water thoroughly (three times, 500 mL of distilled water each time) and dried in a 60°C oven for 3 days.

2.3.2.3.2 Fmoc protected dendrimer

To a solution of the Fmoc protected dendrimer polyurethane in DMF, diethylamine (2.0 mL for 2 g polyurethane) was added with stirring. The reaction was allowed to proceed at room temperature for 1.5 hours. The deprotected dendrimer polyurethane was precipitated in distilled water and washed three times with distilled water (100 mL each time). After drying in an oven at 60°C for 2 days, the deprotected

dendrimer polyurethane was redissolved in DMF and the theoretical maximum amount of SPA-PEG2000 for full dendrimer modification was added with stirring. The reaction was allowed to proceed at room temperature overnight. The final polyurethane product with PEO attached was precipitated in 500 mL of distilled water, washed thoroughly with distilled water (three times, 500 mL each time) and dried in a 60°C oven for 3 days.

2.4 Preparation of Polyurethane Films

10% (wt./vol.) solutions of various polyurethanes in DMF were prepared. 20 mL of this solution was poured into 50 mm glass petri dishes and placed in an oven at 50°C for 5 days to allow the solvent to evaporate. The films were then removed from the dishes, rinsed with methanol, allowed to air dry and stored in plastic petri dishes for further characterization. PEO attached polyurethanes were cast on control polyurethane films because of the low mechanical strength of the polyurethanes with PEO incorporation.

2.5 Characterization

2.5.1 Gel Permeation Chromatography (GPC)

GPC analysis was used to obtain the relative molecular weights and molecular weight distributions of all polymers. The polyurethane was dissolved at a concentration of 0.1% (wt./vol.) in DMF. The analysis was performed using a series of three Waters Styragel® HR4 GPC columns at McMaster University. The retention time of the polyurethane was determined using a Waters 410 Refractometer at room temperature.

Polystyrene standards were used for calibration. A mobile phase consisting of 0.1% (wt./vol.) LiBr in DMF was used.

2.5.2 ^{1}H-NMR spectroscopy

NMR is a useful tool in polymer structure analysis as it is an extremely powerful technique for determining the microstructural details of polymer chemical structure. The source of nuclear magnetic resonance is the Zeeman interaction (i.e. the interaction between the magnetic moment of a nuclear spin and a static magnetic field). Only nuclei with nonzero spin are observable by NMR; this excludes many abundant nuclides, such as ^{12}C or ^{16}O. These elements do have NMR-active isotopes, although they occur naturally only at low concentrations (1.1% for ^{13}C; 0.04% for ^{17}O). The greatest strength of NMR is its sensitivity to subtle details of chemical structure. The exact resonant position, or chemical shift, primarily depends on the electronic environment around the nucleus, with effects being observed over several bond lengths (Brandolini et al., 2000).

^{1}H-NMR spectroscopy was performed using an AV-2000 spectrometer at McMaster University to confirm the polymer structure after dendrimer incorporation, and using a Bruker AMX-500 at the University of Ottawa for the polymer structures after PEO attachment. This was done by using the peaks at 6.13 ppm and 8.37 ppm which are assigned to types of protons in the urea groups formed by the reaction of NH_2 with -NCO of the prepolymer and the peak at 3.51 ppm (-CH_2O-) for PEO. Peak assignments for ^{1}H-NMR measurements made on ED chain extended polyurethanes are summarized in Appendix B. All spectra were recorded in a mixture of deuterated DMSO and chloroform (v:v = 1:1).

2.5.3 FTIR analysis

The synthesized polymers were analyzed using FTIR spectroscopy. Transmission FTIR spectra were obtained using a Bio-Rad FTS-10 FTIR instrument at McMaster University. For analysis purposes the films were cast on a NaCl crystal window. All films were dried in a vacuum oven at 60°C for 24 h prior to recording.

2.5.4 Water Contact Angles

Water contact angles were used to obtain a measure of the hydrophilicity of the polymers with and without dendrimer and PEO modification. Surfaces with a higher hydrophobicity will have a larger contact angle whereas surfaces that are more hydrophilic will have a greater degree of wetting and therefore a smaller contact angle as shown in Figure 2.4. This information is qualitative rather than quantitative since surface chemistry is not specifically determined using this technique. The contact angles were measured by a sessile drop method using a goniometer (Rank Scherr Tumico 22-2000 series 14 inch horizontal beam bench comparator). A 10 μL water droplet was placed on the polymer surface using a microsyringe. For each sample, 3-5 separate drops were examined at room temperature.

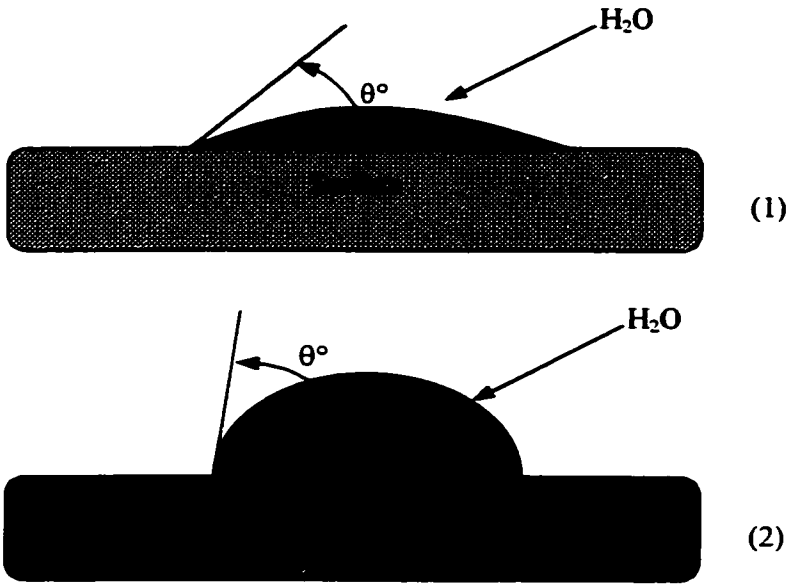

Figure 2.4 Schematic representation of surfaces with different degrees of hydrophilicity. Surface (1) is more hydrophilic than surface (2) and is characterized by a lower water contact angle (θ°).

2.5.5 X-Ray Photoelectron Spectroscopy (XPS)

XPS can be used to obtain quantitative information about the surface (upper 100 nm) chemistry. The surfaces are bombarded with an X-ray beam, the energy from which causes the emission of electrons from core atomic levels of atoms on the surfaces as shown schematically in Figure 2.5.

The energy of the emitted photoelectron corresponds to the binding energy of the core electrons. By measuring the emissions from the surface, it is possible to determine what elements are present and the bond configurations of these elements. A low resolution XPS scan can give quantitative information about the elements present on the surface. High resolution scans are focused on particular elements and their specific bond configurations.

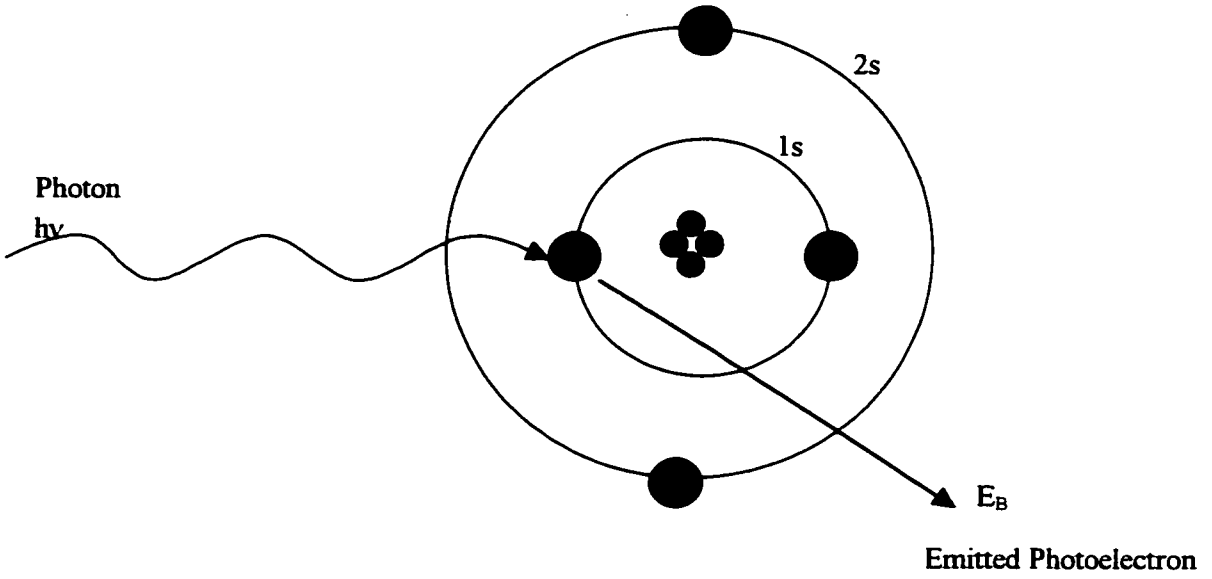

Figure 2.5 Schematic representation of the emission of a photoelectron from a core electron after bombardment with an x-ray.

XPS was used in this work to study the surface chemistry of the dendrimer modified polyurethanes to examine whether any surface enrichment of the PEO occurred. The analysis was performed by the ICPET at the National Research Council of Canada using a Kratos AXIS HS X-Ray photoelectron spectrometer. The samples were cast from 10% DMF solutions and dried in a 60°C oven. After rinsing with methanol and drying in a vacuum oven at 60°C, the samples were placed in the vacuum chamber of the XPS. Low resolution spectra were obtained for all surfaces. High resolution spectra were recorded for carbon, nitrogen and oxygen chemical species.

2.6 Protein Adsorption

When polymeric biomaterials come in contact with blood or protein containing

solutions, the initial and fate-determining step is the adsorption of plasma proteins. This adsorption can lead to a variety of reactions including activation of the intrinsic coagulation pathway or the complement cascade, activation of platelets as well as interactions with other cellular components present in the blood (see section 1.2.1). The effect of dendrimer modification on protein adsorption from plasma was investigated in this study. The adsorption of fibrinogen and other proteins from plasma onto the modified surfaces was examined. All protein adsorption tests were performed at McMaster University. The polyurethane control surface and dendrimer incorporated polyurethanes using t-Boc and Fmoc protecting groups both before and after PEO attachment were compared.

2.6.1 Preparation of ^{125}I labeled Fibrinogen in Plasma

Human fibrinogen was labeled with ^{125}I using the iodogen method. Fibrinogen was diluted with phosphate buffered saline (PBS), pH 7.4, to a concentration of 1.0 mg/mL. A solution of 1,3,4,6-tetrachloro-3a,6a-diphenylcoluril (Iodogen®, Pierce) in chloroform (0.1 mg/mL) was prepared. 100 μL was placed in a 3 mL conical glass vessel. The chloroform was evaporated at room temperature under dry nitrogen. 5 μL of radioactive sodium iodide ($Na^{125}I$, 0.5mCi, Amersham) was added to the glass vessel and stirred for approximately 1 minute. 100 μL of the fibrinogen solution and 100 μL of PBS buffer were added to the reaction vessel and stirred for 15 minutes. The fibrinogen was then removed from the reaction vessel to terminate the iodination reaction. Free iodide was removed by dialysis. The labeled fibrinogen was injected into a SLIDALYZER® dialysis cassette (Pierce) and dialyzed against 500 mL of PBS buffer, at 4°C overnight

with three changes of the dialysate. Next, the ^{125}I labeled fibrinogen was removed from the dialysis cassette and the cassette was rinsed twice with 500 μL of PBS to remove any remaining protein. Fibrinogen concentration was then determined spectrophotometrically. Free iodide was determined by trichloroacetic acid precipitation of the protein. A 0.25% labeled fibrinogen solution was prepared and solutions were prepared by diluting a 1 mg/mL solution. These solutions were used in the adsorption experiment.

2.6.2 Fibrinogen Adsorption

6 mm diameter polyurethane surfaces were placed in the wells of a microtitre plate and 250 μL of the radiolabeled fibrinogen solution was added to each of the wells. The surfaces were incubated at room temperature for three hours, removed from the solution and dip-rinsed three times in PBS buffer. The radioactivity of the surfaces was measured with a gamma counter and the counts were compared to known standards. The adsorbed fibrinogen was evaluated using the following equation:

$$Adsorbed\ Fibrinogen = \frac{CPM_1 \times C_{Fg} \times V}{A \times CPM_2}$$

where: CPM_1 is the radioactivity of the surface [cpm];

C_{Fg} is the concentration of fibrinogen in plasma solution [μg/mL];

V is the volume of reference fibrinogen solution [mL];

A is the surface area of polyurethane disk [mm^2];

CPM_2 is the radioactivity of reference fibrinogen solution [cpm].

2.6.3 Protein Adsorption from Plasma

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were used to examine protein adsorption from plasma to the various surfaces including the polyurethane controls and the dendrimer and PEO incorporated polyurethanes. A more detailed SDS-PAGE and immunoblotting procedure is presented in Appendix C.

2.6.3.1 SDS Polyacrylamide Gel Electrophoresis

SDS-PAGE was used in order to determine the differences in protein adsorption patterns on the different dendrimer modified polyurethane surfaces. This separation technique involves the application of a voltage to proteins loaded onto a polyacrylamide gel of two different concentrations. Proteins are separated according to molecular weight. Dissolution in a 2% SDS solution masks any charge effects that may be present. A 4% polyacrylamide gel was used for "stacking" or concentrating the proteins before separating to achieve sharp bands after separation. A 12% polyacrylamide gel was used for separating the proteins.

The 12% polyacrylamide gel solution was poured into a gel mould and the top was overlaid with water. After polymerizing for approximately one hour, the stacking gel was prepared. The water was removed from the top of the gel and the stacking gel was added. A well comb was then fitted into the top of the gel. The stacking gel was then allowed to polymerize for one hour.

The surfaces were incubated in a solution of citrated pooled normal plasma (>25 donors) for a period of two hours. To remove the proteins adsorbed to the polyurethane surfaces, the surfaces were incubated overnight in 2% SDS at 4°C. The samples and standard markers were then loaded onto the gel. A volume of 0.5 μL of the standards and 125 μL of the eluted proteins were loaded into their respective wells at the top of the gel. The electrophoresis apparatus was assembled and 200 volts were applied across the gel for approximately one hour. Tracking dye was used to follow the progress of protein migration.

After electrophoresis, the proteins were transferred to an Immobilon PVDF transfer membrane. The membrane was pre-wetted with methanol and water, then soaked in transfer buffer (see Appendix D). The gel was applied to the membrane and 200 volts were applied for a period of one hour. The membranes were stained with colloidal gold or dried for immunoblot analysis.

2.6.3.2 Immunoblotting

Immunoblotting was used to determine the specific protein adsorption patterns. Sections of the membrane containing low molecular weight and prestained marker lanes were removed and stained by incubating in Protogold solution (Cedarlane Lab Ltd., Hornby ON) for 1-4 h. The remainder of the membrane was cut into 3 mm strips. To block unbound membrane sites and prevent non-specific binding, the strips were incubated for 1 hour in 5% (wt./vol.) dry skim milk in tris buffered saline (TBS), pH 7.4 with gentle agitation followed by three 5 minute rinses in 0.1% dry skim milk in TBS. Each strip was then incubated with 1% (wt./vol.) dry skim milk and 0.05% (v/v) Tween

20 in TBS with a 1/1000 dilution of the primary antibody (as shown in Table 2.2) to the protein of interest for 1 hour. This treatment was followed by three 5 minute rinses in 0.1% dry skim milk in TBS. Strips were then incubated for 1 hour in 1 mL 1% (wt./vol.) dry skim milk and 0.05% (v/v) Tween 20 in TBS with a 1/1000 dilution of the alkaline phosphatase linked secondary antibody. This was again followed by three 5 minute rinses in 0.1% (wt./vol.) dry skim milk powder in TBS. Finally, the strips were incubated for up to 4 hours with the colour generation solution (Appendix D) to develop the bands. The reaction was terminated by rinsing the strips extensively in distilled water. The strips were then allowed to air dry.

Chapter 3 Results

3.1 Synthesis of polyurethanes

Polyurethane synthesis was done by a conventional two-step approach using two standard chain extenders, ED and BDO. In the first step, prepolymers were formed by reacting MDI with PTMO. In the second step, ED (or BDO) was added so that the molar ratio of MDI:PTMO:ED (or BDO) was 2:1:1. The final reaction mixture was a clear solution often with a yellow tinge, likely the result of reaction of the MDI with air.

Two approaches were employed to synthesize dendrimer modified polyurethanes with incorporated PEO. Approach 1, in which a star PEO dendrimer was first synthesized by reacting SPA-PEG 2000 with poly (propyleneimine octaamine) G_2 dendrimer at a molar ration of 6:1 (PEG:dendrimer) (see section 2.3.1), was unsuccessful. This approach should statistically leave an average of two free amine groups on the dendrimer for further reaction. GPC analysis and a ninhydrin test monitoring the resulting star PEO confirmed the reaction of SPA-PEG2000 and G_2 dendrimer. GPC results, shown in Table 3.1, indicate the incorporation of PEO into the dendrimer since the measured molecular weight of the star PEO (12300 or 10200) is higher than both of the molecular weights of PEO (2000) and that of dendrimer G2 (773). Possibly due to the branches present in the polymers, which could also have blocked further PEO addition to the dendrimer, the measured GPC results were lower than the true values, which should have shown a

molecular weight of approximately 16000 for fully modified dendrimer and 12000 for partially modified dendrimer.

Table 3.1 GPC analysis of PEO modified dendrimer G_2

Sample	Mn	Mw
Fully modified dendrimer (PEG:dendrimer = 8:1)	9400	12300
Partially modified dendrimer (PEG:dendrimer = 6:1)	7700	10200

As observed from a ninhydrin test, the reaction of NH_2 groups of dendrimer G_2 with SPA-PEG2000 was initially very fast. While complete reaction was noted within 3 hours on an 8:1 reaction mixture, the reaction rate was found to drop sharply after the first 1 to 2 hours, suggesting that with the addition of the PEO and the formation of PEO containing star polymers, the remaining free amine groups become buried in the entangled PEO chains, significantly hindering further reaction. Therefore, this is likely the reason for the failure of this approach to result in the expected molecular weight of the PEO dendrimers and the failure to form high molecular weight polymers with PEO incorporated, even with a 9:1 ratio of standard chain extender to the PEO dendrimer. It appears that the free NH_2 groups of dendrimers were sterically "buried" inside after PEO modification, resulting in low reactivity of the "free" NH_2 groups.

To overcome this steric hindrance problem, a modification was made to the method. SPA-PEG2000 and dendrimers were introduced in sequence with the standard chain extender (i.e. ED or BDO) first, SPA-PEG2000 second, and dendrimer G_2 last during the chain extension step. The sequence was chosen in order to minimize the crosslinking caused by the multifunctional dendrimers. The reaction temperature, and

speed of reactant addition affected the chain extension reaction, and an insoluble crosslinked polyurethane resulted after several trials. However, an optimal condition (as shown in Table 3.2) was found to produce DMSO soluble, slightly crosslinked polyurethanes that incorporated dendrimers with PEO attached (denoted PU-ED-PEO^1, where 1 represents approach 1). This product was soluble in DMF or DMSO and had superior mechanical strength, possibly due to some degree of crosslinking.

Table 3.2 Optimal condition for the reaction to synthesize dendrimer incorporated polyurethane with PEO attached (PU-ED-PEO^1) by derived approach 1

Prepolymerization temperature (°C)	50
Prepolymerization duration (min)	120
Chain extension temperature (°C)	25
Chain extension duration (min)	60
Standard chain extender concentration (%,wt./vol.)	2.0
Dendrimer G2 concentration (%, wt./vol.)	2.0
Chain extension sequence	ED/BDO→SPA-PEG→dendrimer
Dropping speed of dendrimer solution (drops/s)	1
Post chain extension temperature (°C)	90
Post chain extension duration (min)	60

Alternatively, approach 2, involving a protection/deprotection strategy to overcome the low reactivity of the free -NH_2 groups after PEO modification, was utilized to produce PEO attached dendrimer modified polyurethanes. The dendrimers were first partially protected with amine protecting groups at a molar ratio of 1:6, leaving on average two free amine groups for the chain extension reaction in the polyurethane synthesis. Two different protecting groups (t-Boc and Fmoc) were used and compared in this study. A purification procedure was applied to t-Boc protected dendrimers to remove any unprotected dendrimers. The purification process makes use of the difference in the

solubility of the dendrimers in water before and after t-Boc protection. The reaction yield of the t-Boc protection was between 60 to 70% after purification. These yields are reasonable since a distribution of the number of the protected amine groups of dendrimer is expected and losses would be incurred during the purification steps and within the original reaction vessel. The small amounts of reactants involved (e.g. around 5 g of total reaction mixture) inevitably contributed to the losses noted.

The t-Boc protected dendrimers were incorporated during the chain extension step with either the ED or BDO at a ratio of 9 moles of standard chain extender to one mole dendrimer and the mixture was reacted as in the case of the controls. The resulting polyurethane (denoted PU-G_2-ED^{2a}, where 2 represents approach 2 and a for t-Boc protecting groups used) had no apparent crosslinking, confirming the absence of unprotected dendrimers following reaction.

Similarly, Fmoc protecting groups were used to synthesize dendrimer modified polyurethane as described in the previous chapter (see section 2.3.2.1). No purification procedure was applied to the protected dendrimers. However, it is unlikely that any of the dendrimers would be completely unprotected and the resulting polymers (denoted PU-G_2-BDO^{2b}, where 2 represents approach 2 and b for Fmoc protecting groups used) showed no apparent crosslinking, suggesting that there were no significant amounts of unprotected dendrimer for the polyurethane chain extension.

In order to introduce PEO chains to the dendrimer modified polyurethanes, deprotection was necessary using different reactions depending on the protecting group in question. An acid treatment procedure was used to remove the t-Boc protecting groups for subsequent PEO attachment. Following reaction with the PEO in this case, a stable

emulsion in water formed when precipitation was attempted. The formation of the emulsion could be either a result of the degradation of polyurethanes following exposure to the acid in the deprotection reaction because the procedure affects urea bonds between the t-Boc and the $-NH_2$ groups in the dendrimer, a significant increase in the hydrophilicity of polyurethane after PEO attachment or a combination of the two effects. In order to examine the effect of the acid treatment on the polyurethane structure, polyurethane control samples chain extended with either ED or BDO were treated by the acid for similar time periods as the dendrimer deprotection reaction and their molecular weights determined by GPC. A comparison of these molecular weights with those of the untreated control polymers is summarized in Table 3.3.

Table 3.3 GPC results (Formic acid treatment of polyurethanes overnight)

Sample	Mn	Mw
ED chain extended control	82800	133100
BDO chain extended control	49400	64700
Formic acid treated ED PU	68900	110000
Formic acid treated BDO PU	30300	48900

From this table, it can be seen that there was a small amount of degradation of both the BDO and ED chain extended polyurethanes by the acid treatment. However, the polymer molecular weight remained significant, making it unlikely for this to be the major contributing factor to the formation of the emulsion. Therefore, it seems more likely that the emulsion formation resulted from changes in the solubility of the polymers by the incorporation of the PEO into the polymer structure. In order to obtain the final

polymer in this case however, it was necessary to evaporate the solvent used in the reaction and purify the resultant polymer by extensive water washing and methanol extraction.

Deprotection by reaction with diethylamine was used to remove the Fmoc protecting groups for subsequent PEO attachment. No emulsion formed following this procedure and the final products with PEO attachment were obtained following precipitation and purification in distilled water.

Films cast from solutions of the various polyurethanes in DMF were clear and transparent with a slight brown tinge, again likely the result of MDI oxidation. All of the films were easy to peel off the glass dishes with the exception of the final polyurethanes with PEO attached, which had seemingly poor mechanical strength and were too weak to be removed from the dishes. Therefore, the PEO attached polyurethanes were cast onto control films, giving better mechanical strength while allowing for surface characterization.

3.2 Chemical Characterization of Polyurethanes

3.2.1 GPC Analysis

GPC analysis was used to obtain the relative molecular weights and molecular weight distributions of the polymers. The GPC results of all the polyurethanes synthesized are summarized in Table 3.4.

Since GPC was calibrated with linear polystyrene chains, the molecular weight information obtained is only applicable to linear polymer chains. In the case of polymers

with branches like the dendrimer modified polyurethanes, the measured values are lower than the real values. Furthermore, as has been previously shown in our laboratory, polystyrene standards do not provide accurate information about polymers with PEO incorporated. However, the GPC results still provide useful information about the polymers made by the various approaches.

Table 3.4 Polyurethanes Synthesized and their Molecular weights

Nomenclature	Chain Extender	Dendrimer	Protection	Deprotection	Mn	Mw
PU-ED	ED	No			82800	133100
PU-G2-ED[2a]	ED	Yes	t-Boc		67800	106300
PU-ED-PEO[2a]	ED	Yes	t-Boc	Formic Acid	62600	100600
PU-BDO	BDO	No			49400	64700
PU-G2-BDO[2a]	BDO	Yes	t-Boc		30400	48800
PU-G2-BDO[2b]	BDO	Yes	Fmoc		76400	117000
PU-BDO-PEO[2b]	BDO	Yes	Fmoc	Diethylamine	86000	135900
PU-ED-PEO[1]	ED	Yes			138700	214800

Note: 1–approach 1; 2–approach 2; a–t-Boc protecting groups;b–Fmoc protecting groups.

It can be seen that, as would be expected from the reactivity of isocyanates with the hydroxy terminated chain extender relative to that of the amine terminated chain extender, the polymer molecular weights were significantly greater in the polyurethane controls chain extended with ED (i.e. PU-ED) compared with those chain extended with BDO (i.e. PU-BDO). The dendrimer incorporated polyurethanes had significantly lower molecular weights than control PUs when t-Boc was used (PU-G_2-ED[2a] compared to PU-ED, and PU-G_2-BDO[2a] to PU-BDO), likely the result of the lower reactivity of the

dendrimers due to steric factors. Furthermore, the dendrimer modified polyurethanes made using the Fmoc protection/deprotection approach had higher molecular weights than those using the t-Boc protection/deprotection approach (PU-G_2-BDO^{2b} compared to PU-G_2-BDO^{2a}), suggesting possible crosslinking in polymer PU-G_2-BDO^{2b} because no purification procedure was applied to the Fmoc protected dendrimers. The GPC results also demonstrated that no unreacted dendrimers remained in the polyurethanes following reaction and purification as there were no other peaks except for the polyurethane peak.

The dendrimer modified polyurethanes from t-Boc protection/deprotection approach showed a slightly decreased molecular weight after PEO attachment (PU-ED-PEO^{2a} compared to PU-G_2-ED^{2a}). While an increase was expected due to incoporated PEO, it is possible that degradation of the polymer backbone by the acid treatment used for deprotection affected the resultant molecular weights of the polymers. On the other hand, when the Fmoc protection/deprotection approach was used, the molecular weight increased after PEO attachment, as expected, suggesting that the PEO chains were reacting with the polyurethane backbone and no degradation occurred during deprotection.

3.2.2 ^{1}H-NMR Spectroscopy

^{1}H-NMR spectra were used to confirm the structure of the polymers based on peak assignments. The ED chain extended polyurethanes before and after dendrimer modification are shown in Figures 3.1 and 3.2, respectively.

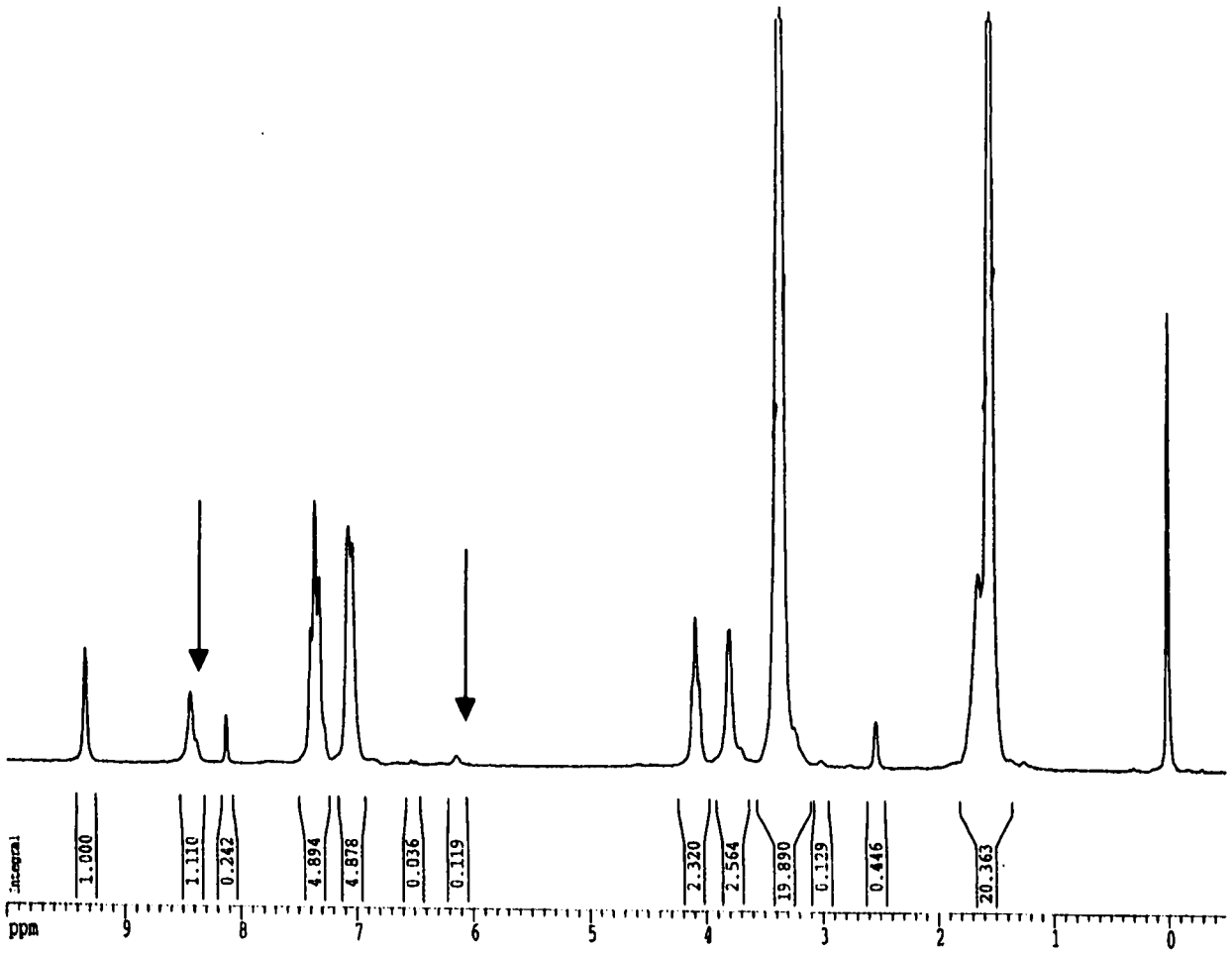

Figure 3.1 ^{1}H-NMR spectrum of ED chain extended polyurethane control (PU-ED). Of interest are the peaks at 6.13 ppm and 8.17 ppm (as shown), indicative of the formation of ure bonds.

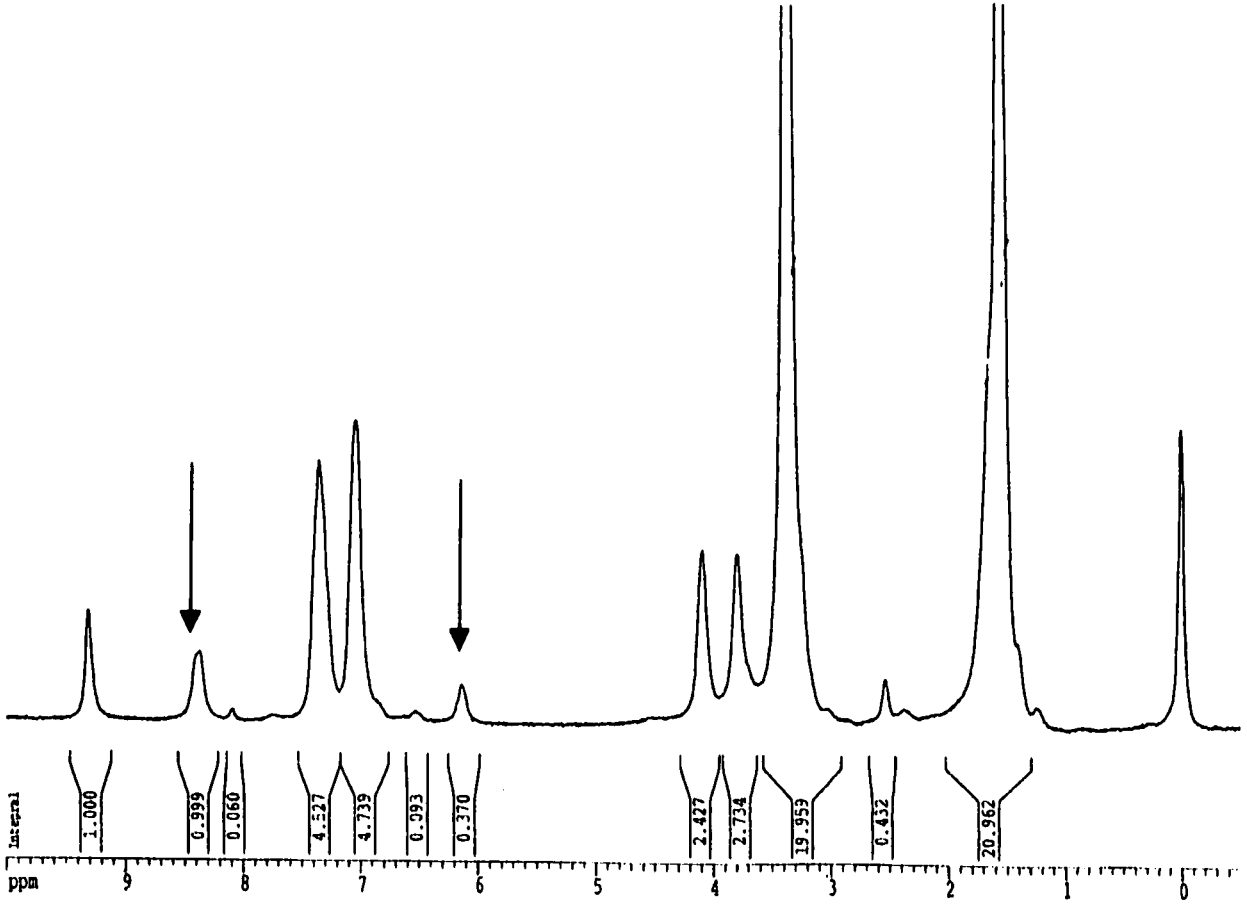

Figure 3.2 ^{1}H-NMR of dendrimer modified polyurethane with ED as chain extender (PU-G_2 ED^{2a}). The peaks at 6.13 and 8.17 ppm have increased in intensity relative to the ED control polymer as expected from the increased numbers of urea linkages due to the presence of the dendrimers.

Peak assignments for ^{1}H-NMR measurements made on ED chain extended polyurethanes are summarized in Appendix B. Of specific interest are the peaks at 6.13 and 8.37 ppm which are assigned to types of protons in the urea groups formed by the reaction of NH_2 (from ED or dendrimer) with -NCO of the prepolymer. These were found to increase with incorporation of the dendrimer prior to deprotection, as expected, because the protection reaction generates a urea linkage.

In order to make it clearer that the increase of the peak at 6.13ppm and 8.37ppm was the result of dendrimer incorporation, BDO was used as the chain extender in the polyurethane synthesis. Unlike ED, BDO contains OH groups that react with -NCO groups of prepolymers to form a urethane linkage. Therefore, the BDO control polymer lacks the urea peaks at 6.13ppm and 8.37ppm as shown in Figure 3.3. However, after incorporation of the dendrimer incorporation, those two peaks reappeared (see Figure 3.4).

The presence of PEO in the PEO incorporated polyurethanes is demonstrated by ^{1}H-NMR by the presence of a peak at 3.51 ppm in Figures 3.5, 3.6 and 3.7 for the PU-ED-PEO^{2a}, PU-BDO-PEO^{2b} and PU-ED-PEO^1 polymers, respectively.

Quantitative analysis of the spectra show that relative to the dendrimer at 6.13 and 8.37 ppm, PU-ED-PEO^{2a} has the fewest PEO amounts incorporated while PU-ED-PEO^1 has the most PEO chains. A summary of the estimated amount of PEO attached to the dendrimers based on peak integration is shown in Table 3.5.

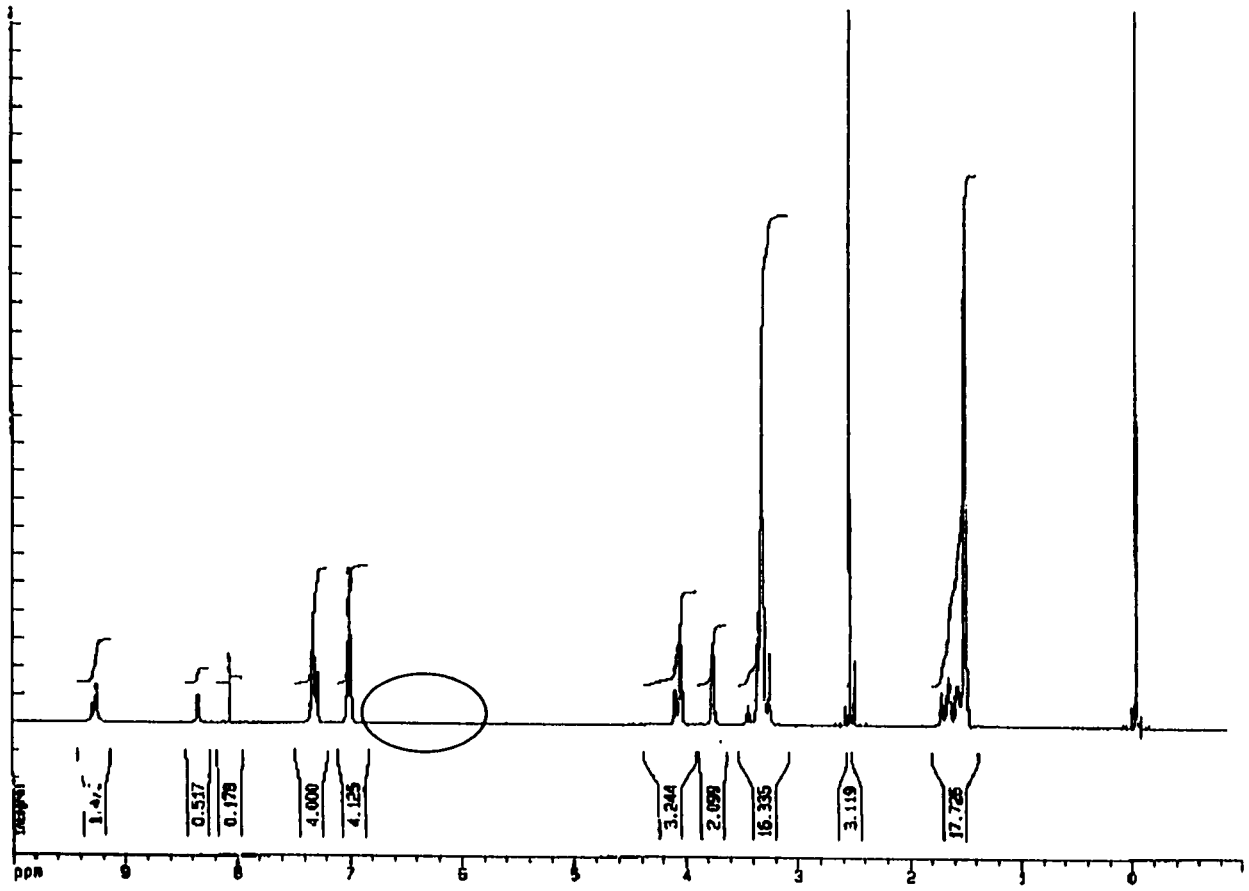

Figure 3.3 ^{1}H-NMR spectrum of BDO chain extended polyurethane control (PU-BDO) Of interest are the lack of urea peaks as noted.

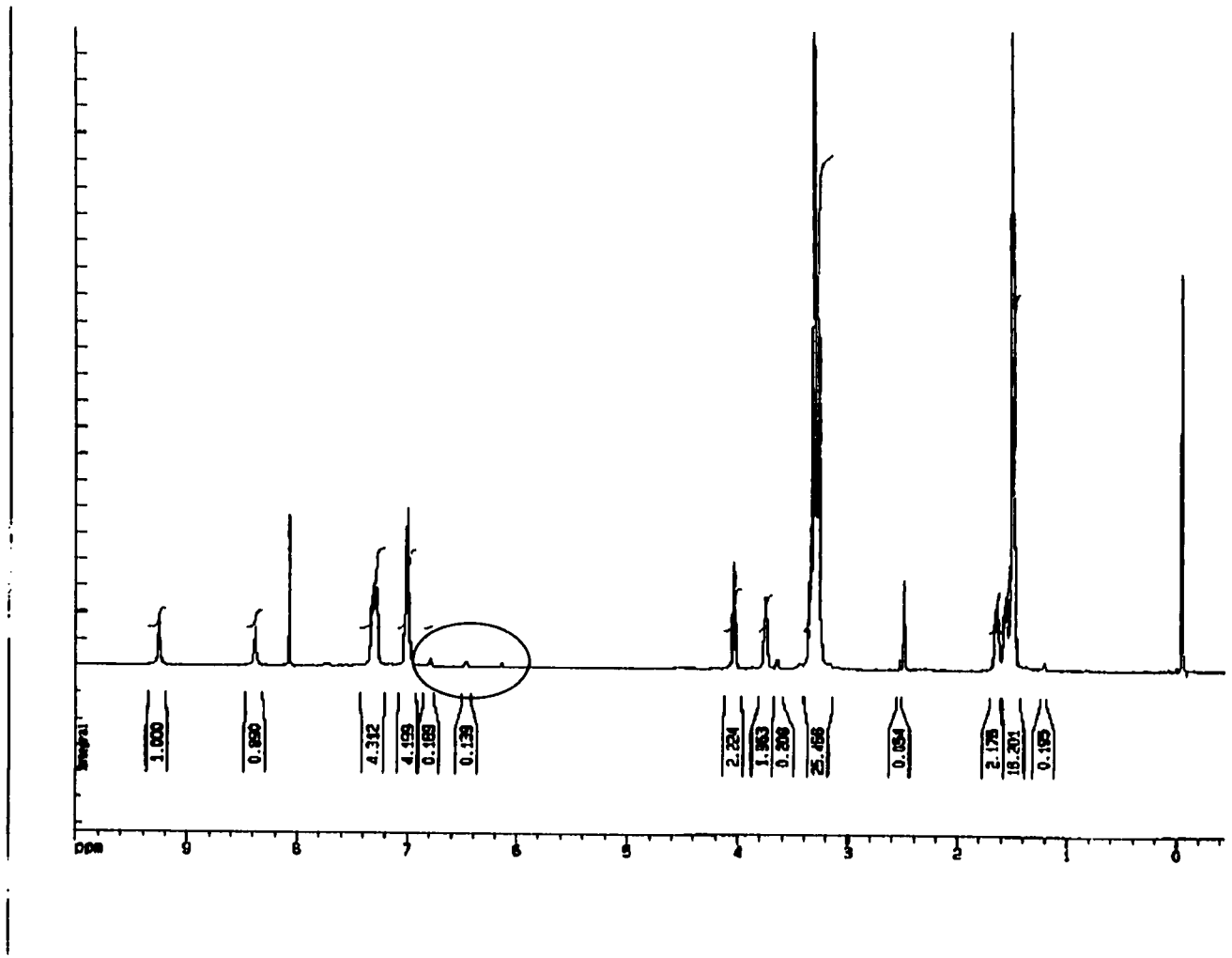

Figure 3.4 ^{1}H-NMR of dendrimer modified polyurethane with BDO as chain extender (PU G_2-BDO^{2b}). The reappearance of the urea peak as expected indicates the presence of the dendrimer in the polymer structure.

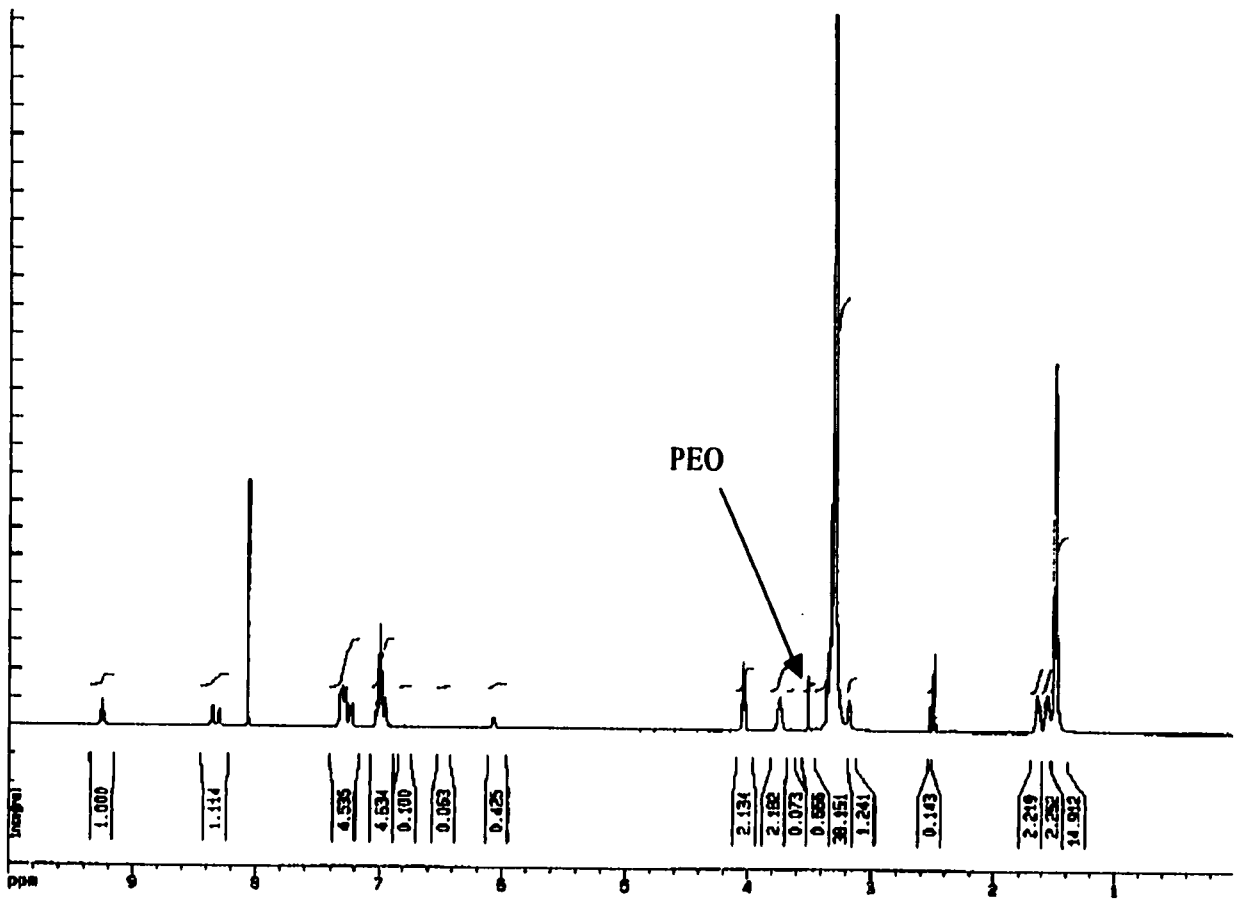

Figure 3.5 ^{1}H-NMR of dendrimer modified polyurethane with ED as chain extender and PEO attached (PU-ED-PEO^{2a})

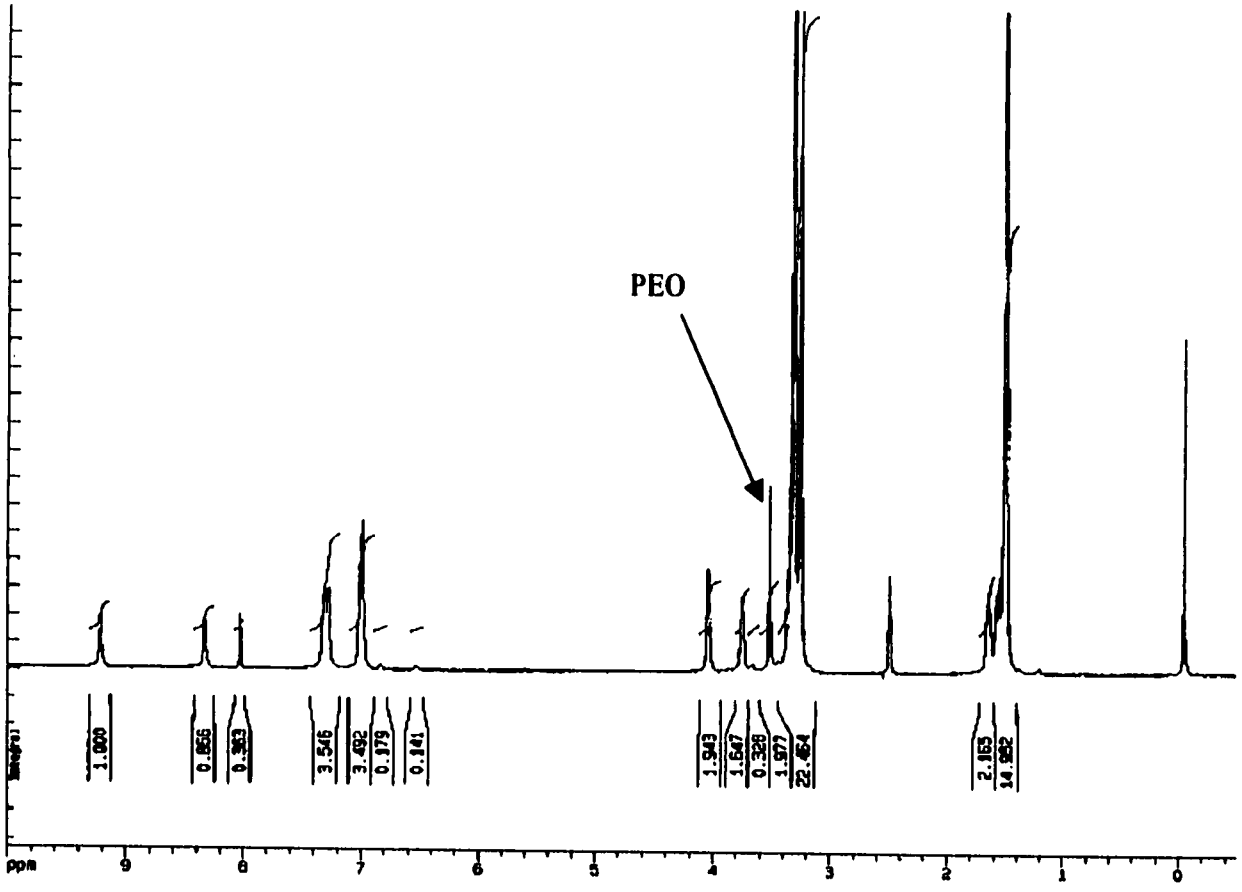

Figure 3.6 ^{1}H-NMR of dendrimer modified polyurethane with BDO as chain extender and PEO attached (PU-BDO-PEO^{2b})

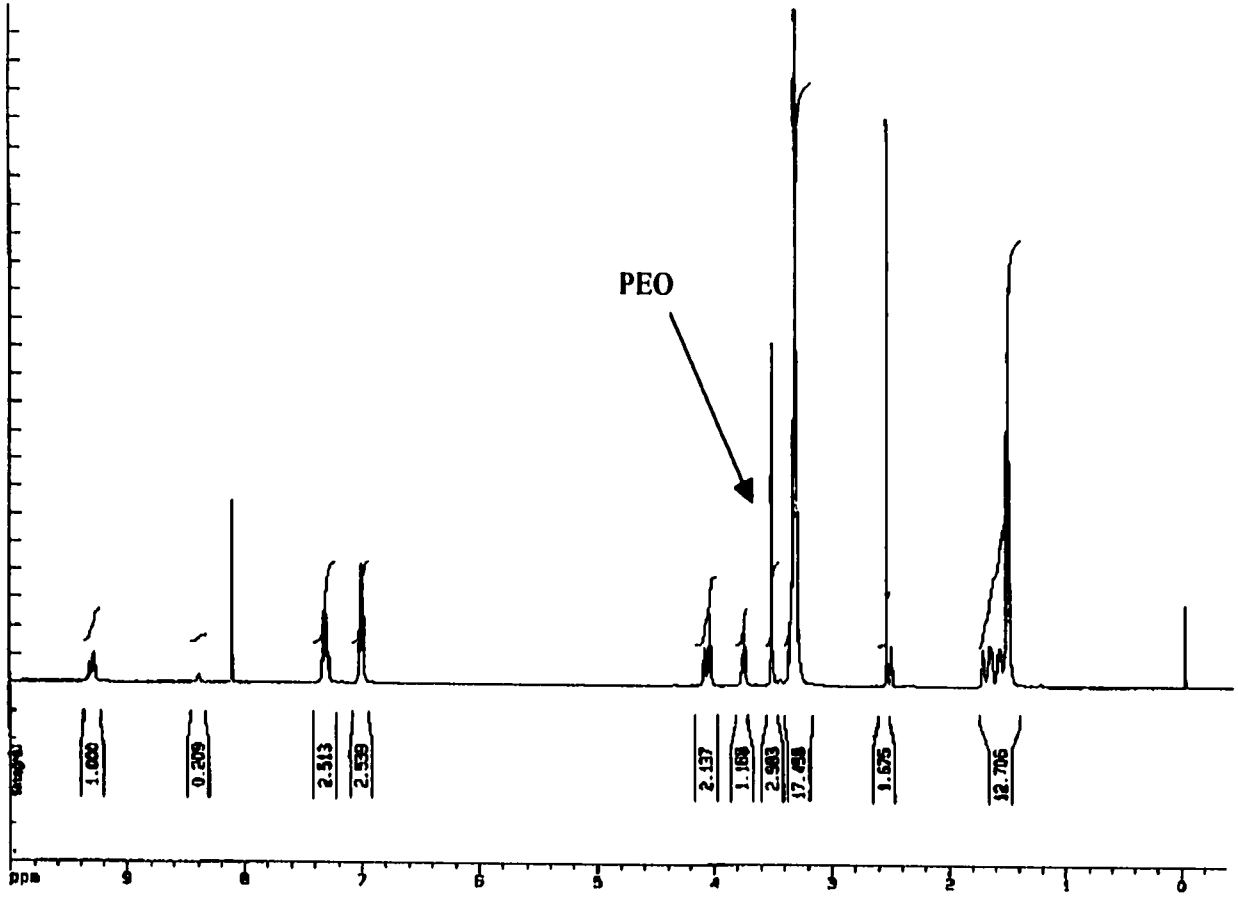

Figure 3.7 ^{1}H-NMR of dendrimer modified polyurethane with ED as chain extender and PEO attached (PU-ED-PEO^{1}) by derived approach 1

Table 3.5 PEO amount in polyurethanes as determined by ^{1}H-NMR

Polymer	PEO amount (g / g of dendrimer G_2)	Theoretical PEO amount (g / g of dendrimer G_2 if all 6 arms were coupled with PEO)
PU-ED-PEO[2a]	1.57	15.52
PU-ED-PEO[1]	12.36	15.52
PU-BDO-PEO[2b]	10.02	15.52

3.2.3 FTIR analysis

A typical FTIR spectrum of polyurethane control chain extended with ED is shown in Figure 3.8.

Comparatively, the polymers with the incorporated dendrimer, shown in Figure 3.9 show increases in peaks at 3315, 1708 and 1647 cm^{-1}, which indicate increases in hydrogen bonding of N-H and C=O of urethane and urea probably due to the incorporation of dendrimers.

The FTIR spectrum of a polyurethane control chain extended with BDO (PU-BDO) is shown in Figure 3.10. There was a decrease in the peaks at 3315, 1708 and 1647cm^{-1} relative to the PU-ED in Figure 3.8 indicating lower hydrogen bonding because of the presence of -OH in BDO instead of $-NH_2$ in ED. Dendrimer incorporated polyurethane with BDO (PU-G_2-BDO[2b]) is shown in Figure 3.11.

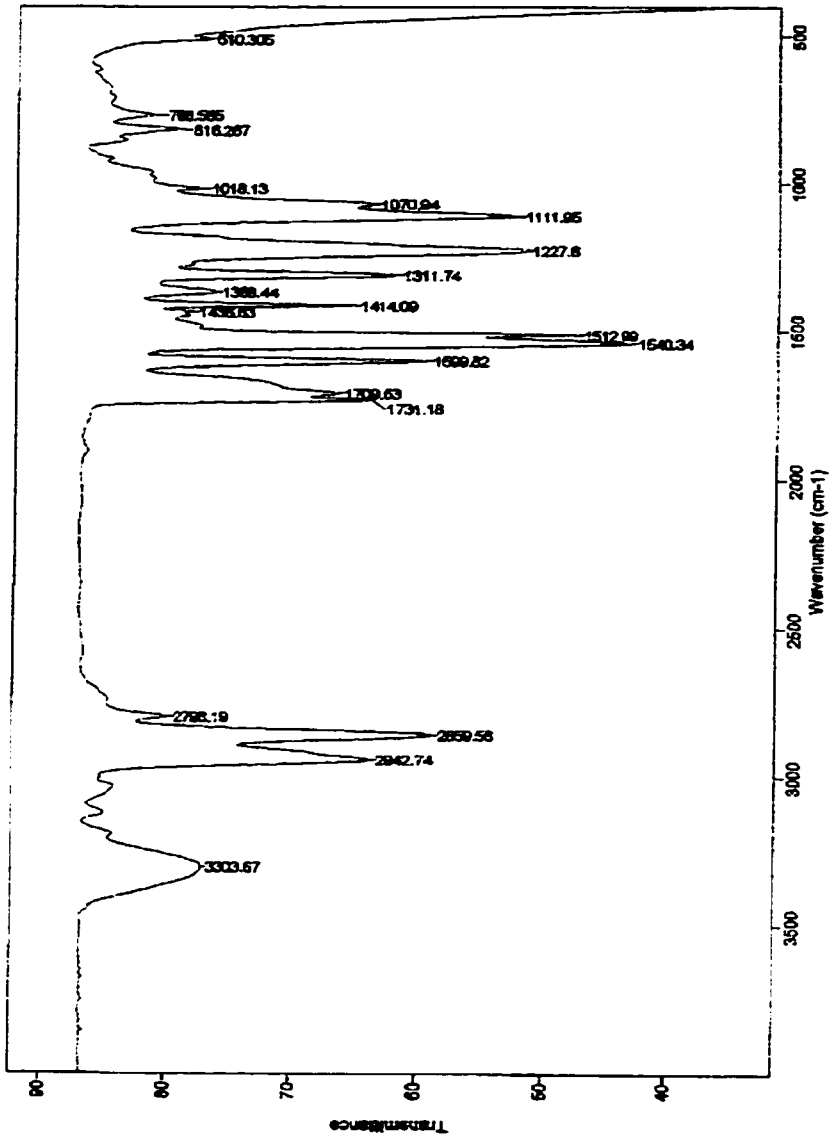

Figure 3.8 FTIR of ED chain extended polyurethane control (PU-ED)

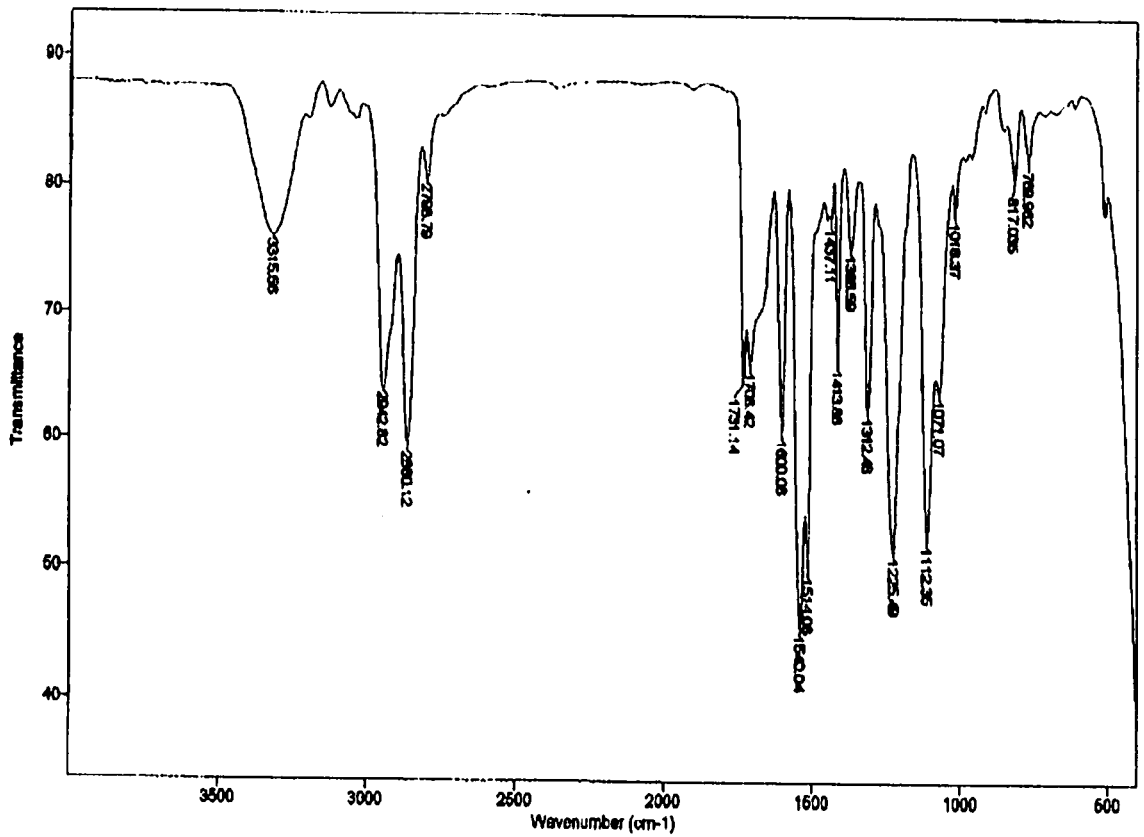

Figure 3.9 FTIR of dendrimer modified polyurethane with ED as chain extender (PU-G_2-ED^{2a}). Increases in peaks at 3315, 1708 and 1647 cm^{-1} indicate increases in hydrogen bonding of N-H and C=O of urethane and urea probably due to the incorporation of dendrimers.

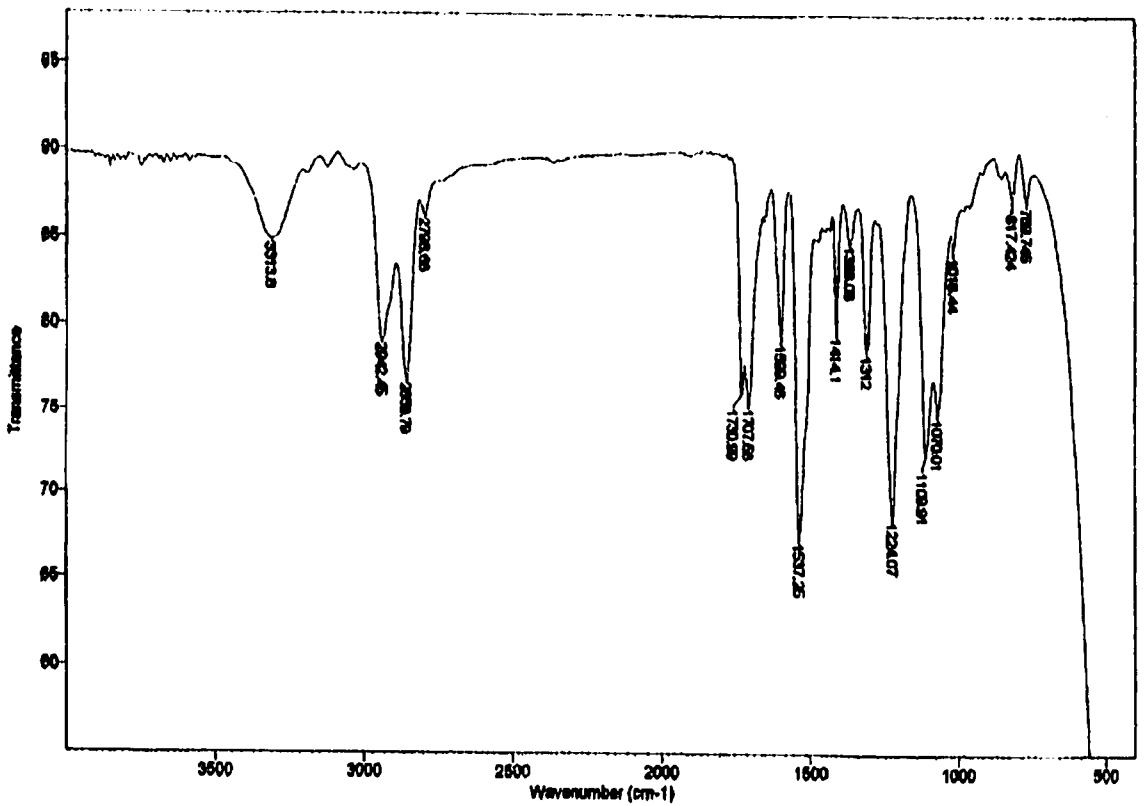

Figure 3.10 FTIR of BDO chain extended polyurethane control (PU-BDO). A decrease in the peaks at 3315, 1708 and 1647cm^{-1} indicate lower hydrogen bonding because of presence of -OH in BDO instead of -NH in ED.

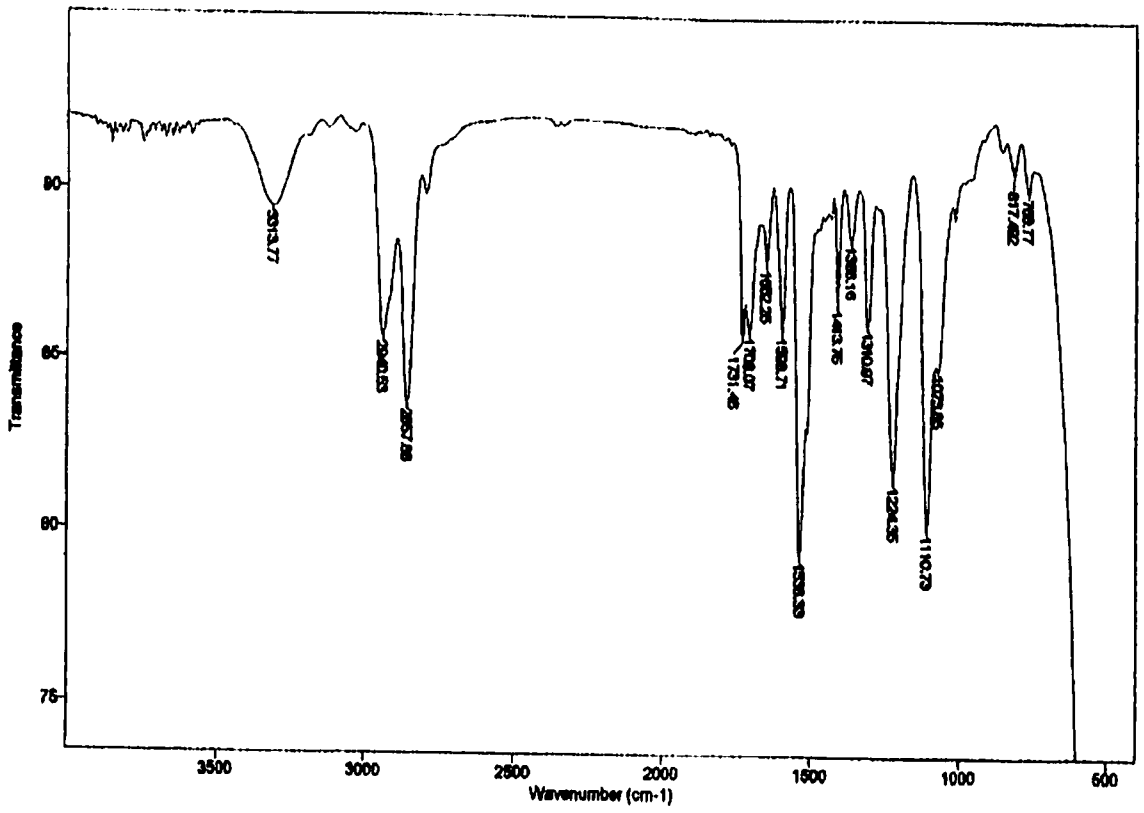

Figure 3.11 FTIR of dendrimer modified polyurethane with BDO as chain extender (PU-G_2-BDO^{2b}). An increase in the peak at 1647cm^{-1} indicate increased hydrogen bonding likely due to the presence of the dendrimer in the polymer structure.

After chain extension with the BDO dendrimer combination (PU-G_2-BDO^{2b}), there was an increase in the peak at 1647cm^{-1} indicating increased hydrogen bonding likely due to the presence of the dendrimer in the polymer structure.

With PEO attached, FTIR of the dendrimer modified polyurethane with ED as chain extender (PU-ED-PEO^{1}), shown in Figure 3.12, shows decreased peaks at 3315, 1708 and 1647cm^{-1}. This indicates lower hydrogen bonding, likely the result of the presence of fewer amine groups as a result of the attached PEO. However, the FTIR spectrum of the dendrimer incorporated polyurethane with BDO as chain extender showed little difference before and after PEO attachment, likely the result of the lower levels of hydrogen bonding that would be expected in these polymers (the spectrum of the PU-BDO-PEO^{2b} is not shown because of its similarity to Figure 3.11).

Taken together, the NMR and FTIR results provide strong evidence that the dendrimers and the PEO have been incorporated into the polymer structure and that differences in the attachment methods resulted in differences in the amount of PEO incorporated.

3.3 Surface Characterization of Polyurethanes

NMR and FTIR provide information about the bulk characteristics of the polymers. However in biological applications, the interface with the biological solution is of significant importance. In order to characterize the surfaces of the various polymers, two techniques were used: water contact angles and XPS.

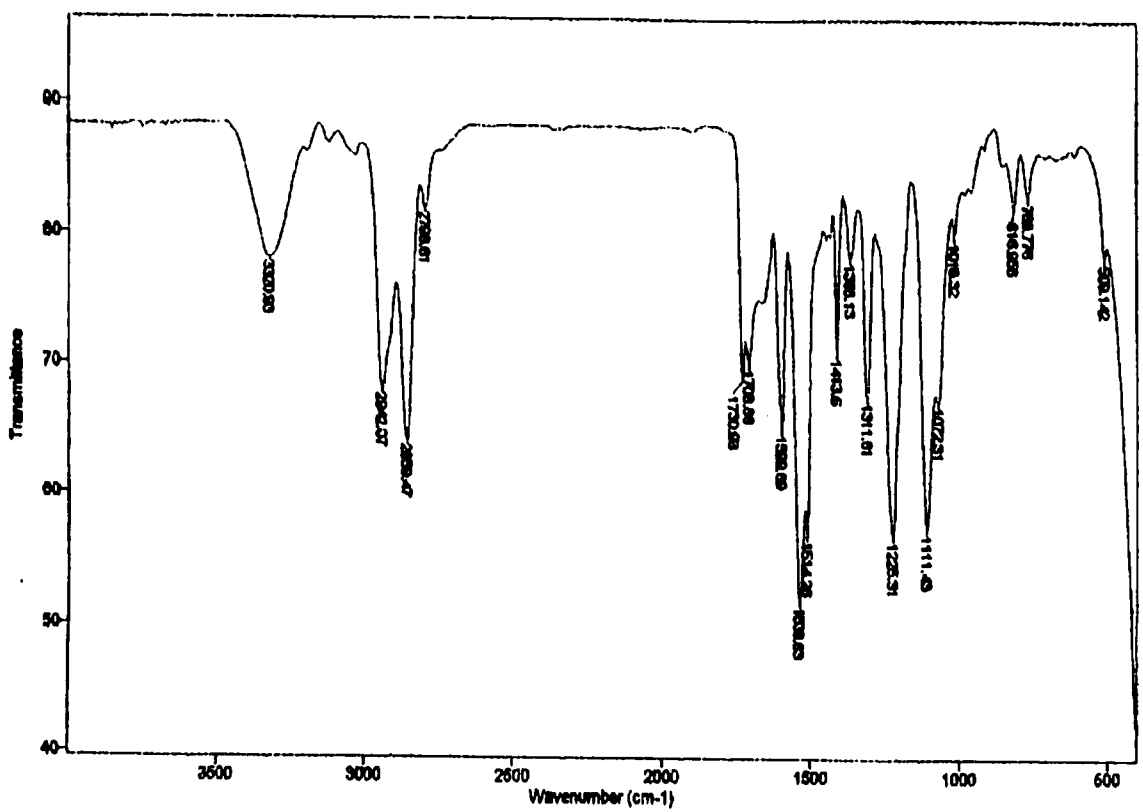

Figure 3.12 FTIR of dendrimer modified polyurethane with ED as chain extender and PEO attached

3.3.1 Water Contact Angles

Water contact angles were used to obtain a measure of the hydrophilicity of the polymer surface with and without dendrimer and PEO modification. The results are summarized in Figure 3.13.

The water contact angles measured on the two control polymers (i.e. PU-ED and PU-BDO) were similar at approximately 70°. Following incorporation of the dendrimer (PU-G_2-ED[2a] and PU-G_2-BDO[2b]), there was a small decrease in the measured water contact angles. This was not significant in the case of the ED polymer (PU-G_2-ED[2a] compared to PU-ED) as might be expected from the similarity of the chemical structures of the ED and the dendrimer. However the decrease was significant in the case of the BDO chain extended polyurethane (PU-G_2-BDO[2b] compared to PU-BDO). Following incorporation of the PEO into the polymer structure (i.e. PU-ED-PEO[2a] and PU-BDO-PEO[2b]), the contact angles decreased to approximately 45°. This decrease was significant ($p > 0.99$) for both the BDO and ED chain extended polyurethanes. However, these water contact angles are higher than those noted in other studies on PEO modified surfaces which average between 15 and 30° depending on the underlying substrate (Santerre et al., 1992). However, the results agree with other researchers' contact angle data after PEO attachment (Park, et. al. 1999).

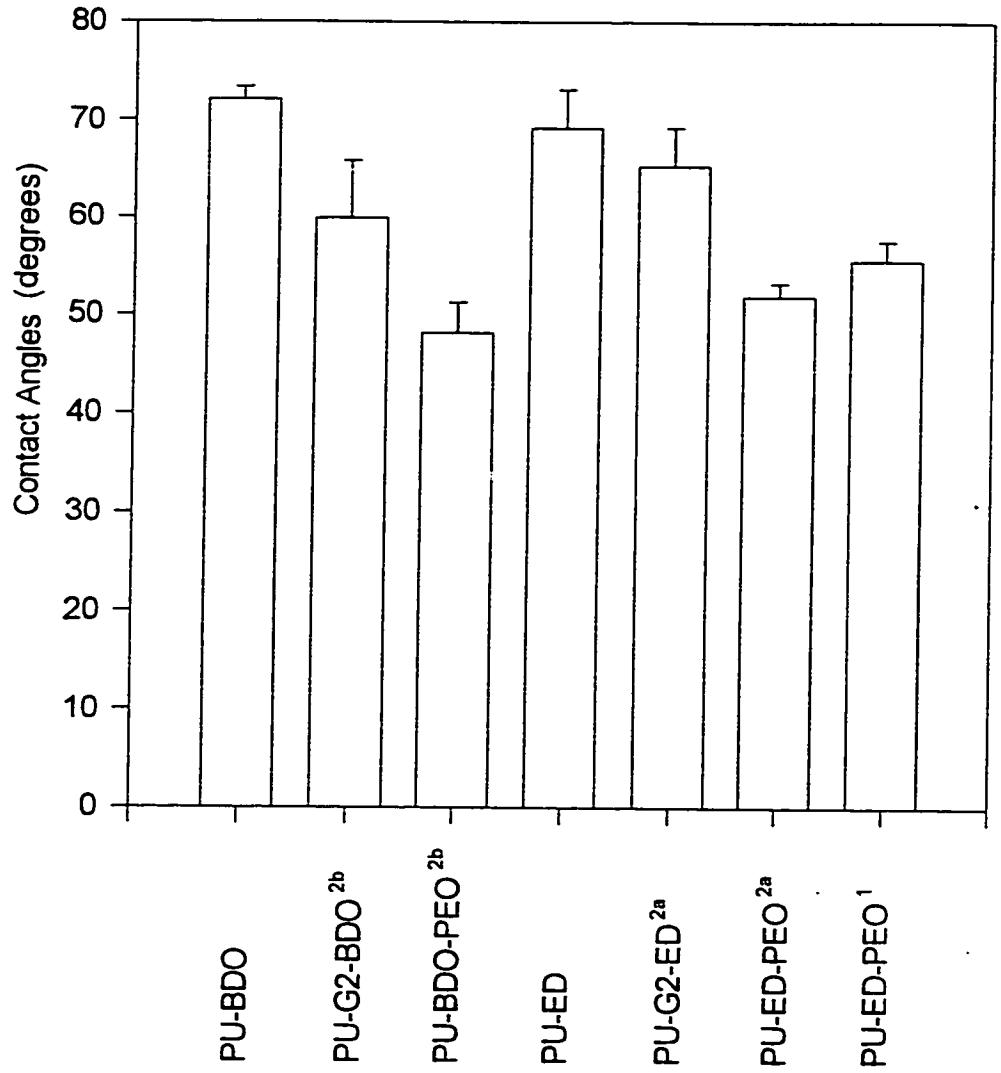

Figure 3.13 Water contact angles measured on the polyurethanes. Significant decreases in the water contact angles were noted on the surfaces following PEO incorporation, suggesting some surface enrichment of PEO.

3.3.2 X-Ray Photoelectron Spectroscopy (XPS)

XPS was used to determine the chemical compositions of the polyurethane surfaces. Low-resolution spectra and high-resolution spectra for carbon, nitrogen and oxygen were obtained for all surfaces. The results are summarized in Table 3.6.

Table 3.6 Summary of high-resolution XPS results for control polyurethane surfaces, dendrimer modified surfaces and PEO attached surfaces

Sample	C1s					O1s	N1s
	Total	284.8 C-C	286.3 C-N	289.2 C-OR	289.6 C(OOR)		
PU-ED	76.3	50.6	24.6	0.4	0.68	21.4	2.28
PU-G2-ED[2a]	76.3	50.5	24.5	0.62	0.72	21.1	2.65
PU-ED-PEO[2a] (TFA)	78.0	51.8	22.1	4.02		18.2	3.86
PU-ED-PEO[2a] (FA)	75.7	46.1	26.8	2.02	0.73	20.3	4.04
PU-BDO	77.2	57.9	17.6	1.65		21.3	1.50
PU-G2-BDO[2b]	77.8	56.2	20.1	0.90	0.66	19.4	2.83
PU-BDO-PEO[2b]	76.8	47.7	28.1	0.48	0.98	19.7	3.44

XPS results provide evidence both for dendrimer and PEO incorporation into the polymer structure and for surface enrichment of these groups at the polymer surfaces. Specifically, there was an increase in the nitrogen atomic concentration on the dendrimer and PEO incorporated polymers relative to the controls (compare PU-G_2-ED[2a] and PU-ED-PEO[2a] to PU-ED, and compare PU-G^2-BDO[2b] and PU-BDO-PEO[2b] to PU-BDO). High resolution carbon scans provided further evidence of PEO and dendrimer incorporation and surface enrichment. In the ED chain extended polymers, there was a

significant increase in the atomic concentration of carbon at 289.2 eV, representative of carbon atoms in ether structure (PEO or PTMO). However, this was not seen on the BDO chain extended polymers (PU-BDO-PEO[2b]), possibly the result of the migration of the PEO chains away from the surface due to the hydrophobic XPS environment. However, there was a significant increase in the contribution of C-N at 286.3 on these polymers, likely from the presence of the dendrimers. There are no trends in the oxygen signal, signifying incorporation or surface enrichment of PEO, again likely the result of the hydrophobicity of the XPS environment.

It should also be noted that there was a significant amount of silicon contamination, likely the result of the use of a silicon oil bath during polymer synthesis. This signal was eliminated from Table 3.6 in order to better evaluate the contributions from carbon, nitrogen and oxygen. Trace amounts of tin were found in the samples chain extended with BDO, likely the result of contamination from the catalyst, DBTDL, used during the chain extension step.

3.4 Biological Characterization of Polyurethanes

3.4.1 Fibrinogen Adsorption

Radio labeled (^{125}I) fibrinogen adsorption experiments were used to characterize protein interactions with the various polymers. Dialysis was used to remove any free iodide following protein labeling as excessive amounts of free ^{125}I binding to the surfaces

could result in erroneous protein adsorption results. In this study, the free iodide in the fibrinogen solution was determined by trichloroacetic acid precipitation of the protein to be 0.47% of the total radioactivity of the fibrinogen solution. Therefore, free iodide is not expected to make a significant contribution to the radioactivity noted on the various surfaces.

The adsorbed fibrinogen on the polyurethane surfaces was determined using a gamma counter and the results are summarized in Figures 3.14 and 3.15.

From Figures 3.14 and 3.15, it can be seen that the amount of fibrinogen adsorbed on the polyurethane surfaces decreased after dendrimer addition (compare PU-G_2-ED[2a] to PU-ED, and compare PU-G_2-BDO[2b] to PU-BDO) and even more after PEO incorporation (i.e. PU-ED-PEO[2a], PU-BDO-PEO[2b] and PU-ED-PEO[1]). The effect of dendrimer and PEO incorporation on fibrinogen adsorption seemed more apparent when BDO was used as chain extender. This correlates well with the water contact angle measurements and the chemical characterizations. On the ED chain extended polyurethanes, there was no significant difference between the amounts of fibrinogen adsorbed when two different acids (FA and TFA) were used for deprotection purposes. Furthermore, there was no significant difference before and after the PEO was attached to the polyurethane when t-Boc was used as the protecting group (PU-ED-PEO[2a]), consistent with the GPC and NMR results that showed a lower level of PEO attachment.

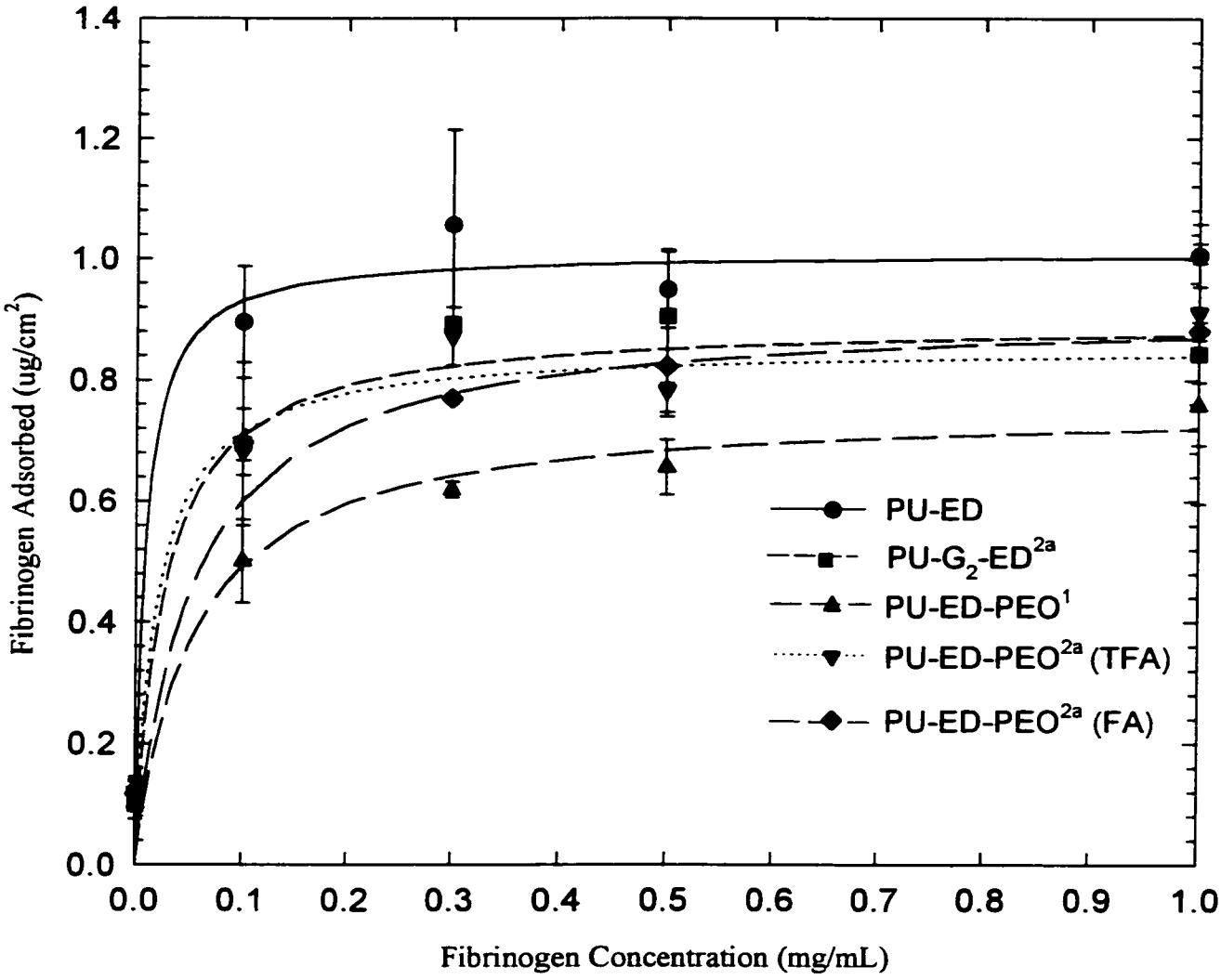

Figure 3.14 Comparison of fibrinogen adsorption on ED series polyurethane surfaces. After dendrimer incorporation, fibrinogen adsorption decreased, further decrease was observed after PEO was attached.

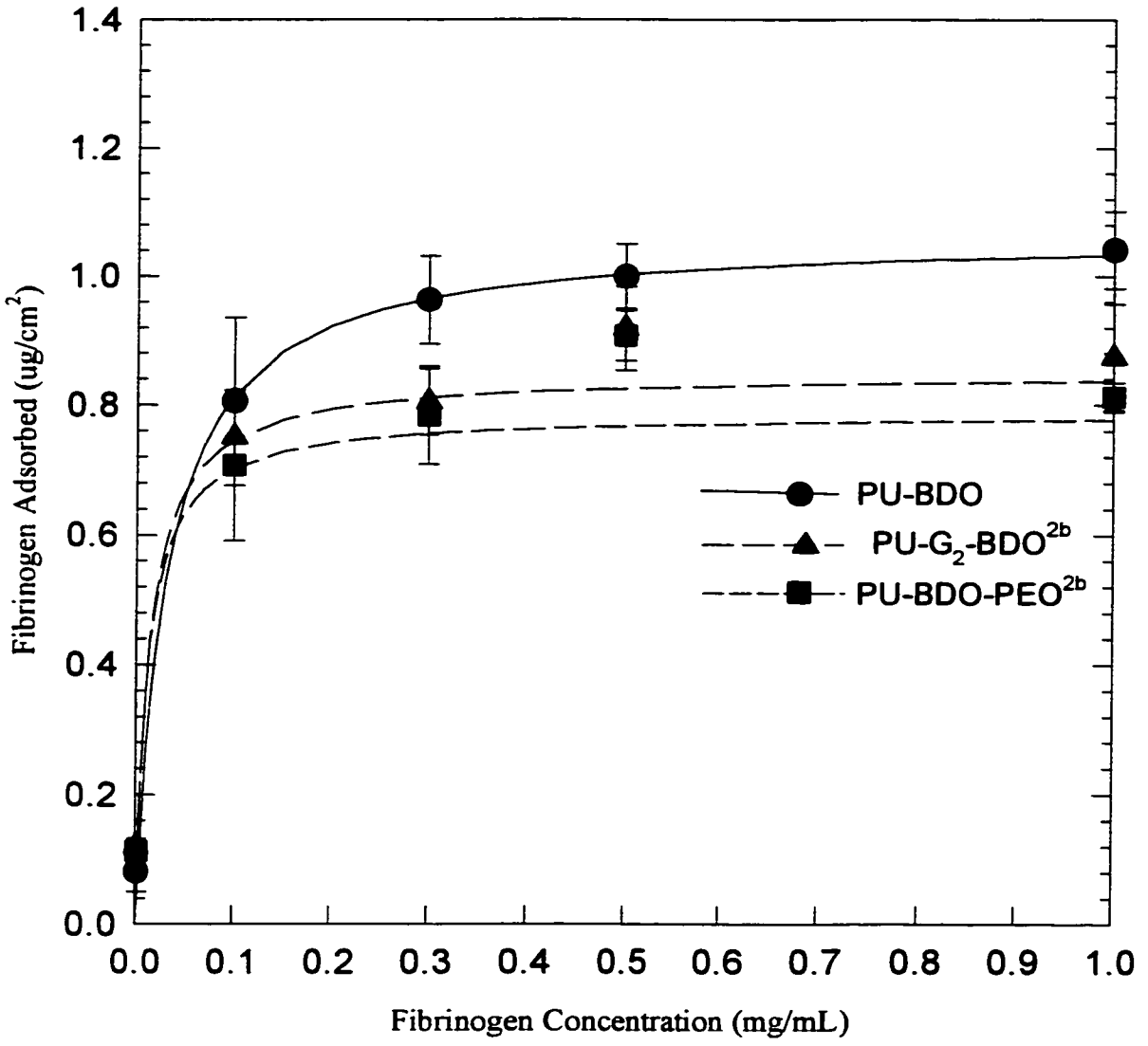

Figure 3.15 Comparison of fibrinogen adsorption on BDO series polyurethane surfaces. Fibrinogen adsorption decreased after dendrimer incorporation and further decreased after PEO was attached.

Interestingly, the polyurethane synthesized from derived approach 1 (PU-ED-PEO[1]) showed the greatest decrease in fibrinogen adsorption relative to the control, with an almost 40% decrease in the amount of adsorbed fibrinogen, again consistent with the GPC and NMR results, which showed higher levels of PEO attachment on these polymers relative to the others examined. It is likely that this approach is more direct and can result in the incorporation of more PEO chains through reaction with the dendrimer (refer to Tables 3.5 and 4.1).

However, the problem of crosslinking was difficult to overcome in this approach because of the multifunctional nature of the dendrimers. A number of variables, including the reaction temperature and the speed of reactant addition affected the chain extension reaction. Nevertheless, a DMSO soluble product was obtained after careful selection of optimal conditions. This product possessed superior mechanical strength and extremely high molecular weight, possibly due to the presence of some crosslinking, in addition to an apparent increase in protein resistance. It should be noted that unlike the other polymers, this polymer was not transparent.

3.4.2 Protein Adsorption from Plasma

SDS PAGE and immunoblotting analyses of the eluates following protein adsorption from plasma were performed to determine the patterns of plasma protein adsorption on the various surfaces. Immunoblotting results are summarized in Table 3.7.

Table 3.7 Summary of immunoblotting results for the various polyurethane surfaces (x represents the intensity of the bands observed from the blots)

Sample / Protein	PU-ED	PU-G_2-ED[2a]	PU-ED-PEO[1]	PU-ED-PEO[2a](FA)	PU-ED-PEO[2a](IFA)	PU-BDO	PU-G_2-BDO[2b]	PU-BDO-PEO[2b]
Factor XI						x		
Factor XII	x		x					x
Prekallikrein	x							
HMWK	x	x	x	x		x	x	x
Fibrinogen	xx	x	x	x		x	x	x
Plasminogen			x					
ATIII								
C3	xx	xx	x	x	x	xx	xx	xx
Transferrin	xx	x	x	x	x	x	x	x
Fibronectin								
Albumin	xxx	xx	xx	xx	x	x	x	x
IgG	x	x	x	x	x	x	x	x
Alpha-2-Macroglobulin								
Vitronectin	xxx	xx	x	x	x	x	x	x
Prothrombin						x		
Beta-2-microglobulin								
Haemoglobin	x		x	x	x	x		x
Factor B	x	x	x	x	x	x		
Factor H	x	x	x	x	x	x	x	x
Factor I					x	x		x
Apolipoprotein	xx	x	x	x	xx	x	x	xx

It can be seen from Table 3.7 that, consistent with the fibrinogen adsorption results, there was a decrease in the adsorption of several of the proteins to the surfaces following incorporation of the dendrimers and following PEO attachment. Specifically, on both the ED and BDO chain extended polyurethanes, decreases in the amounts of adsorbed fibrinogen, C3, transferrin, albumin, vitronectin, apolipoprotein A1 as well as the complement proteins were noted on these surfaces. However, the banding patterns indicated that there were no changes in adsorption of some of the other proteins, possibly the result of the relatively low amounts of adsorption noted on the control polymers.

Immunblotting results for the various polyurethane surfaces before and after incorporation of dendrimers and PEO are shown in Figures 3.16 to 3.23.

In general, protein adsorption from plasma on polyurethane surfaces was found to decrease after dendrimer incorporation and/or PEO attachment. The decrease seemed to be more apparent in the ED series, possibly because polyurethane control with ED (i.e. PU-ED) had a higher initial amount of adsorbed proteins.

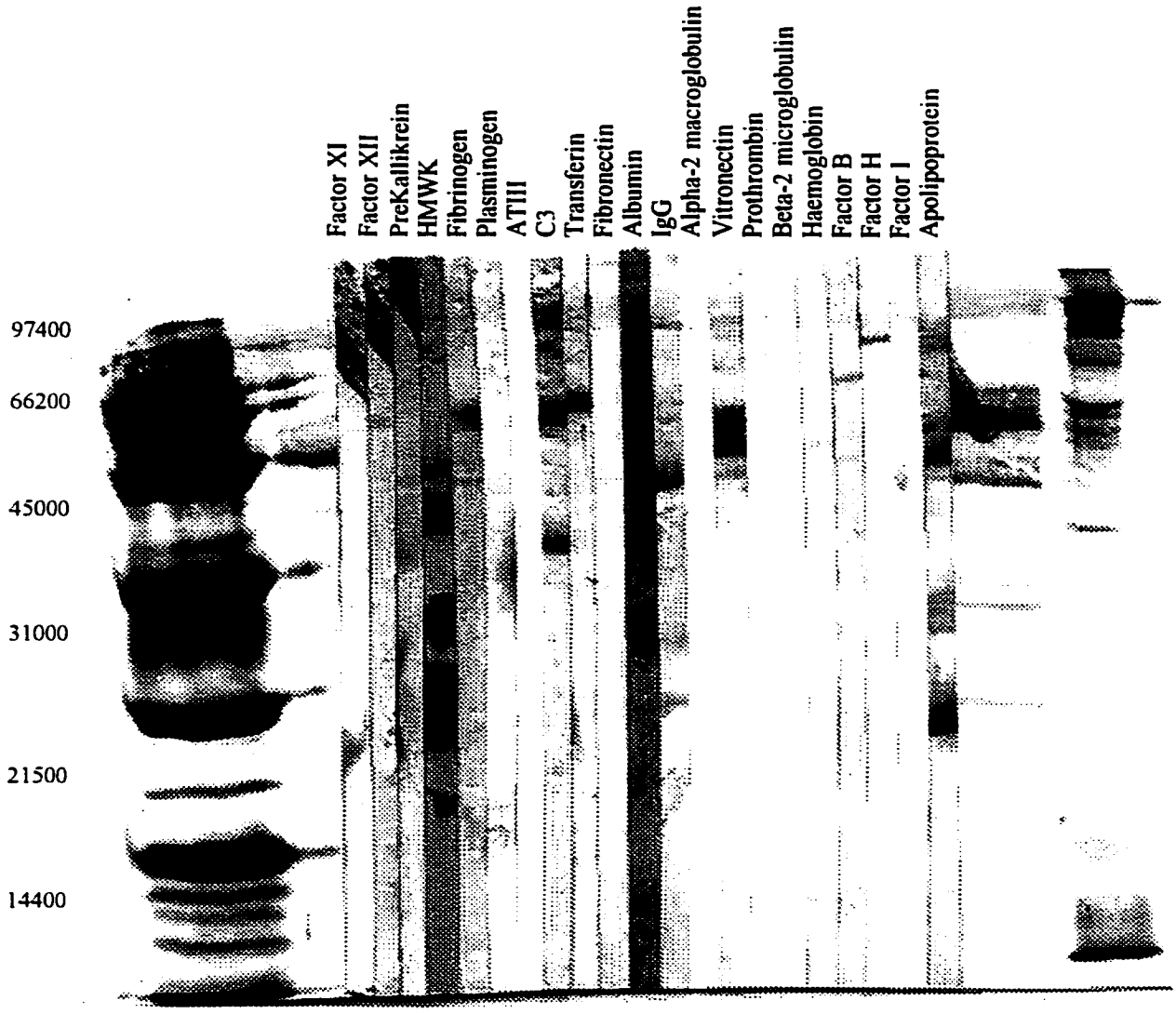

Figure 3.16 Immunoblotting results for plasma protein adsorption to the ED chain extended polyurethane control (PU-ED). The two outside lanes represent gold stained gels of plasma and the molecular weight markers.

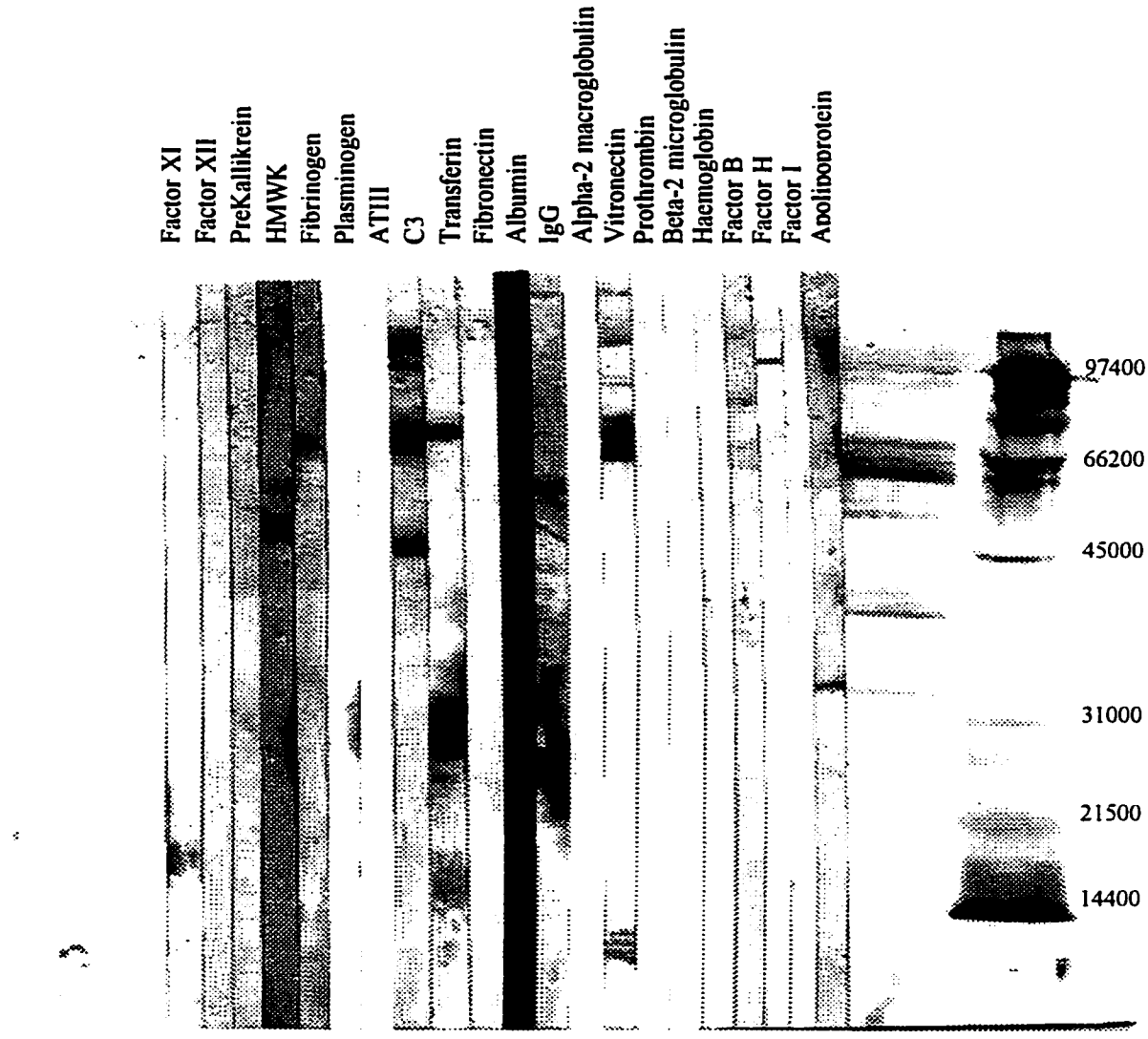

Figure 3.17 Immunoblotting results for plasma protein adsorption to the dendrimer modified polyurethane chain extended with ED (PU-G_2-ED^{2a}). The number and amounts of proteins adsorbed from plasma decreased after dendrimer incorporation.

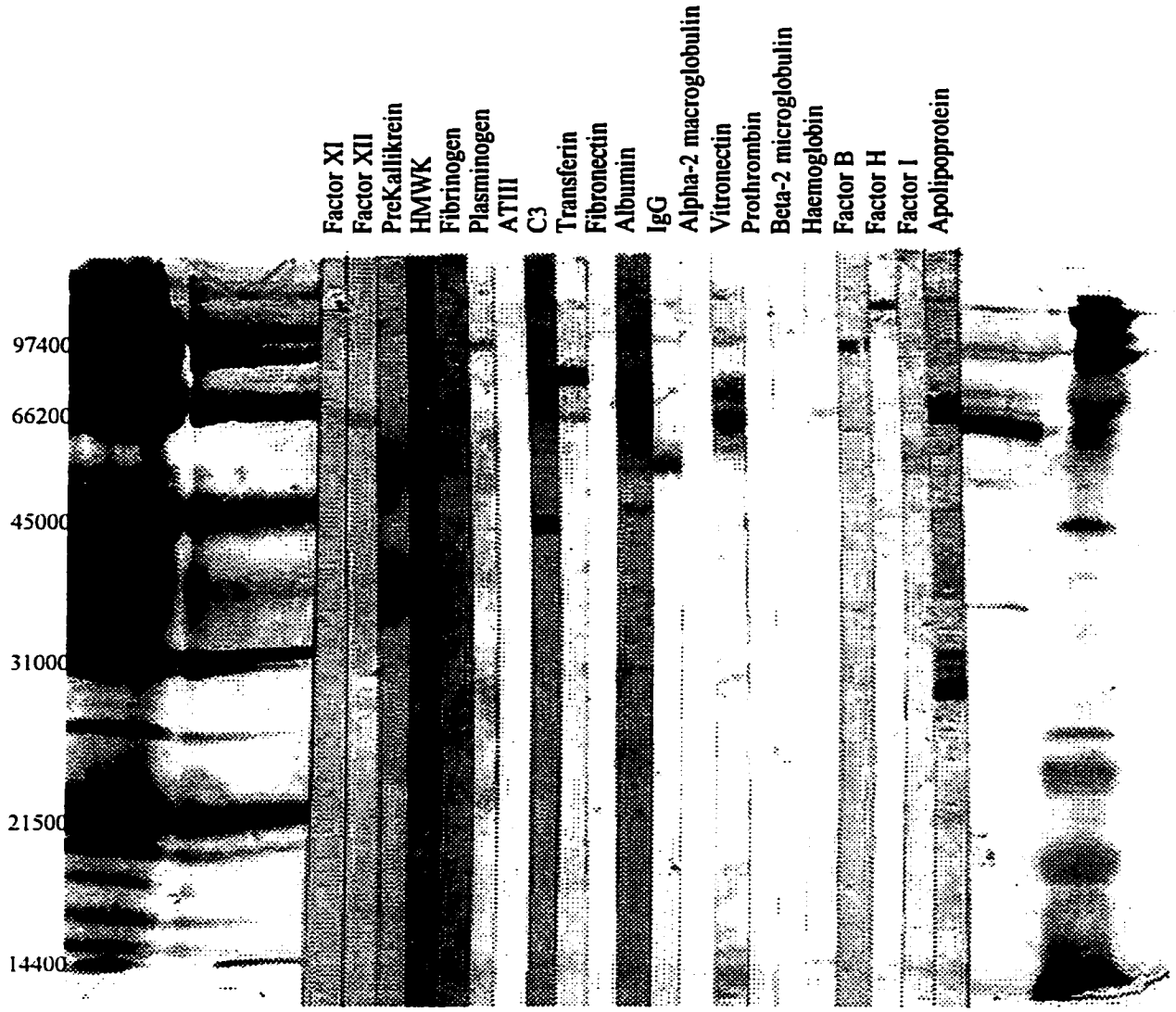

Figure 3.18 Immunoblotting results for plasma protein adsorption to the dendrimer modified polyurethane with PEO attached synthesized by derived approach 1 (PU-ED-PEO[1]). The amounts of some proteins adsorbed from plasma decreased further after PEO incorporation due to enrichment of PEO on surfaces.

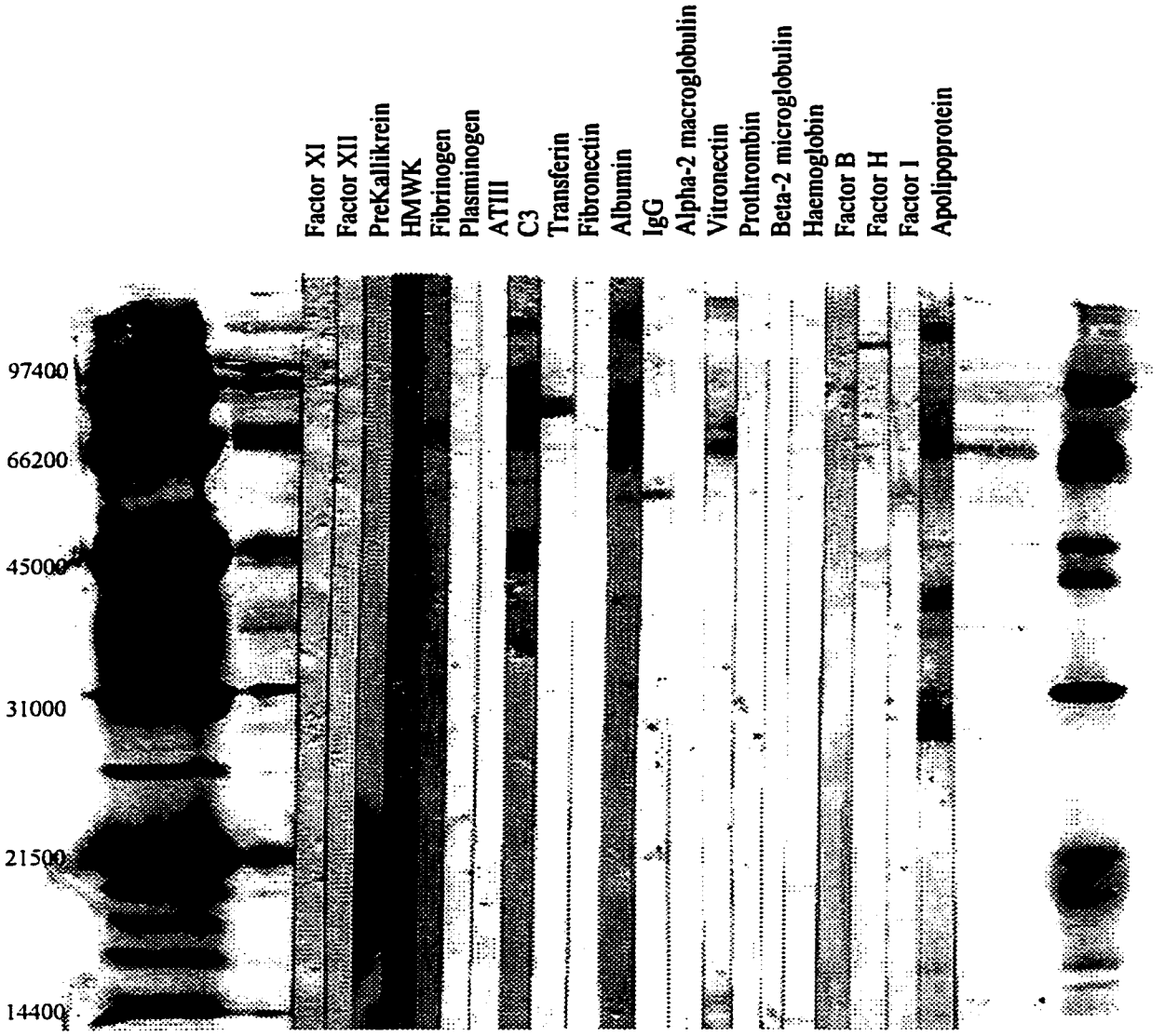

Figure 3.19 Immunoblotting results for plasma protein adsorption to the dendrimer modified polyurethane with PEO attached synthesized by approach 2 using formic acid for deprotection (PU-ED-PEO^{2a}). The amounts of some proteins (ex. C3 and Vitronectin) adsorbed from plasma decreased further after PEO incorporation due to enrichment of PEO on surfaces.

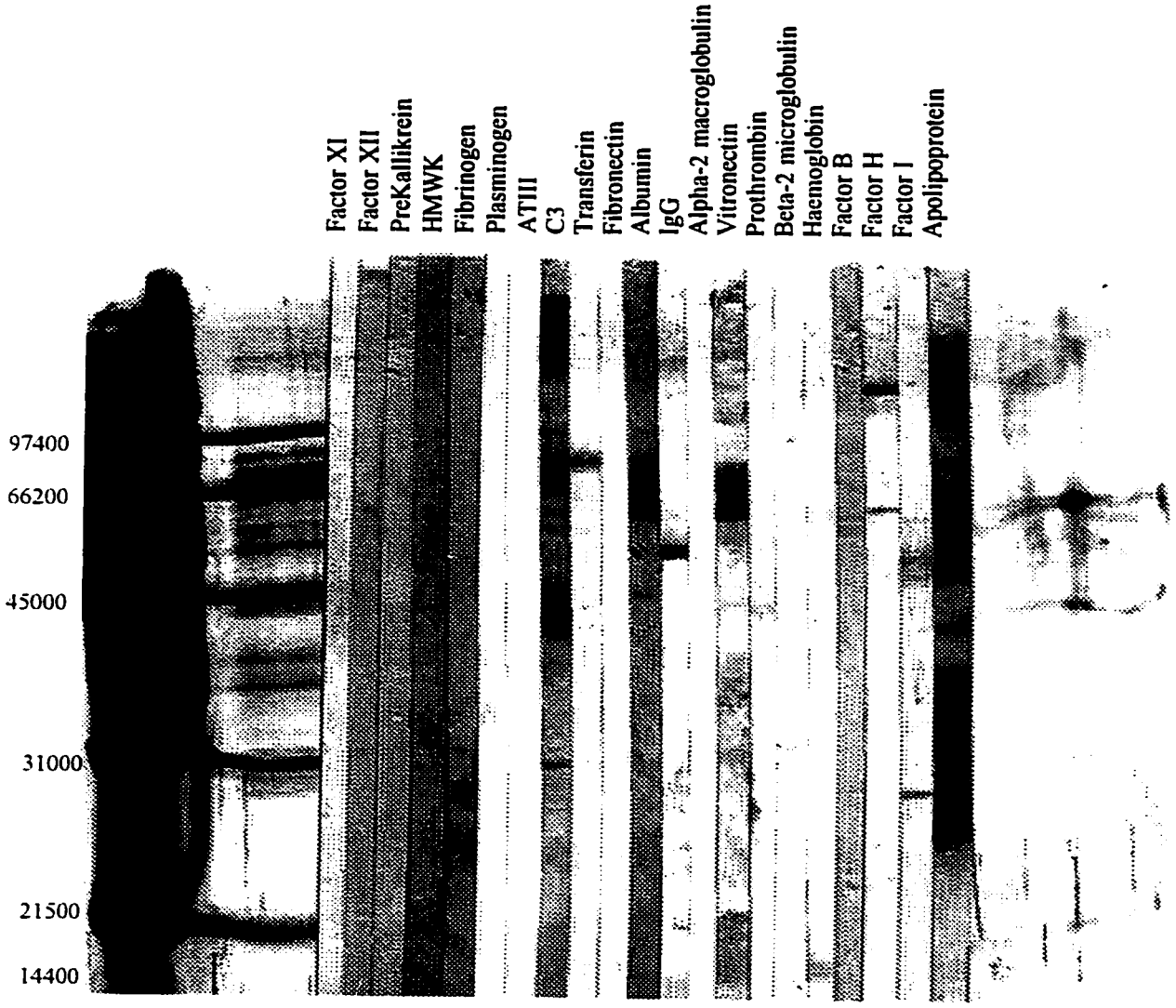

Figure 3.20 Immunoblotting results for plasma protein adsorption to the dendrimer modified polyurethane with PEO attached synthesized by approach 2 using trifluoroacetic acid for deprotection (PU-ED-PEO^{2a}). The amounts of some proteins (ex. C3 and Vitronectin) adsorbed from plasma decreased further after PEO incorporation due to enrichment of PEO on surfaces.

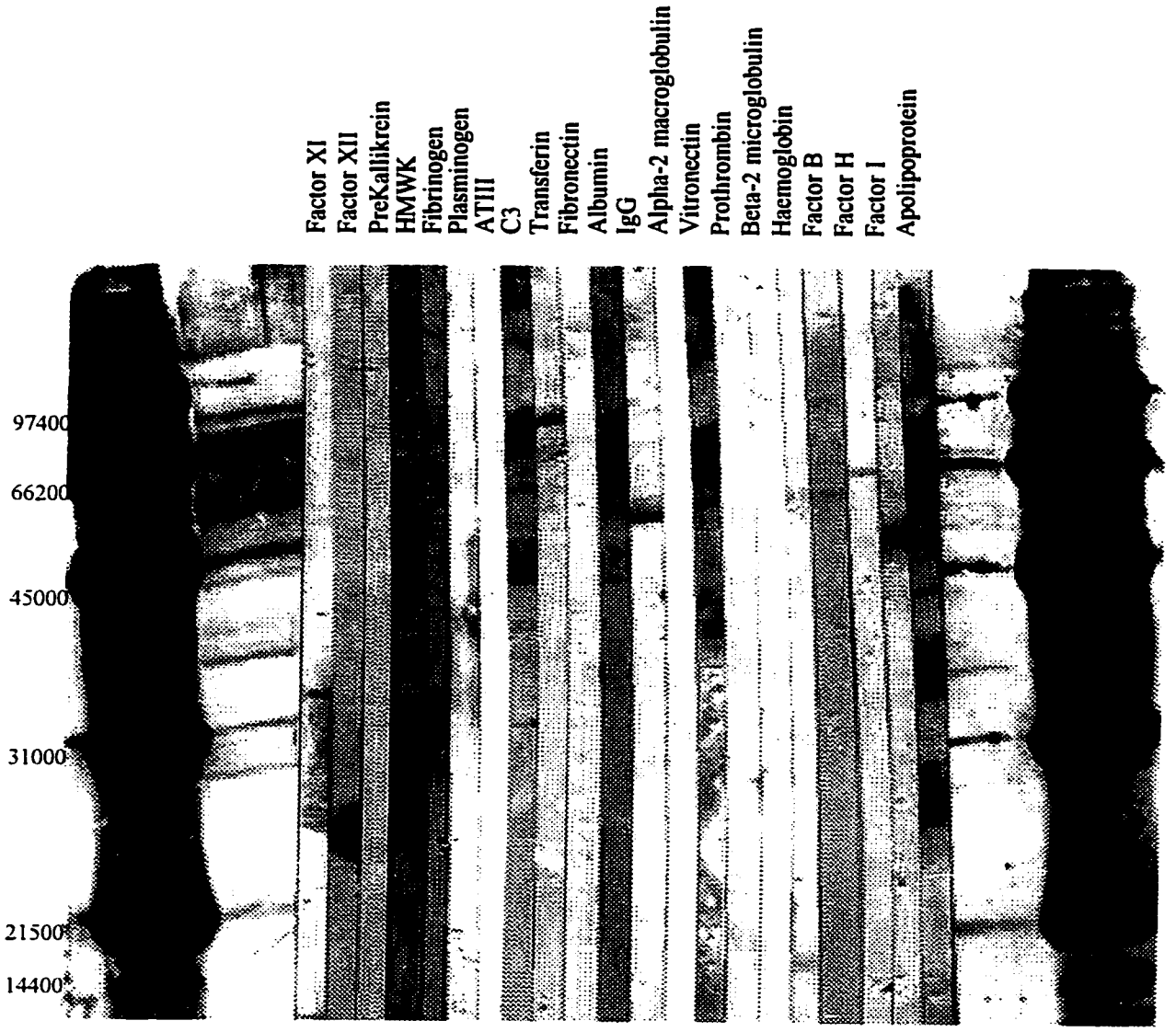

Figure 3.21 Immunoblotting results for plasma protein adsorption to BDO chain extended polyurethane control (PU-BDO). The two outside lanes represent gold stained gels of plasma and the molecular weight markers.

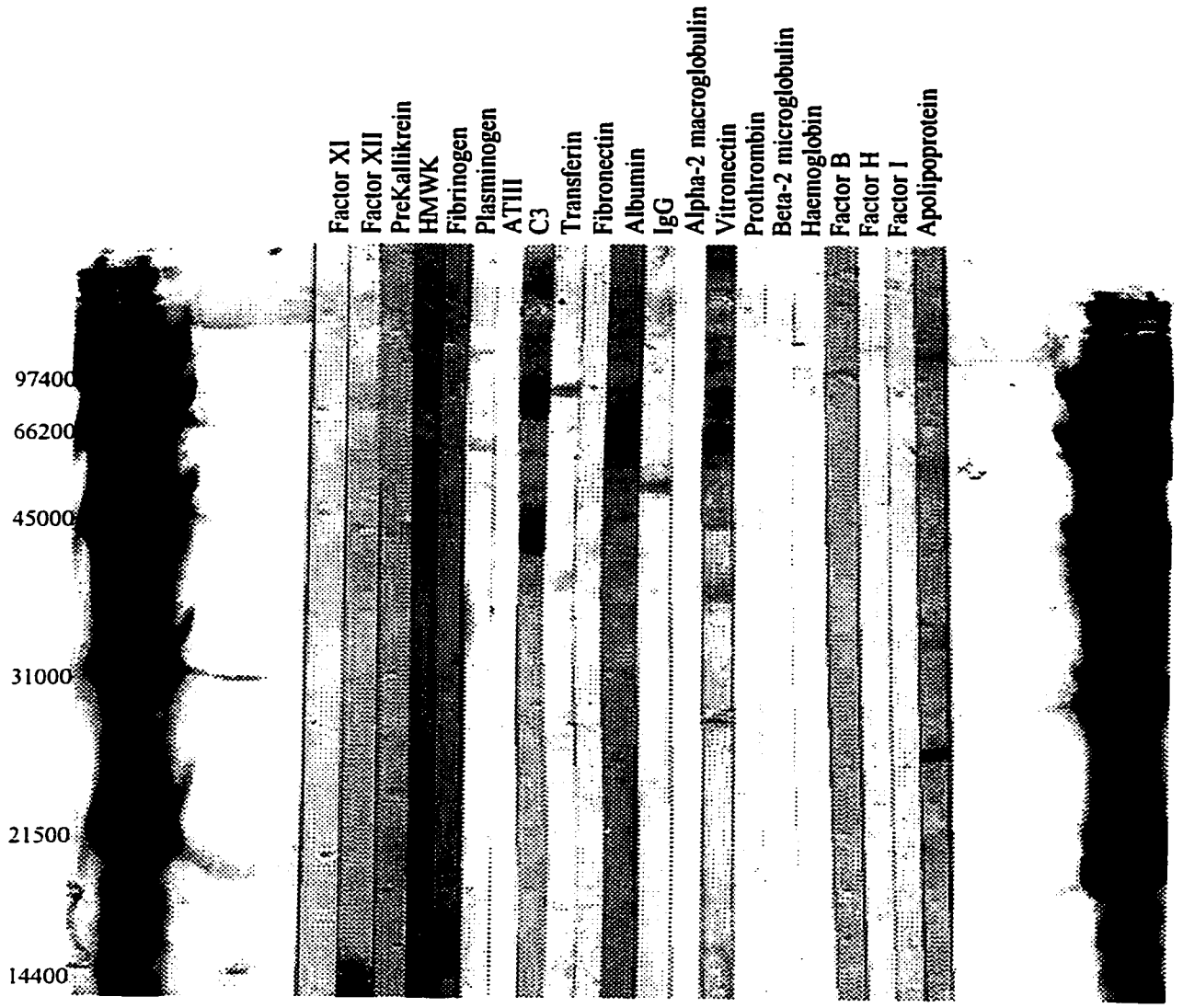

Figure 3.22 Immunoblotting results for plasma protein adsorption to the dendrimer modified polyurethane chain extended with BDO (PU-G_2-BDO^{2b}). No significant decrease of protein adsorption from plasma was observed after dendrimer incorporation, possibly due to low initial amount of proteins adsorbed onto polyurethane control with BDO.

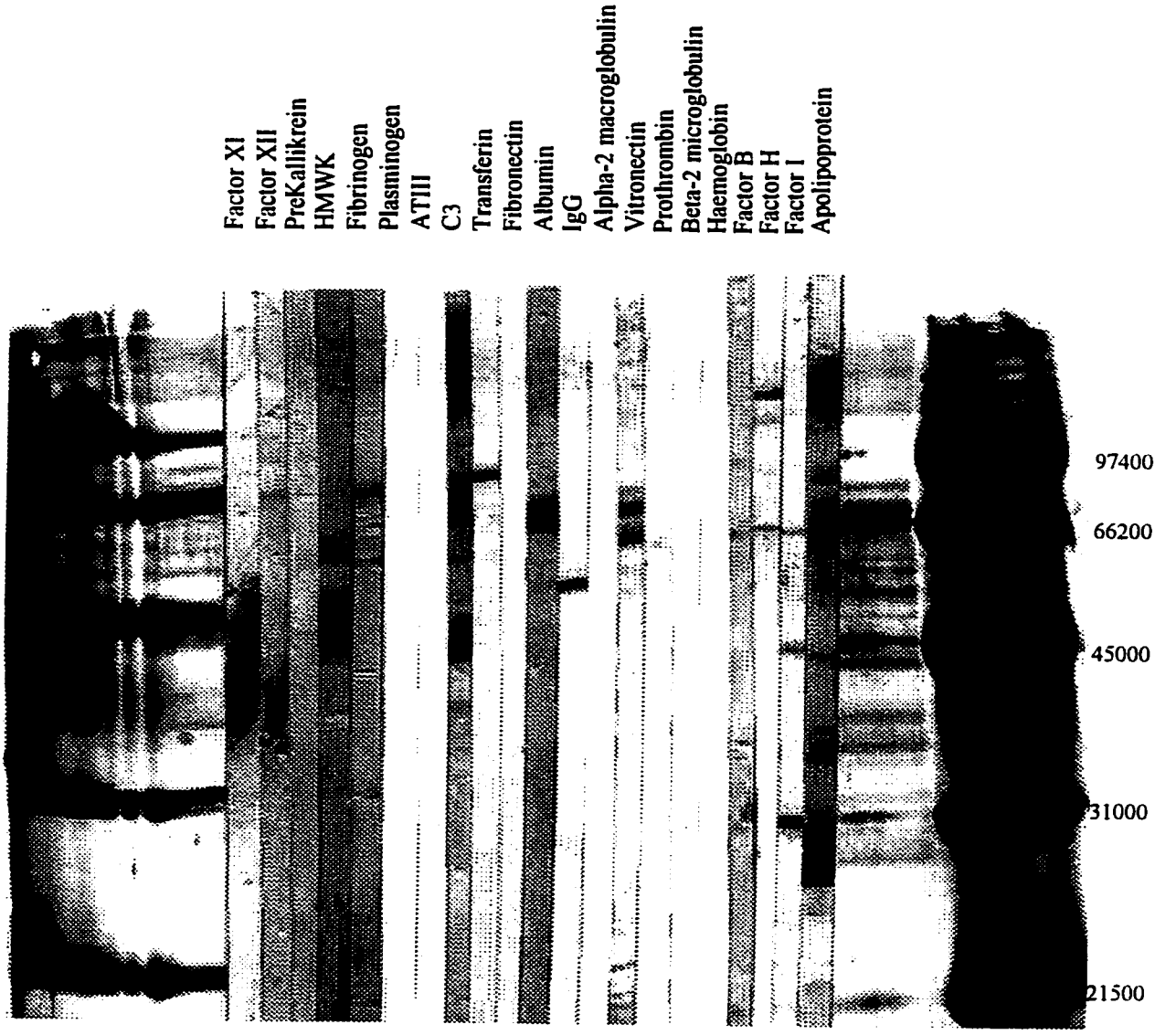

Figure 3.23 Immunoblotting results for plasma protein adsorption to the dendrimer modified polyurethane with PEO attached (PU-BDO-PEO^{2b}). No significant decrease of protein adsorption from plasma was observed after PEO incorporation, possibly due to low initial amount of proteins adsorbed onto polyurethane control with BDO.

Chapter 4 Discussion

4.1 Synthesis of polyurethanes

Two approaches were applied to synthesize dendrimer modified polyurethanes with PEO attached in an attempt to increase the level of functionalization of these polymers. In both approaches, dendrimers were incorporated into polyurethane backbones at the chain extension step using a conventional two step polymerization method to synthesize all polyurethane samples.

In approach 1, PEO was first incorporated into the G_2 dendrimer by reaction of SPA-PEG2000 with dendrimer as illustrated in Figure 4.1.

A molar ratio of 6:1 was selected, leaving two free amine groups in the dendrimer for further chain extension of the polymer. This ratio was selected to minimize the potential for crosslinking, although a lower PEO:dendrimer ratio may have resulted in a greater level of success in these polymers. An 8:1 ratio was used to obtain an estimate of the required reaction time by ninhydrin analysis of the reaction mixture. GPC analysis of the PEO modified dendrimers was performed and suggested the successful incorporation of PEO into dendrimers (see Table 3.1). It was found that all or part of the dendrimer arms could possibly be modified with PEO. Rather, it is expected that a reaction occurred

between the prepolymer and one of the reactive amine groups in the dendrimer, but that steric effects terminated the polymers at this point, leaving relatively low molecular weight products. The results of the ninhydrin analysis to determine the required reaction time, which showed a sharp drop in the rate after the first two hours of reaction, provided further evidence of these steric effects.

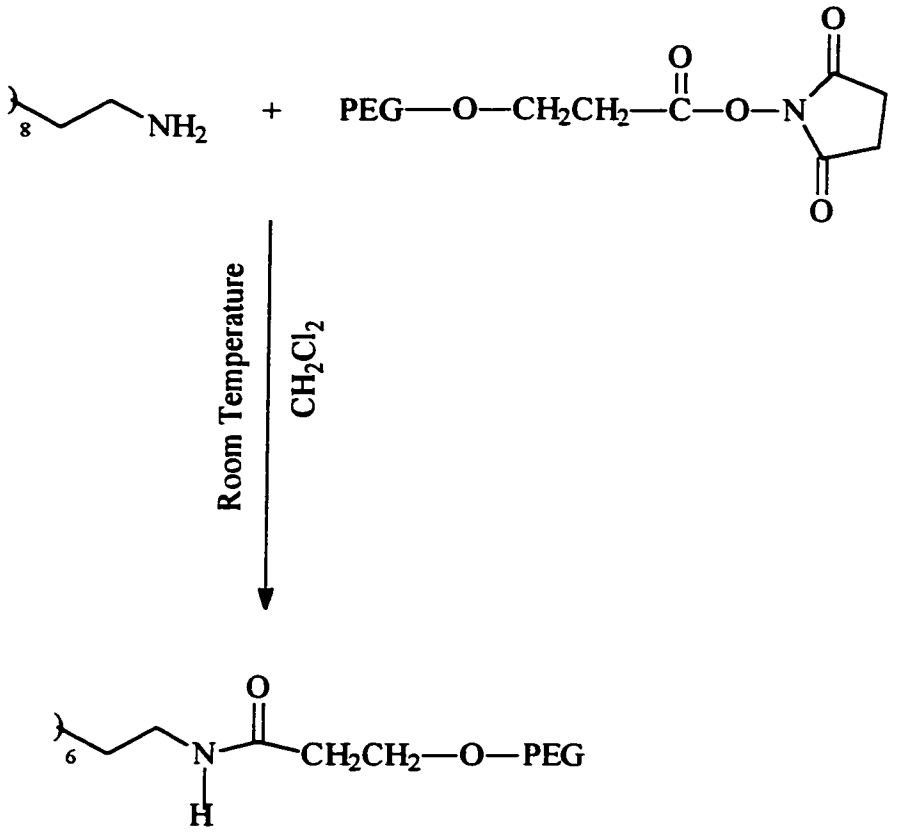

Figure 4.1 Reaction of dendrimer G_2 with SPA-PEG2000. SPA-PEG2000 reacts with dendrimer G_2 in a molar ratio of 6:1 leaving two free amine groups on dendrimer

An alternate approach (derived approach 1) was undertaken to overcome this steric hindrance problem. In this approach, the standard chain extender ED, SPA-

PEG2000 and dendrimer G_2 were added in a sequence chosen to minimize the crosslinking caused by the multifunctional dendrimers, but to allow PEO incorporation. ED was added first to the prepolymers in order to consume part of the reactive -NCO groups while chain extending the prepolymer chains to produce higher molecular weight polymers. SPA-PEG2000 was subsequently added to compete against the remaining -NCO groups of prepolymers in reacting with the G_2 dendrimer. The dendrimer was added very slowly, dropwise, with the assumption that large amounts of SPA-PEG would react with the dendrimer on a molecular scale in the solution so that the majority of the arms of the dendrimer would be coupled with SPA-PEG but that two or three would be available to react with -NCO groups of the prepolymers forming high molecular weight polyurethanes. However, crosslinking was unavoidable in this case because of the multifunctional nature of the dendrimers. It was found that reaction conditions including temperature, and the rate of reactant addition affected the chain extension reaction. Using an optimal condition as summarized in Table 3.2 (see section 3.1), soluble, slightly crosslinked dendrimer incorporated polyurethanes with PEO attached were produced (PU-ED-PEO[1]). The product was soluble in DMF, DMSO and had superior mechanical strength, possibly due to some degree of crosslinking, suggesting that these latter reactions were minimal. However, the reproducibility of this reaction procedure was poor.

Approach 2 was developed and used to produce a crosslink-free (or low crosslink density) polyurethane with incorporated dendrimer and PEO attached.

In this approach, the protecting groups t-Boc or Fmoc, were used to block a fraction of the reactive sites on the dendrimers (statistically 6 out of 8) leaving 2 free amine groups for the polyurethane chain extension.

Low molar ratios of dendrimer to standard chain extender were selected in these studies to examine the potential for synthesis. While it is expected that higher molar ratios will result in better biological properties, a further deterioration of mechanical properties should result and it will be necessary to form blends with, or coatings on, standard polyurethanes.

In order to introduce PEO chains into the dendrimer incorporated polyurethanes, deprotection was necessary using different reactions depending on the protecting group. When acid treatment was used to remove t-Boc groups from the dendrimers, an adverse effect of acid on polymer molecular weight was found, as determined by GPC (refer to Table 3.3). While the polymer molecular weight remained significant, making it unlikely for this to be the major contributing factor to the formation of the emulsion in water after synthesis, there was significant polymer degradation, with a 16% decrease in the molecular weight in the case of ED chain extended polymers and 38% when the polymers were chain extended with BDO. Therefore, while it seems more likely that the emulsion formation resulted from changes in the solubility of the polymers by the incorporation of the PEO into the polymer structure and necessitated purification by solvent evaporation, the degradation of the polymers upon exposure to the acid is significant and should be further investigated. Polyurethane degradation, by enzymatic or other means has been well characterized (Santerre et al., 1993) and represents a significant limitation to their widespread use in certain biomedical applications.

4.2 Chemical characterization of polyurethanes

GPC, ^{1}H-NMR and FTIR were used in this study to confirm the structure of polyurethanes after dendrimer and PEO modification. Water contact angles and x-ray photoelectron spectroscopy were used to determine the chemistry of the surface of the polymers.

^{1}H-NMR and, to a lesser extent FTIR, provide clear molecular evidence that the dendrimers and PEO have been incorporated into the polyurethane structure. While the FTIR results were less conclusive than those obtained by NMR, this was likely a reflection of the low ratio of dendrimer to standard chain extender used in this work. GPC results also provide evidence of the successful synthesis of polyurethanes with dendrimer incorporated and PEO attached. Given the increase in the molecular weight and an average PEO molecular weight of 2000, a total of 10.22 g of PEO per gram of dendrimer were incorporated into the polymer backbone for PU-BDO-PEO[2b]. An estimate of the amount of PEO incorporated in the various PEO incorporated polyurethanes is shown in Table 4.1.

Table 4.1 Estimate of amount of PEO in polyurethanes determined by GPC

Polymer	PEO amount (g / g of dendrimer G_2)	Theoretical PEO amount (g / g of dendrimer G_2 if all 6 arms were coupled with PEO)
PU-ED-PEO[2a]	2.53	15.52
PU-ED-PEO[1]	16.44	15.52
PU-BDO-PEO[2b]	10.22	15.52

This table gives a semi-quantitative estimate of the amount of PEO incorporated into the polyurethane structure. The results indicate that the Fmoc protection/deprotection approach was much more effective than the t-Boc approach. The estimated amount of PEO attached to PU-ED-PEO[1] is greater than the theoretical value, likely due to differences in the actual and measured molecular weights as a result of polymer crosslinking. The PEO incorporated polyurethane synthesized by the simultaneous reaction of the dendrimer, PEO and chain extender with the prepolymer (denoted PU-ED-PEO[1]) had an extremely high molecular weight, consistent with the observation that the polymer was likely slightly crosslinked. When compared with other results including the amount of PEO attached as determined from the NMR measurements, it can be seen that the reliability of the estimate is reasonable as summarized in Table 4.2.

Table 4.2 Comparison of estimated PEO amount on polyurethanes resulted from GPC and ^{1}H-NMR

Polymer	PEO amount estimated from GPC (g / g of G_2 dendrimer)	PEO amount estimated from NMR (g / g of G_2 dendrimer)
PU-ED-PEO[2a]	2.53	1.57
PU-ED-PEO[1]	16.44	12.36
PU-BDO-PEO[2b]	10.22	10.02

Based on these two analyses, the results indicate that the greatest amounts of PEO are incorporated into the surface prepared by simultaneous incorporation of the chain extender, PEO and dendrimer. While the BDO chain extended polymers reacted with the protected dendrimers and subsequently modified with PEO are followed by ED chain

extended polymers under similar conditions. The literature suggests that the levels of protein adsorption to PEO modified surfaces are dependent on such factors as the density of PEO and the PEO molecular weight (Merrill, 1993; Silver et al., 1994). Therefore, the protein adsorption results correlate well with the NMR and GPC results, with lower levels of fibrinogen adsorption noted on the surfaces that showed higher amounts of attached PEO.

Comparing the two protection/deprotection schemes examined, it can be seen that protection with Fmoc resulted in polyurethanes with more attached PEO. Compared with the theoretical PEO amount if all 6 arms of dendrimers were coupled with PEO, only 1 out of 6 arms of dendrimers in PU-ED-PEO[2a], which used t-Boc for protection, reacted with PEO while 4 out of 6 arms of dendrimers in PU-BDO-PEO[2b], using Fmoc, had PEO chains. However, this is still less than the theoretical maximum, demonstrating the problems associated with reacting high molecular weight entities and obtaining significant reaction yields. Alternatively, it is possible that the deprotection reaction was incomplete, again due to steric factors. In comparison, the polymer synthesized by in situ addition of the various reactants in sequence, PU-ED-PEO[1], showed a greater than theoretical level of PEO addition by GPC and closer to theoretical addition by NMR. The greater than theoretical amount of incorporated PEO determined by GPC is likely the result of an overestimate in the amount of PEO due to crosslinking in this polymer. The PEO amount estimated from NMR results suggests that approximately 5 of 6 dendrimer arms were coupled to PEO.

Water contact angle measurements showed significant decreases from 70° to 60° after dendrimer incorporation in the case of the BDO chain extended polyurethane, with further decreases to about 45° after PEO attachment. These results show that the surfaces were becoming increasingly hydrophilic with the various modifications. These water contact angles are higher than those noted in other studies on PEO modified surfaces which average between 15 and 30° depending on the underlying substrate (Santerre et al., 1992). However, the results agree with other researchers' contact angle data after PEO attachment (Park, et. al. 1999). The reason for this discrepency is that it is expected that higher densities of PEO could be obtained with specific surface modifications. Furthermore, the amounts of dendrimer used in these studies were significantly lower than the theoretical maximum. It is also possible that the polymers are absorbing water, resulting in the decrease in the water contact angles (Santerre et al., 1992).

It is interesting to note that the water contact angle results show a strong correlation with the results obtained by GPC and NMR, which demonstrate that the BDO chain extended polyurethane using Fmoc as a protecting group had more attached PEO than the ED series using t-Boc. However, PU-ED-PEO[1] did not show the decrease in the water contact angle that would be expected. This can possibly be due to the presence of crosslinking in the polymer, which may reduce the mobility of the PEO chains, making it more difficult for the chains to reach the surface.

XPS analysis of polyurethane surfaces further confirmed the incorporation of dendrimer and PEO and suggests that some surface enrichment of these groups does

occur. However, due to the highly hydrophobic nature of the XPS, it is likely that the results are not indicative of the surface composition in an aqueous environment, as would be the case in a blood contacting application. That is probably the reason for the increase in nitrogen concentration observed on some of the surfaces after PEO attachment. However, there was an increase in the ether contribution to the high resolution carbon envelope in the ED chain extended polymers, as would be expected with a PEO containing surface. XPS results clearly demonstrate that, while there is PEO on the surface of these polymers, the surface is not completely saturated. While surface modification methods may be used to obtain higher PEO densities, it is possible that the use of higher generations of dendrimers and lower dendrimer to standard chain extender ratios coupled with either coating or blending could result in polyurethanes that are easy to produce and of equal quality to those obtained by more tedious surface modification techniques.

4.3 Biological characterization of polyurethanes

Protein adsorption on polyurethane surfaces after dendrimer and PEO modification were examined by adsorption of radio labeled fibrinogen adsorption as well as by SDS-PAGE and immunoblotting.

It was expected that protein adsorption on polyurethane surfaces would decrease significantly with the amounts of dendrimer incorporated and PEO attached. Fibrinogen was selected for the protein adsorption test because of its pivotal role in the coagulation

cascade (refer to section 1.2.1) and its relative abundance in plasma.

Adsorption of fibrinogen decreased on dendrimer modified polyurethane surfaces relative to the controls for both the ED and BDO series of polymers. After PEO was attached, a further decrease was observed, as expected. Furthermore, the trend and value of fibrinogen adsorbed agreed well with the results of Santerre et al. (1992). An effect of PEO amount in the polymer was also observed. PU-ED-PEO[1], which had the greatest amount of PEO, adsorbed the least fibrinogen. As well, the PU-BDO-PEO[2b] polymer showed a larger decrease in fibrinogen adsorption and a larger amount of incorporated PEO relative to the PU-ED-PEO[2a] polymer. Significant reductions in protein adsorption were observed despite the relatively low efficiency of PEO attachment and relatively low levels of dendrimer and PEO in the polymers. It therefore seems likely that the protein adsorption could be further decreased if a higher molar ratio of dendrimer to standard chain extender were used or if the reaction conditions of attaching PEO are modified to increase the efficiency. On the other hand, these mild reaction conditions can be used to modify the dendrimers with other biologically active moieties of interest, including peptides, without loss of activity. The adsorption of fibrinogen from PBS buffer precluded the presence of any Vroman effects on these polymers. It may be useful to examine ^{125}I fibrinogen adsorption from plasma to examine the significance of this effect on these surfaces.

The immunoblotting results offered qualitative information about the amount of protein adsorbed to the polyurethane surfaces, as well as the composition of the adsorbed

protein layer. Modification of the polymer structure to incorporate dendrimers and PEO did not result in significant differences in the patterns of protein adsorption, although the results seem to suggest that significantly less of the various contact activation proteins were adsorbed on the experimental surfaces relative to the controls. However, there were generally low amounts of proteins adsorbed on the polyurethane controls, particularly the BDO control (PU-BDO). Therefore, the decreases in protein adsorption on these surfaces were not as significant on these surfaces in particular. Nevertheless, the trend on all of the surfaces was a general decrease in the intensity of the bands associated with several proteins after dendrimer and PEO modification.

Again, it should be noted that a relatively low generation of dendrimer was used in these studies and that the amount of dendrimer relative to the amount of standard chain extender was quite low. Therefore, it is likely that greater amounts of surface PEO are possible using alternative reaction mixtures, including higher generations of dendrimer. Furthermore, it has recently been shown by Tan and Brash (2001) that polyurethanes synthesized in a similar fashion to pluronics can be used to obtain significant reductions in protein adsorption to surfaces and good adhesion to polyurethane substrates. Using similar polymers, but with dendrimers and PEO, it may be possible that even greater amounts of surface PEO and therefore, greater reductions in protein adsorption could be obtained. However, it is clear from these results that polyurethanes with incorporated dendrimers can be synthesized and used to improve biological interactions through the presence of higher levels of biologically relevant molecules.

Chapter 5 Conclusions and Recommendations

5.1 Incorporation of dendrimers and PEO in polyurethanes

Two approaches for incorporating dendrimers into polyurethane backbones were designed and used to synthesize dendrimer modified polyurethanes:

- Approach 1 was unsuccessful. No high molecular weight polymers were produced. The results suggest that the remaining free NH_2 groups of dendrimers were sterically "buried" inside after PEO modification, causing low reactivity of the "free" NH_2 groups.
- Derived approach 1 produced a DMSO soluble but slightly crosslinked polyurethane with both dendrimer and PEO incorporated (denoted as PU-ED-PEO^{1}).
- Approach 2 utilized a protection/deprotection strategy to get PEO attached dendrimer modified polyurethanes (PU-ED-PEO^{2a} and PU-BDO-PEO^{2b}).

An estimate of the amount of attached PEO was obtained from GPC and NMR results. The results indicated that only 1 out of 6 arms of the dendrimer was coupled with PEO in PU-ED-PEO^{2a} and 4 out of 6 arms were attached with PEO in PU-BDO-PEO^{2b}. In PU-ED-PEO^{1} it was estimated that almost 5 out of 6 arms of the dendrimer were

coupled with PEO.

5.2 Surface characterization of polyurethanes

Two techniques were used in this study to characterize the polyurethane surfaces:

- Water contact angles decreased significantly from 70° to 60° after dendrimer incorporation and decreased further to about 45° after PEO attachment showing the increased hydrophilicity of the polymer surfaces after modification.
- XPS results provided further confirmation of the incorporation of dendrimer and PEO into the polyurethanes synthesized by the two approaches in this study and indicated the presence of dendrimers on the polymer surfaces. However, the results also showed PEO migration away from the surfaces due to the highly hydrophobic nature of the XPS environment.

5.3 Biological characterization of polyurethanes

Biocompatibility of the polyurethanes was characterized with adsorption of radiolabeled (^{125}I) fibrinogen and immunoblotting. Fibrinogen adsorption on dendrimer modified polyurethane surfaces decreased in both the ED and the BDO series. After PEO was attached, a further decrease was observed as expected due to the high mobility of the PEO chains and their reported ability to repel proteins from surfaces. The immunoblotting results suggest that there were qualitatively small differences in the relative amounts of protein adsorption on the dendrimer incorporated and PEO attached

polymers.

In this work, a new method was developed to incorporate dendrimers into the backbone of polyurethane to increase the density of reactive sites for further modification. Furthermore, this is the first example of application of dendrimers for polyurethane modification to improve its biocompatibility up to present. These results demonstrate the potential of dendrimers for increasing the levels of biologically relevant molecules in biomaterials and for use in biomaterials applications.

5.4 Recommendations for future study

Several recommendations for future studies can be made based on the results of this work:

- Low molar ratios of dendrimer to chain extender were selected in this study to examine the potential for synthesis. It is likely that increasing the amounts of dendrimer incorporated into the polyurethane structure would have adverse effects on the polymer molecular weights and likely on the mechanical properties of the polymers. However, it is also likely that the incorporation of greater amounts of dendrimer and PEO would result in significantly better biological results. This variable should be examined in future work to determine the feasibility of incorporating higher amounts of dendrimer. It may be possible, for example, to overcome the limitations imposed by the mechanical properties by increasing the amount of crosslinking in these polymers or by coating or blending techniques, thereby obtaining the mechanical properties of the substrate.

- Generation 2.0 dendrimer (G_2) was used in this work and proved to be useful for increasing the density of functional groups in polymers. Other generations such as G3 and G4 could be investigated for their potential for this application, particularly given the possibility that these higher generations would result in significantly higher levels of biologically relevant molecules.
- The results demonstrated that there was a low efficiency of PEO attachment after deprotection of dendrimers possibly due to the lack of optimization of the selected deprotection and PEO attachment reaction conditions used. Therefore, it would be worthwhile to investigate alternative deprotection methods in order to improve the efficiency of PEO attachment.
- It would be useful to develop polyurethane pluronic equivalent polymers with terminal dendrimer PEO combinations to further increase PEO surface density.
- PEO attachment to the dendrimers was studied in this work and was shown to be successful in reducing the protein adsorption on polyurethane surfaces. However, many other biological molecules could be introduced into the polyurethane structure by reaction with incorporated dendrimers. It would be valuable to examine polymers synthesized with cell adhesion peptides or other biologically relevant molecules including heparin and growth factors attached to either the dendrimers or to the ends of the PEO chains to further investigate the possibility of this technique for generating superior biomaterials.

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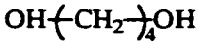
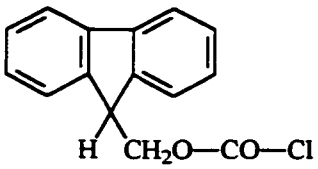
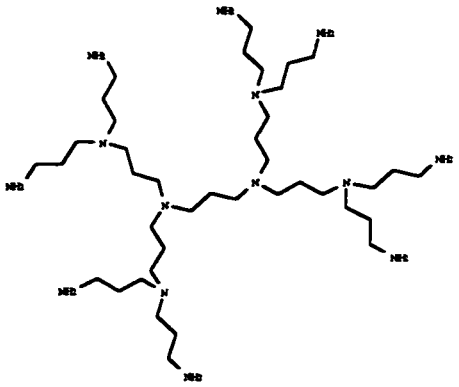
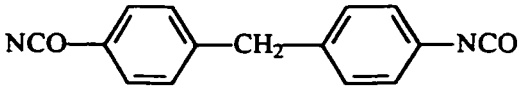
Appendix A: Chemical Structures of Ingredients

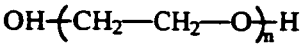
Butanediol 

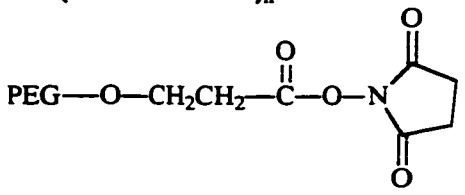
Ethylene Diamine 

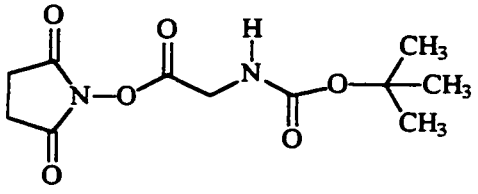
9-Fluorenylmethyl Chlorocarbonate 

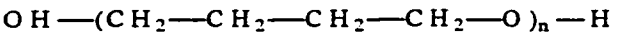
Poly (propyleneimine) dendrimer G_2 

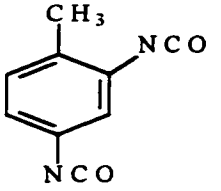
MDI 

Poly (ethylene Oxide) 

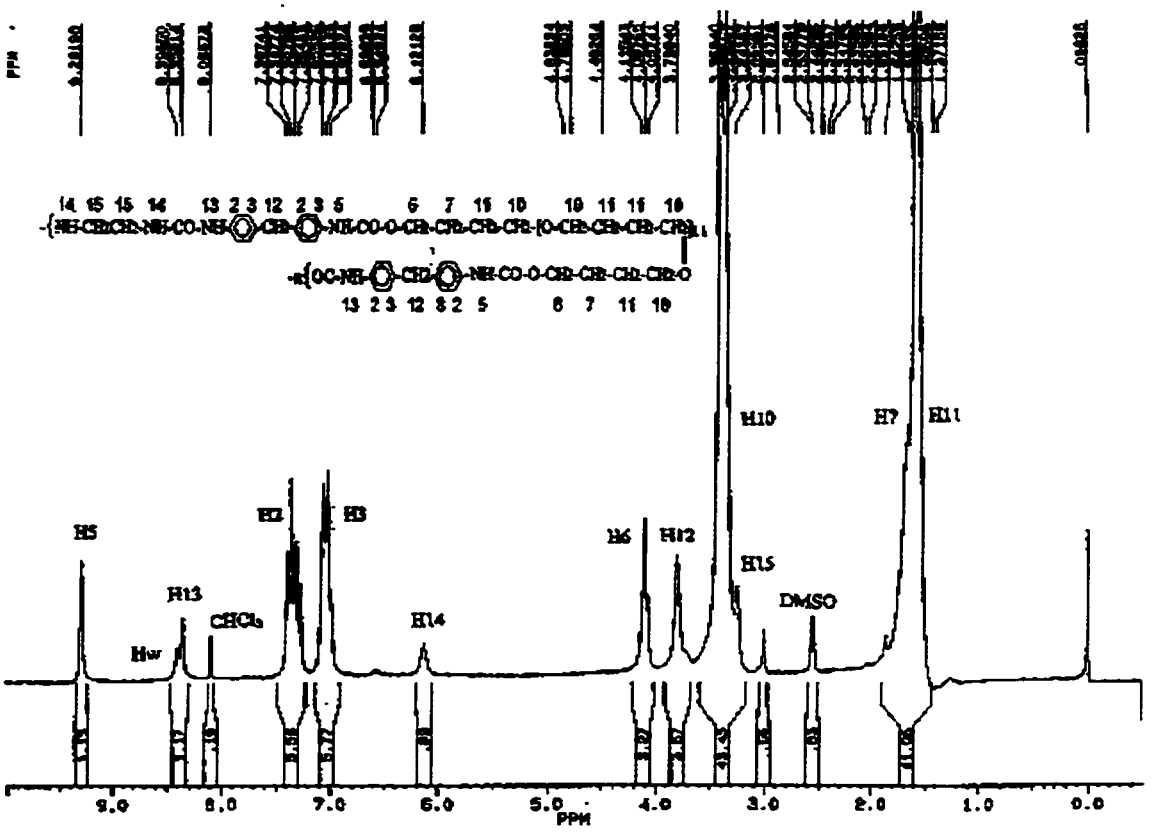
SPA-PEG 

t-Boc protected L-alanine N-hydroxy-succinimide ester 

PTMO 

TDI 

Appendix B: Peak Assignments for ^{1}H-NMR Spectrum of Polyurethane Chain Extended with ED. Taken from (Tan J and Brash JL, 2001) with permission.



Proton NMR spectrum of PU in a mixture of deuterated DMSO and chloroform (1/1, v/v).

Appendix C: SDS-PAGE and Immunoblots Procedures

Polyacrylamide gel preparation (12% separating gel, 4% stacking gel)

The acrylamide/bis solution is prepared by dissolving the following reagents in distilled water, diluting to 100 mL and filtering the final solution:

Acrylamide	29.2 g
N,N'-Methylenebisacrylamide	0.8 g

The reagents for the 12% separating gel were mixed and degassed for 15 min at room temperature:

Distilled water	3.35 mL
1.5 M Tris, pH 8.8	2.5 mL
10% (wt./vol.) SDS	0.1 mL
30% (wt./vol.) Acrylamide/Bis	4.0 mL

Immediately prior to casting the gel, the following reagents are added to initiate polymerisation in the above mixture:

10% (wt./vol.) ammonium persulfate (fresh)	50 μL
TEMED	5 μL

The casting plates were successively cleaned with distilled water and 95% ethanol. Once dry, the plates were inserted into the casting assembly. The assembly was then secured to the casting stand. Using a syringe, the gel plates were filled with polymerising 12% acrylamide solution, leaving enough space to pour the stacking gel. After 2 min, a small quantity of water was layered over the gel. The gel was allowed to polymerise for 1 h.

The reagents for the 4% stacking gel were mixed and degassed for 15 min at room temperature:

Distilled water	3.0 mL
0.5 M Tris, pH 6.8	1.2 mL
10% (wt./vol.) SDS	0.1 mL
30% (wt./vol.) Acrylamide/Bis	0.65 mL

Immediately prior to casting the gel, the following reagents are added to initiate polymerisation in the above mixture:

10% (wt./vol.) ammonium persulfate (fresh)	25 μL
TEMED	5 μL

Using a syringe, the remainder of the gel plates was filled with polymerising 4%

acrylamide solution. An appropriate comb was added and the gel allowed to polymerise for 1 h.

Sample preparation

The sample buffer used in sample preparation consists of the following reagents, mixed and stored at 4°C in 225 μL aliquots:

Distilled water	4.0 mL
0.5 M Tris, pH 6.8	1.0 mL
10% (wt./vol.) SDS	1.6 mL
Glycerol	0.8 mL

Immediately prior to use, the following reagents are added to an aliquot, yielding tracking dye (TD):

2-Mercaptoethanol	30 μL
0.5% (wt./vol.) Bromophenol blue	30 μL

Samples and standards used for SDS-PAGE only are prepared as follows:

0.5 μL SDS-PAGE MW Standards, Low Range, 10 μL TD

10 μL Protein sample, 10 μL TD

7.5 μL Prestained SDS-PAGE Standards, Low Range

Samples and standards used for immunobloting are prepared as follows:

1 μL SDS-PAGE MW Standards, Low Range, 10 μL TD

80-150 μL Protein sample, 10 μL TD

7.5 μL Prestained SDS-PAGE Standards, Low Range

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

Electrophoresis

Once the gel polymerisation was complete, the combs were gently removed and the wells rinsed with distilled water. The gels were removed from the casting stand and placed into the clamp assembly. The assembly was then placed into the buffer chamber. A 5X stock solution of electrophoresis buffer was prepared by mixing the following reagents in distilled water and diluting to 1 L (Note: the pH of this solution should be 8.3 ± 0.3):

Tris Base	15 g	
Glycine	72 g	
SDS		5 g

Just prior to use, the 5X stock solution was diluted to 1X with distilled water. The upper buffer chamber was filled to a level 3 mm below the edge of the outer (long) glass plate with electrophoresis buffer. The lower buffer chamber was filled to a level that covered the bottom 1 cm of the gel. The comb was subsequently removed and the well flushed with transfer buffer. The sample was then loaded into the wells and a potential difference of 200 V applied across the gel for approximately 1 h. When performing an immunoblot, a small quantity of pyronin Y dye (dissolved in sample buffer) was layered into the wells just before the tracking dye had reached the bottom of the separating gel. Electrophoresis was stopped once the pyronin Y dye had reached the top of the separating gel.

Gel equilibration

Transfer buffer was prepared by mixing the following reagents in distilled water and diluting to 1 L (Note: the pH of this solution should be 8.3 ± 0.3):

Tris Base	3.03 g
Glycine	14.4 g
Methanol (HPLC grade)	200 mL

The gels were removed from the electrophoresis assembly and equilibrated in fresh cold (4°C) transfer buffer for 30 min.

Electrophoretic transfer

Immobilon (PVDF) membranes were cut to gel-size, prewetted in methanol (1-3 seconds), incubated in water (1-2 min) and soaked 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. The membranes can then immediately be stained with colloidal gold or dried and used for immunoblot analysis.

Gold staining

The PVDF membranes were washed two times in phosphate buffered saline (PBS), pH 7.4. PBS was prepared by mixing the following reagents in distilled water, adjusting the pH to 7.4 and diluting to 1 L:

Na_2HPO_4	1.32 g	
$NaH_2PO_4 \cdot H_2O$	0.345 g	
NaCl		8.5 g

The membranes were then incubated in 0.3% (v/v) Tween 20 solution in PBS for 1 h at 20°C to block unbound membrane sites. This was followed by three further washings of 5 min with this blocking solution. The membranes were then rinsed in water three times for 1 min.

The membranes were then placed in Protogold solution and stained for 1 to 4 h. Following the staining, the membranes were rinsed extensively with distilled water and air dried.

Immunoblotting

The sections of the membrane containing MW markers lanes and a small section of the sample lane were removed to be stained with the gold staining procedure described above.

The remainder of the membrane was sliced into 3 mm strips. The strips were pre-wetted in methanol, rinsed in distilled water and placed into plastic wells. In order to block unbound membrane sites and prevent non-specific binding, the strips were incubated for 1 h in 5% (wt./vol.) dry skim milk in TBS, pH 7.4 with gentle agitation. This treatment was followed by three 5 min rinses in 0.1% (wt./vol.) dry skim milk in TBS.

Each strip was then incubated for 1 h in 1 mL 1% (wt./vol.) dry skim milk and 0.05% (v/v) Tween 20 in TBS with a 1/1000 dilution of the primary antibody to the protein of interest. This treatment was followed by three 5 min rinses in 0.1% (wt./vol.) dry skim milk in TBS. Each strip was then incubated for 1 h in 1 mL 1% (wt./vol.) dry skim milk and 0.05% (v/v) Tween 20 in TBS with a 1/1000 dilution of the alkaline phosphatase-linked secondary antibody. Again followed three 5 min rinses in 0.1% (wt./vol.) dry skim milk in TBS. Finally, the strips were incubated for up to 4 h with a solution to develop the colour reaction and detect the bands. The buffer for this solution

is prepared by dissolving the following reagents in distilled water, adjusting the pH to 9.8 and diluting to 100 mL:

$NaHCO_3$	840 mg
$MgCl_2 \cdot 6H_2O$	20 mg

The final solution is prepared by mixing 1 mL NBT stock (30 mg NBT in 1 mL 70% DMF in distilled water) and 1 mL BCIP stock (15 mg BCIP in 1 mL DMF) in 100 mL buffer: This reaction was terminated by rinsing the strips in distilled water twice for 5 min.

Appendix D: Reagents for SDS-PAGE and Western Blotting

Colour generation reagent for Western Blotting

The colour generation buffer is prepared by dissolving the following reagents in distilled water , adjusting the pH to 9.8 and diluting to 100 mL:

$NaHCO_3$	840 mg
$MgCl_2 \cdot 6H_2O$	20 mg

The final solution was prepared by mixing 1 mL NBT stock solution (30 mg NBT in 1 mL 70% DMF in distilled water) and 1 mL BCIP stock (15 mg BCIP in 1 mL DMF) in 100 mL buffer.

Tris-buffered Saline (TBS)

50 mM Tris

150 mM NaCl

Adjust pH to 7.4

Phosphate Buffered Saline (PBS)

Na_2HPO_4	1.32 g
NaH_2PO_4	0.345 g

NaCl	8.5 g

Mix in distilled water, adjust pH to 7.4 and dilute to 1 L.

Electrophoresis Buffer

Tris Base	15 g
Glycine	72 g
SDS	5 g

Mix in distilled water and dilute to 1 L. The pH should be 8.3 ± 0.3 Prior to use the 5X stock solution is dilute to 1X with distilled water.

Transfer Buffer

Tris Base	3.03 g
Glycine	14.4 g
Methanol (HPLC grade)	200 mL

Mix with distilled water and dilute to 1 L. The pH should be 8.3 ± 0.3.