POLYMER MATRICES FOR CELL ENCAPSULATION

A THESIS PRESENTED

BY

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DECLARATION

I, Sheela George, hereby declare that I had personally carried out the work depicted in the thesis entitled **"POLYMER MATRICES FOR CELL ENCAPSULATION"** except where external help sought are acknowledged.

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This is to certify that **Ms. Sheela George**, in the Division of Biomaterials Group of this Institute, has fulfilled the requirements of the regulations relating to the nature and the prescribed period of research for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The work relating to her thesis entitled " POLYMER MATRICES FOR CELL ENCAPSULATION" was carried out under my direct supervision.

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.....to my beloved daughter

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SYNOPSIS

SYNOPSIS

Encapsulation of cells within a synthetic semipermeable membrane prior to transplantation is an innovative technique to prevent the immunorejection of cells and avoid the use of toxic immunosuppressive drugs. Such therapy has been targeted at several hormone deficiency and neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington chorea as well as the control of chronic pain. The encapsulation of hepatocytes for the production of a bioartificial liver and islet cells for bioartificial pancreas are the other emerging applications. However, to date, bone marrow transplantation is the only cellular transplantation technique that is currently perfectly standarised and clinically practiced. The broader clinical use of organ/ cell transplantation is hampered by the shortage of human organ donors as well as the need for a permanent immunosuppressive drug therapy in order to avoid immune rejection. Immunoisolation of cells by semipermeable polymeric membranes has been conceived with the aim of permitting the transplantation of xenogenic cells, from species with large phylogenic separation from humans, without recourse to immunosuppressive therapy.

Diabetes Mellitus afflicts an estimated 80 million people worldwide; 5 to 10% are classified Type I and are dependent upon exogenous insulin for life. Despite attempts to control blood glucose levels with diet, exercise, and exogenous insulin therapy, however, patients continue to develop serious vascular and neurological complications, including blindness and renal failure highlighting the need for alternate therapy. In the long term, the alternate therapy of pancreatic islet transplantation in humans is limited by relatively low availability of human islets and the above mentioned immunorejection and associated immunosuppression drug therapy. A possible solution might be to transplant xenogenic islets by a permselective synthetic membrane, which isolates the transplant from its environment. In that case, the membrane material allows the placement of living tissues in an immuno-protected recipient without the need of immunosuppressive drug therapy. These membranes would permit low molecular weight entities such as glucose

and insulin to pass through but not the host immune effectors. Several polymeric membranes such as poly (2-hydroxyethyl methacrylate), poly (vinylchloride- co- acrylic acid) and cellulose acetate have been proposed as immunoisolation matrices for islet cells. Fibrotic reaction, biocompatibility and reduced permeation are some of the factors limiting their widespread use.

The present study envisages the need to synthesise a polymeric matrix that would overcome these limitations and have adequate physicochemical characteristics for use as immunoisolation matrix membrane for islet cells. The excellent physicochemical and biocompatibility aspects of many polyurethane polymers have prompted their widespread use in various medical devices. Polyurethanes have, however, not been extensively explored for immunoisolation purposes.

This thesis deals with the synthesis, characterisation and biocompatibility studies of novel polyurethanes with varying hard segments for immunoisolation of islet cells. Surface modified polyurethanes prepared by grafting with vinyl monomers have also been proposed for immunoisolation. Permeation, biocompatibility and islet cell viability studies have been used to establish the immunoisolatory character of the membranes and their comparative efficiency for the intended application.

The introductory chapter begins with the brief background of cell encapsulation, dealing with islet cells in particular, thus leading to the concept of bioartificial pancreas. This chapter also deals with the advantages and disadvantages of some of the polymer matrices used so far for immunoisolation purposes. The usage of polyurethanes as biomaterials and in various medical devices has also been considered.

This chapter also deals with the aims and objectives of the present study. Briefly, the present study aims at synthesis of the polyurethane membranes and their characterization using various physicochemical and biological assays, for qualifying the

membranes as potential islet cell immunoisolation matrices. Surface modification of the polyurethane by radiation grafting of vinyl monomers has also been attempted to elucidate some of the mechanisms governing the islet cell viability.

Chapter 2 deals with the materials and methodology of the investigation. The methods for the preparation of synthetic nonporous elastomeric polyurethane membranes with varying percentages of hard segments are presented in section 2.1. Polyurethanes were synthesised using diisocyanate, such as toluene diisocyanate and polyols such as poly (tetramethylene oxide) glycol, and poly (propylene oxide) glycol. The chain extender used for the above synthesis was 1,4 butanediol. The synthesised membranes were characterised using various techniques such as Infrared spectroscopy (IR), Scanning electron microscopy (SEM), Mechanical properties, Contact angle studies, Thermal analysis, WAXD, Optical Microscopy as well as permeation studies.

The next section of this chapter deals with the materials and methods used for surface modification of a polyurethane membrane. Various hydrophobic and hydrophilic monomers were grafted on the surface of the membrane by radiation grafting technique. The surface modified membranes were characterised using similar physicochemical techniques.

The methodology of biocompatibility studies and blood compatibility are discussed in the third section of this chapter.

The *in vitro* biocompatibility evaluation of the synthesised membranes were carried out using fibroblast cells such as L929, NIH 3T3 and VERO cells in order to understand the general response of the materials for use in medical devices. Islets isolated from BALB/ c mice were used to study the specific response of the materials for islet cell application.

The preliminary studies of blood compatibility of the promising compositions of synthesised as well as the surface modified polyurethane membranes were analysed. The degradation of the promising candidate membranes as well as grafted membranes in response to different catalytic enzymes such as trypsin, papain, bromalein etc.were studied to assess the stability of these materials.

Chapter 3 deals with results and discussions. This chapter contains five sections. Section 3.1 deals with the chemistry of polyurethane synthesis. The reaction mechanism of the formation of linear segmented polymer with different percentage of hard segments and their physicochemical characterisation studies are described. The effects of hard segment on the various physicochemical characteristics are also discussed. The Infrared (IR) study shows that the synthesised membrane is of polyurethane type. Infrared spectral analysis showed extensive hydrogen bonding and phase mixing in linear polyurethane. The Scanning electron microscopy (SEM) studies show smooth nonporous membranes at all compositions. The polyurethane membranes possess adequate mechanical properties and the tensile strength, percentage elongation, modulus are discussed in terms of their variation in the composition (e.g. type of the polyol used etc.). The surface parameters are dependent on the type of the polyol used suggesting the hydrophilicity/ hydrophobicity of the system. A variation of the surface parameters such as surface free energy with respect to the composition was observed.

Differential Scanning Calorimetry (DSC) analysis provided glass transition temperature of soft segment and hard segment while Thermogravimetric Analysis (TGA) indicated the thermal stability of the synthesised polyurethane membranes. The changes in thermal properties of the polyurethanes with respect to their composition are also discussed in this section. Wide angle X-ray diffractometer (WAXD) profile suggested that polyurethane synthesised using PTMG as glycol was more crystalline in comparison to the polyurethanes synthesised using PPG as glycol. This enhanced crystallinity is

further confirmed by polarised optical microscopy studies wherein spherulite like structures were observable in the PTMG system. Permeation studies using model compounds were carried out to assess the immunoisolatory behaviour. The membranes were found to be readily permeable to glucose and insulin and impermeable to albumin and immunoglobulin. The membranes were hence found to be selectively permeable and immunoisolatory.

Section 3.2 deals with the physicochemical characterisation of the surface-modified grafted membranes. The Infrared (IR) studies confirmed the grafting of various monomers onto the polyurethane. SEM analysis showed an altered morphology indicating that surface modification and some extent of phase separation had taken place on grafting. The tensile strength of the grafted samples were reduced in comparison to the ungrafted samples. The grafted membranes showed better permeation of the low molecular weight substances as compared to the ungrafted membranes. The grafted membranes were impermeable to albumin and immunoglobulins emphasizing their immunoisolatory nature.

The morpological features of the grafted membranes were probed using WAXD, polarized microscopy, and thermal analysis. WAXD analysis shows low intense peak corresponding to the PTMG unit cell indicating that the crystallinity of the ungrafted membranes is maintained in the grafted samples and also confirming that only surface grafting had taken place. Polarized optical microscopy studies of the grafted samples reveals that by grafting the materials are tending towards the amorphous nature. Thermal stability of the polyurethanes were not found to be affected by grafting.

Section 3.3 has two sub parts. The first part of this section focuses on the cytotoxicity of the synthesised membranes in response to the fibroblast cells. Some of the compositions synthesised were found to be noncytotoxic towards the fibroblast cells and only these compositions were further tested with NIH 3T3 cells. Digital image analysis

(DIA) studies showed intact morphology to isolated mouse islets cultured on one of the polyurethane compositions. Viability of cells on the polyurethane membranes was comparable to similar islets cultured on tissue culture polystyrene (TCPS) control. Islets also retained their functionality as judged by insulin secretion in response to in vitro glucose challenge (16.0 mM). An attempt was made to correlate the physicochemical properties with that of the viability of islets. The synthesised PU membranes were seen to have varying hydrophilicity and surface free energy values. Viability with pancreatic islet cells showed that hydrophilicity and least surface free energy were the determining factors in deciding the biocompatibility of polyurethane membranes. Hence these studies indicate that hydrophilicity/hydrophobicity and the surface free energy as important parameters while considering the membranes as suitable candidates for immunoisolation purposes.

The second subsection deals with the biological evaluation of the grafted membranes with fibroblast cells (VERO cells) and isolated islets. Some of the compositions of the grafted membranes were better than the ungrafted membrane.

Section 3.4 deals with the results obtained from the in vitro blood compatibility evaluation of promising synthesised and surface modified polyurethane membranes. The preliminary blood compatibility studies, which were done for promising synthesised and grafted membranes, showed that the membranes can be additionally considered as blood compatible.

Section 3.5 deals with the stability of the synthesised and the surface modified membranes in different enzymes. Infrared analysis studies of changes in mechanical properties of the enzyme treated membranes were carried out to assess the enzymatic biodegradation of the materials. Some of the compositions were found to be stable in *invitro* studies.

Finally chapter 4 contains the summary, conclusions of the studies. Future aspects of the investigations are also proposed.

CHAPTER 1
INTRODUCTION

INTRODUCTION

Cell transplantation procedure is an innovative technique for greater understanding of cell function and the potential therapeutic use of transplanted cells for treating various forms of illnesses. Along with serving as a model for assessing cellular physiology, transplantation of normal healthy cells offers the replacement of specific biological deficiencies or as a form of auxiliary support for a failing organ. There is hope that for long term, cell transplantation procedures may also have the potential for the development of artificial organ support system for sustaining patients with severe and chronic diseases such as diabetes, liver failure, endocrine and exocrine disorders, neurological abnormalities and congenital metabolic defects. However, the complications due to tissue rejection, without adequate immunosuppression, limit the usage of such therapy. A possible solution proposed to eliminate the immunological complications following transplantation, is encapsulation of cells within a synthetic semipermeable membrane prior to transplantation. By cell encapsulation we mean that the enclosed material/cells might be protected from destruction and from participation in immunological processes, while the enclosing membrane would be permeable to small molecules of specific cellular product which could then enter the general extracellular compartment of the recipient. The idea of using encapsulation of living cells, creating a bioartificial organ, was envisaged in 1964(Chang). A large amount of work has been reported on encapsulation of islet cells, thus leading to the concept of bioartificial pancreas.

1.1. Bioartificial or Hybrid Artificial Pancreas- The concept of Immunoisolation:

Diabetes mellitus afflicts an estimated 80 million people worldwide; 5 to 10 % are classified Type I and are dependent upon exogenous insulin for life (Cooper et al & Colton et al, 1991). Despite attempts to control blood glucose levels with diet, exercise, and exogenous insulin therapy, however, patients continue to develop serious vascular and neurological complications, including blindness and renal failure. The development of these complications is partially due to inadequacy of the metabolic control that can be achieved by medical therapy (Tchobroutsky, Diabetologia, 1978). The most sophisticated method of insulin delivery currently available for clinical use is continuous insulin infusion by a pump, which though may be implanted subcutaneously, does not achieve perfect metabolic control (Reach, 1990), probably because of the absence of closed-loop regulation of insulin delivery by the concomitant blood glucose level.

Because of these difficulties, whole -organ and segmental pancreas transplantation has been undertaken with increasing success in some centres (Shaffer et al, 1992). Pancreas transplantation has achieved excellent stabilization of blood glucose control, and insulin independence has been achieved, but reversal or stabilization of the diabetic complications remains unproven (Gaber et al, 1991). Furthermore, the disadvantages of donor organ shortage, major surgery, and chronic immunosuppression remain.

Islet transplantation has been studied extensively as an alternative to whole organ pancreas transplantation and has a number of practical and theoretical advantages. There are about 1 million islets of Langerhans in a human pancreas. They have a diameter of about 150 mm, and 60% of the islet mass is made up of beta cells that secrete insulin. About 10%, or 10,000 islets (a volume of less than 1 ml), should

be enough to provide normoglycemia in a 70 kg human (Colton et al, 1991). Pancreatic islets can be isolated using collagenase digestion to separate the endocrine from the exocrine tissues, followed by the purification by handpicking the islets from the tissue suspension or by density gradient separation using Ficoll or albumin (Warnock et al, 1988). There remain some difficulties in large animal models and in humans, particularly with the purification stage. The islets can be transplanted into the liver via the portal vein or by a subcapsular injection into the kidney or spleen. Rejection of the islets remains a problem and it is not entirely successful in clinical conditions (London & Bell, 1992).

Recent work, therefore, has been concentrated on the development of a bioartificial pancreas. This approach is based on the concept of immunoisolation. Living pancreatic islet cells are isolated from the immune system by an artificial coat or membrane with semipermeable properties. The membrane pore size is defined such that small molecules such as glucose, insulin, and nutrients can pass through freely, but antibodies and white blood cells are excluded. Immune recognition and response cannot occur. Several attempts at the development of a hybrid artificial pancreas have been made, with varying degree of success. Each islet or group of islets is surrounded by a thin membrane either as microcapsule (Altman & Gallete, 1986) or macrocapsule (Lim & Sun, 1980) and may be extravascular or intravascular (Sullivan et al, 1999 & Chick et al, 1977).

1.2 Polymers as a Matrix for cell encapsulation:

A biomaterial can be defined as a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body (Williams et al, 1992).

The term 'biomaterial' includes all materials used for medical applications that are interfaced with living systems or other systems developed for extracorporeal uses. The natural tissues in our body get damaged due to diseases, trauma or ageing. Allografts or xenografts appear to be ideal logical materials for replacements. Shortage of organs for implantation and the need for chronic immunosuppression however makes them less reliable. Therefore, a variety of other materials have been tried as biomaterials. These include metals, glasses, polymers (both natural and synthetic), ceramics, carbon and composites made from various combinations of these (Hoffman, 1984). Since polymers can be tailor made to match the mechanical and physical characteristics of many parts of a body, they find maximum applications as biomaterials. Among polymers, synthetic polymers make up by far the broadest and most diverse class of biomaterials, making the medical market the fourth largest area of plastic application (Lantos, 1988). The main reasons for this wide applicability of polymers are the availability of synthetic polymers in wide variety of chemical compositions and physical properties, their ease of fabrication into complex shapes and structures, their easily taliored surface properties and favourable cost performance ratio (Szycher & Robinson, 1980). Although several synthetic polymers are available, only ten or twenty polymers are mainly used in medical device fabrications from disposable to long term implants. This is because, the success of a biomaterial in the body depends on factors such as the material properties, design and biocompatibility and hence these aspects should be rigorously considered. Polymers to be qualifying as biomaterials should possess certain requirements (Lyman D.J, 1966). The biomaterial

1. Should be reproducibly produced as pure materials.
2. Should be fabricated into the desired form without being degraded or adversely changed.
3. Should have the required chemical, physical and mechanical properties.
4. Sterizable without effecting the properties.

5. Should not have their physical, chemical and mechanical properties adversely altered by the biological environment unless purposely designed as degradable materials.
6. Should have no adverse effects upon the recipient of the implant.
7. Should not induce thrombosis or intima formation or interfere with the functioning.
8. Should not alter configuration or stability of any cellular elements or soluble materials in blood that would lead cell fragility, ageing, allergic hypersensitive or toxic reactions.
9. Should not activate the complement system.
10. Should not induce adverse inflammatory and foreign body reactions.
11. Should not be toxic, carcinogenic or mutagenic.

Though no man made materials exist at present satisfying all these requirements, polymers such as silicones, polyurethanes etc. from a broad sense satisfy several of the above mentioned aspects.

1.3.1 Some Polymer Matrices used for Cell Encapsulation:

The various types of polymer matrices used for cell encapsulation purposes are as follows :

1) Alginate- Polylysine alginate microcapsule system (Lim and Sun, 1980)
2) Chitosan alginate System (Shiotani et al, 1989 and Mcknight et al, 1988)
3) Polyacrylate Microcapsules (Buchwald, 1987; Sefton 1982, 1987; Sugamori, 1987)
4) Agarose Microcapsules (Iwata et al, 1989)
5) Polyacrylonitrile, Cellulose polymer or Amicon XM-50 (Altman et al, 1986)
6) AN 69 Membranes (Honiger et al, 1994)

Before going into the details of each matrix, it is very essential to consider the requirements of a membrane for qualifying as a cell encapsulation matrix.

1.3.1 **Requirements of an immunoisolatory membrane** (Robert et al, 1993):

1) It should protect the implanted cells from macrophages and other host immune system elements.
2) Maintain cell viability for extended periods.
3) Permit free passage of nutrients, secretagogues and cell products.
4) Present the blood and the tissues with a biocompatible surface.
5) Be constructed of biostable materials.
6) Facilitate surgical implantation and cell reseeding.
7) Be easily fixed in place and easily removed if needed.

These membranes are used in different configurations for immunoisolation of donor islets, which are given below -

i) Vascular Devices
ii) Macrocapsule System
iii) Microcapsule System

i) Vascular devices

In the form of vascular implant (Sullivan et al, 1991; Chick et al, 1977), the islets can be distributed in compartments separated from the blood stream by a semipermeable membrane, the entire device being implanted as a shunt (i.d -1mm) in the vascular system. The cells enclosed in these devices can survive for longer times due to the diffusion of nutrients in these chambers as the blood is constantly flowing.

Though vascular device is a fairly good encapsulation procedure; one of its major disadvantages is that it is prone to thrombosis of blood especially at anastomoses.

ii) Macrocapsule system

A concept of hollow fibre macroencapsulation has been proposed with the islets being encapsulated in diffusion chambers, by Altman and Gallete, 1986. In this system, the cells are placed in sheaths, rods or discs (diameter >0.5 mm to 1 mm). The main advantages are: (1) It is easy and simple to enclose the required cells in the closed chamber and at a time a number of cells can be incorporated inside the chamber. (2)

During cell necrosis, it is easier to retrive the entire chamber and prevent the accumulation of degraded cell debris.

The disadvantages are: (1) The cells enclosed inside the chamber does not survive for long as the inner cells are deprived of the nutrients for long term use. (2) There is aggregation or clogging of the cells inside the macrocapsule. (3) Surgical technique may be required to remove the system.

iii) Microcapsule system

The islets can be included in microcapsules (microencapsulation), and injected intraperitoneally, subcutaneously or into other sites. Lim and Sun (1980) first introduced the concept of microencapsulation of islet cells. In microcapsule system, the cells are placed in injectable spherical beads of diameter < 0.5 mm. Device biocompatibility is critical because insulin reactions can block the flow of nutrients and waste to and from the capsules. Microcapsules are generally made of hydrogels or

polysaccharides such as agarose or alginate because of extremely mild conditions required for gel formation. The technique is safe and simple for injecting the immunoisolated cells in various sites.

The disadvantages are: (1) The microcapsules are very fragile and hence difficult to handle. (2) Considered less biocompatible as they can be engulfed by macrophages depending on the size. (3) Though injectable in the blood stream, the microcapsules are not target specific. (4) The microcapsules are irretrievable once injected. Degraded and dead islets could be accumulated in the system and and be antigenic. How the accumulation of such microcapsules could affect the physiological system is not clearly understood.

The microencapsulated islets (Lim & Sun, 1980; O'Shea et al, 1984) and the macroencapsulated islets (Lanza et al, 1992 & 1993) reversed experimental diabetes in rats for more than a year. Vascular devices (Lanza et al, 1994 & Maki et al, 1996) have been used successfully for a period of > 7 months using porcine islets after which thrombosis limited their further use.

1.3.2 Matrices for Cell Encapsulation:

1) Alginate -Polylysine alginate microcapsule system:

Microencapsulation of islets for transplantation was first introduced by Lim and Sun, in 1980 using alginate polylysine capsules. With some modifications, for e.g. concerning the concentration of different solutions, this technique is still widely used. While several investigators have reported prolonged graft survival of alginate - polylysine encapsulated islets in the rodents, there has been no successful reversal of diabetes by a single intraperitoneal injection of encapsulated islets in large animal

model. When human or canine islets (Camillo, 1992) were encapsulated and xenotransplanted into streptozotocin - induced diabetic rats normoglycemia (plasma glucose <200 mg/dl) was restored in the recipient rats within 1-2 days, confirming islet cell viability.

The major obstacle preventing this microcapsule from being used in large animal model has been the fibrous overgrowth associated with the capsule membrane. In some rodents also long term success has not been achieved (O'Shea et al, 1984; Lim & Sun, 1980; Weber et al, 1990).

2) Chitosan -Alginate System:

Chitosan is a natural polycationic polymer and is extremely abundant in nature e.g. crustacean shells, insects and fungi. The polysaccharides is highly versatile and can be used for cell immobilization (Shiotani et al, 1989 and Mcknight et al, 1988). Chitosan capsules can be made by direct gelling of sodium alginate droplets in a solution containing chitosan and calcium or formed in a liquefied core gelation by adding cell containing drops of a solution of either an anionic composition to a solution of an ionic polymer of opposite charge. The interface of the ionic polymer forms a semipermeable membrane surrounding the liquid drops. This process has been used successfully for the encapsulation of viable cells with little cellular damage (Rha et al, 1984). Chitosan derivatives are stronger and more flexible than those prepared with Polylysine (Mc Knight et al, 1988).

Cell viability, however, cannot be retained in a long term with this capsule, possibly as a result of small contaminating molecules, short chitosan chains or unreacted nitrite diffusion through to the cells.

3) Polyacrylate Microcapsule Membranes:

Cells can be encapsulated in water-soluble hydrophilic polyacrylates (such as Eudragit RL) by coaxial and interfacial precipitation (Buchwald, 1987; Sefton 1982, 1987; Sugamori, 1987). Despite exposure to organic solvents (diethyl phthalate) and nonsolvents (hexadecane, corn oil, mineral oil), erythrocytes (Sun & Shea, 1985), fibroblast (Sun & Shea, 1985) and pancreatic islet cells (Sun & Shea, 1985) have been shown to survive upto 6 months in vitro, with the latter cells secreting insulin and responding to glucose in both static glucose challenges and perfusion assays as well and as long as control islets which are not encapsulated. Unlike the alginate – polylysine and alginate -chitosan systems, the in vivo stability of a polyacrylate is good. The Eudragit RL polymer used for these studies is not biocompatible and induces an inflammatory response when implanted into the intraperitoneal cavity of animals.

4) Agarose Microcapsules:

Agarose is the gelling component in agar, and is a binary linear copolymer that forms strong transparent thermoreversible gels at concentrations of polymer >0.2%(Sugamori, 1989). Gel -entrapment or encapsulation of a wide range of cells has been successfully carried out using agarose microcapsule, either by mixing the cells with a warm aqueous solution of agarose and letting the gel set by cooling (Matsunuga, 1980), or by emulsifying the polymer suspension in paraffin oil, or various nontoxic seed oils, and then solidified by cooling in an ice bath (Nilsson, 1983). Iwata et al (1989) examined whether xenotransplantation of microencapsulated islets into diabetic animals could reverse the diabetic state. Longest glycemic period is 53 days.

Lack of control of microcapsule morphology and permeability are some of the problems that need to be solved. But agarose seems to a good material and is being explored as a matrix for embedding the islets with a protective polyacrylamide outer layer.

5) Polyacrylonitrile, Cellulose polymer or Amicon XM-50:

It is an acrylic copolymer, XM -50, made of polyvinyl and acrylate. Tubular membranes (0.5 to 1.2mm outside diameter, 50 to 125 mm wall thickness) were sealed at both the ends with similar liquid polymer able to solidify in a humid atmosphere. The asymmetric copolymer had an inner skin and a reticular outside structure. The molecular weight of exclusion is 50,000 daltons. Macrocapsules were implanted in rats and even after one year polymers were not degraded. With Amicon XM-50, tissue reaction is mild. Preservation of the structure of cells after several months in the peritoneal cavity is indicative of the compatibility of polymer with the tissues.

There is inadequate mass of insulin producing tissue because of incomplete filling of the capsule, small size of the implant, functional variability between implants and occasionally excessive fibroblast organization and collagen disposition around the wall of the implant. Recurrence of hyperglycemia after explantation of the capsule clearly demonstrates that the graft was responsible for the normoglycemia (Altman et al, 1986).

6) AN 69 Membrane:

Several teams have used the polymer AN69 (polyacrylonitrile- sodium methallylsulfonate) for encapsulating hepatocytes (Balladur, 1995) or islets (Kesseler et al, 1991, 1992 & Honiger et al, 1994). If normal glycemia is defined as a fasting

plasma glucose level inferior to 250 mg/dl, the free islet transplantation reverses the hyperglycemia immediately but for 3 weeks only. After 3 weeks, animals become hyperglycemic with glucose concentrations rising from 240 ± 20 to 420 ± 4 mg/dl. At the opposite, AN 69 encapsulated islets recipients remain at normoglycemia for at least 70 days. The AN 69 as evaluated by macroscopic observation, is satisfactory. The host reaction to the implant is reduced to a thin layer of fibroblasts.

Hence, considering these matrices biocompatibility, reduced permeation, fibrotic reaction etc. are some of the factors limiting their widespread use. Hence there is an urgent need to develop a polymer which would overcome these limitations and have the desired physicochemical properties. One such polymer is the family of polyurethanes, which is a very versatile polymer as is evident from the various applications in medical devices (Table 1.I). Polyurethanes have however, not been extensively explored for immunoisolation purposes.

1.4 Polyurethanes as Biomaterials:

Among the polymers for biomedical applications, polyurethanes are an interesting family of materials with broad possibility for processibility, superior physical and mechanical properties and biocompatibility that made them so appealing for biomedical applications. The polyurethane used in various biomedical applications is given in Table1.I (Lelah & Cooper, 1986). Biomedical applications of polyurethanes go back to the early 60s, when polyester urethane foams was used for in situ bone fixation, while polyether urethane coatings was applied to cardiovascular implants. In both the cases the outcome was, however, poor due to the premature degradation of the polymer. The initial failure, however, did not impede continuous efforts to develop materials with good characteristics including mechanical. Since 1975 pacing leads with polyurethane insulation have been used in humans. In 1982 the Jarvik-7 total artificial

heart with blood sacs from polyurethanes was first implanted in a human (Lelah & Cooper, 1986). The tremendous popularity of polyurethane as biomaterials can be traced to the specific morphological features leading to the unparalled properties.

Table 1.I.

Various Applications of Polyurethane

Total Artificial Heart	Heart valves
Vascular Prostheses	Pericardial patchs
Vascular stents	Intraortic balloons
Mammary implants	Ureteral prostheses
Fallopian tubings	Adhesives
Orthopaedic casting tapes	Dialysis membranes
Filters in oxygenators	Meniscus reconstruction membranes
Endovascular embolization	Pacing lead insulation
Angioplasty balloons	Gastric balloons and feeding tubings
Catheters and cannulas	Sutures, ligaments
Wound dressings and drape	Blood bags
Peripheral nerve repair device	
Shock absorbing elements for root	
Liners in dentistry and periodontal membranes	
Implants for craniofacial and maxillofacial applications	
Oesophageal and tracheal prostheses	
Roller pump tubings in artificial heart or blood pumps	
Enveloping organs for soft organ fixation	
Endotracheal tubing	

In the broadening scope of artificial organs, polyurethanes also have been incorporated into designs for an artificial pancreas. The utilization of polyurethane to encapsulate or support Islets of Langerhans is being approached. Zondervan et al (1992) crosslinked an aliphatic polyurethane with dicumyl peroxide in order to retard degradation of the polymer following implantation. Results with encapsulated Islets of Langerhans showed a good insulin response to glucose, although the speed of response was slower than that of free cells. Ward et al (1993) has fabricated polyurethane into hollow fibres by a dip casting method. The polymer was synthesized from an aromatic diamine- extended hard segment, and an alkylene oxide soft segment. In vitro culture of pancreatic cells were maintained after 6 months. Seeded implanted in mice, a low level of rejection was observed. The surrounding tissue was tubes were vascularized, nonfibrous and not strongly attracted to the implant. The membrane was permeable to glucose and insulin but not to immunoglobulins. The permeability characteristics were believed to be governed by activated diffusion.

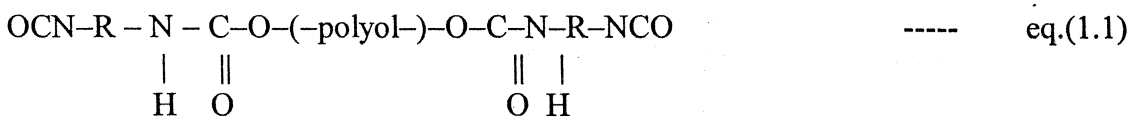
1.4.1 Chemistry of Polyurethanes:

Worldwide interest focussed in polyurethanes since its discovery by Bayer et al in 1937. The reaction of aliphatic diisocyanate and glycol led to the formation of polyurethane suitable for the production of plastics and fibres. Further work using aromatic diisocyanates with high molecular weight glycols resulted in the formation of the first polyurethane elastomer. Chronology of the history of polyurethanes has been documented elsewhere (Saunders & Frich, 1962; Wright & Cumming, 1969). The detailed description of chemistry of polyurethanes has been reported widely (Saunders, 1969; Bruins, 1969 & Oertel, 1985).

For synthesis of PU, mixing all the reactants at once-a one shot process provides the fastest, simplest and most economical manufacturing technique. In one step process, the entire polymer is formation is carried out by simultaneously mixing together polyol, diisocyanate and chain extender in the presence of suitable catalyst. On the other hand, two step process gives a much over toxicity, reactivity, properties and finished product quality. In the two step process, the polymer is formed in two stages. Initially the diisocyanate and polyol are reacted together to form an intermediate oligomer. The reaction is

m OCN – R – NCO + HO–(– polyol–) –OH ⟶

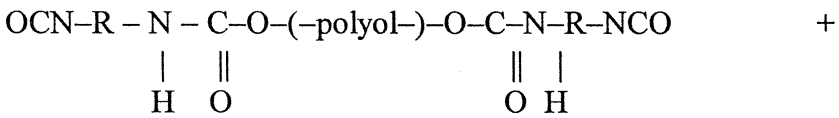
Diisocyanate **Polyol**



----- eq.(1.1)

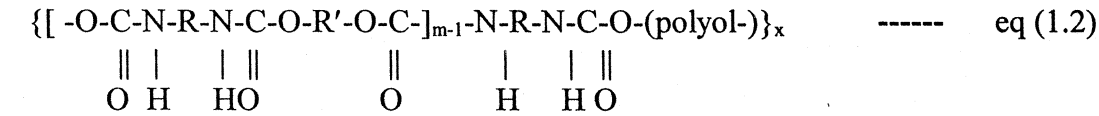
Prepolymer

The prepolymer thus formed is normally a thick, viscous liquid or low melting solid. The prepolymer is then converted into the final high molecular weight polymer by further reaction with a diol chain extender. The chain extension reaction is shown below eq.(1.2)



Prepolymer

Chain extender



Polyurethane

General equation for polyurethane synthesis

1.5.1 Properties of polyurethanes: Correlation with structural features

All these years have witnessed an exponential growth of literature addressing the chemical structure of polyurethanes and resultant properties. The following sections briefly review the physical and morphological characteristics of polyurethanes.

1.5.1.1 Microphase separation:

The thermodyanamics of phase separation of block copolymers has been the subject of widespread attention (Kause, 1969 & 1973; Helfard, 1974; Mier, 1979;

Helfard & Wasserman, 1978). Several theoretical models have been framed to understand the thermodyanamic principles governing the phase separation in polyurethanes. The two phase structure of polyurethane basically determines most of the properties though several factors such as segment polarity, block length, concentration of the hard segment etc. have a bearing on realization of such a two phase system (Sung & Schneider, 1978; Sung et al, 1980; Van Bogart et al, 1979).

1.5.1.2 Hydrogen Bonding in polyurethanes:

Hydrogen bonding plays a major role in properties of polyurethanes (Sung et al, 1980 and Brunett et al, 1982). Hydrogen bonding in PUs results from the –NH groups as donor and the -C= O and the ether oxygen groups as acceptor. H-bonds may be in the hard segment or in the soft segment. Relative amounts of the two types of hydrogen bonds are determined by the extent of microphase separation (Sung & Schneider, 1978). Increased phase separation favours interurethane H-bonding (Srichatrapimuk & Ferguson).

1.5.1.3 Polyurethanes as implants:

Today polyurethanes are believed to be indispensable and the materials find a wide variety of medical applications. Few applications are mentioned in Table1.I. Wide applicability of polyurethanes is believed to be due to their favourable interactions with the biological environment.

1.5.1.3.1 Blood-polyurethane interaction:

Interaction of polyurethane with blood has received intensive attention since the use of polyurethanes as biomaterials in the late 60's (Bruck et al, 1973; Boretos &

Pierce, 1968; Kambic et al, 1976 & Didishein et al, 1979). It has been generally accepted that protein adsorption is the first event that occurs after blood contact a polymer surface leading to the formation of protein layer at the blood polymer interface (Baeir, 1975). Lyman et al (1971) and Brash et al (1974) have shown that the surface, adsorbed more albumin adhere less platelet, and thus passivate the surface. However, fibrinogen has been shown to enhance platelet adsorption and thrombogenecity while gamma globulin activate the reaction (Young et al, 1982). Several investigations have shown the dynamic nature of protein adsorption on polyurethane surface (Bellissimo & Cooper, 1984 and Young & Cooper, 1983). Most of these cases show better performance of polyurethanes as compared to other polymers, in terms of blood compatibility.

1.5.1.3.2 Polyurethane-Platelet Interaction:

Though protein adsorption studies can serve as an index of thrombogenicity of polyurethanes, more elaborate studies in terms of platelet adhesion, platelet activation etc. are necessary to define blood compatibility aspects. Several researchers studied platelet adhesion and activation on polyurethanes resulting in the correlation of several structural parameters of the polymers with platelet interactions (Lelah et al, 1985; Whichter& Brash, 1978; SaDa Costa et al, 1981; Didi et al, 1979 and Takahara et al, 1983). The platelet interactions in a nutshell, suggest polyurethanes tend to be more blood compatible. More hydrophilic polyurethanes are more inert to blood components than hydrophobic materials.

1.5.1.3.3 Polyurethane-tissue interactions:

Over the past two decades, considerable progress has been made in understanding biocompatibility aspects of a wide variety of polymers including polyurethanes (Calnan, 1963 and Marchant et al, 1983). Majority of these studies was based on histological observation and morphological evaluation that were directed towards cellular ageing action immediately adjacent to the implant.

1.6 Surface Modification of Polyurethanes for Biomedical Applications:

The most important challenge in biomaterial research, is the development of materials that maintain mechanical integrity and biocompatibility together. It is well known that the surface composition is inevitably different from the bulk and it's the surface of the biomaterial that comes first in contact with living body. The introduction of polymeric device into a living system creates an interface between a material and tissue. The surface characteristics like surface tension, surface free energy, surface ionic groups, hydrophilicity/ hydrophobocity etc. of the material, therefore affect tissue-polymer interaction. It has been suggested that polymers with critical surface tension in the range of 20- 30 dynes/ cm should show minimum adverse biological reaction (Baier, 1969). A variety of polyurethanes have their surface tension in the range of 30-70 dynes/ cm and critical surface tension 27-19 dynes/ cm (Lelah & Cooper, 1986), favouring blood compatibility. Several reports have appeared correlating surface morphology of polyurethanes and blood compatibility (Goldberget et al, 1984 and Gogolewski, 1984). Hydrophilic surface are believed to be more blood compatibile (Bruck, 1979), although it has also been suggested that a good hydrophilic/ hydrophobic balance is required for optimal blood compatibility (Ratner et al, 1979). Anionally charged surface show a good blood compatibility, since repulsive interactions are operative between the surface and the platelets, which also possess an anionic charge (Swayer & Srinivasan, 1967). Their surface chemistry and structure thus largely control the biological responses towards a biomaterial.

The rationale of surface modification of biomaterials is to retain the key physical properties of a biomaterial like durability, functionality etc. while modifying only the outermost surface to influence the biointeraction. Surface modification of biomaterials is commonly employed to improve blood compatibility and tissue compatibility, to control protein adsorption, to influence cell adhesion and migration to improve lubricity, to improve wear resistance, to alter transport properties etc. According to Ikada (1994) almost all next generation biomaterials clinically used should have excellent properties both in bulk and surface and thus surface modification as in many cases essential for a material to be applied in medicine. Polymers can be surface modified by physicochemical methods as well as by incorporation of biologically active molecules.

1.6.1 Graft coploymers of polyurethanes:

Physicochemical surface modification can be carried out by changing physically or chemically the atoms or molecules on the existing polymer surface or by overcoating the existing surface with a material having different composition. Physicochemical surface modification of polymers can be broadly divided into:

i) Surface confined chemical reaction
ii) Non-covalent coating of the surface
iii) Covalent grafting of polymers

The current hypothesis shows that hydrogels or hydrophilic surfaces exhibit good blood compatibility. However, very hydrophobic inert surfaces such as fluoro polymers or silicones are also known to be biocompatible. Thus, physicochemical

methods are used to increase the hydrophilicity or hydrophobicity of the surfaces to improve biocompatibility. Among these covalent grafting of vinyl monomers has been vastly practiced (Bruck et al, 1973) and perhaps the simplest way to tailor the surface to modulate biological responses. Surface modified polymers by grafting different entities mainly hydrophilic are increasingly being studied for their improved blood contacting properties (Jansen & Ellinghorst, 1979 and Fischer et al, 1982). Chemical modification is an effective means to alter biological means and offers a number of advantages in biomedical fabrication. By varying only the outermost composition at the surface of the material, the properties as well as the fabrication methods of the implant can remain unaffected while modulating the chemistry and properties that direct biological reaction (Llanos & Sefton, 1993; Matsuda & Sugawara, 1995). Among many synthetic methods, free-radical polymerization has almost exclusively been used for surface grafting. To initiate the radical polymerization from a substrate surface. The active species can be produced by high energy radiation or by chemical procedures such as redox systems (Feng et al., 1985). Figure 1.2 illustrates schematically some of these processes. The energy sources commonly used to produce active species on polymer surfaces are: The energy sources commonly used to produce active species on polymer surfaces are: 1. Ionizing radiation such as gamma rays (Krishnan et al., 1990; Hari & Sharma, 1991), 2. UV radiation (Mori et al, 1982), 3. Photoiniated chain transfer reaction (Allmer et al, 1990), 4. High energy ion beams (Svorcik et al., 1997), 5. Low temperature plasmas (Fugimoto et al., 1993) and 6. Ozone gas (Fugimoto et al., 1993a). The usual source of ionizing radiation is cobalt^{60}, produced by neutron irradiation of naturally occuring cobalt ^{59} in a nuclear reactor. Sources of UV radiation are commonly deuterium or high pressure mercury lamps. Some of the beneficial aspects resulted by chemical modification on existing biomaterials are listed in Table 1.II(Lelah et al, 1985; Larsson et al, 1980; Evangelista & Sefton, 1986, Bruck, 1977, Bruck, 1979).

Table 1.II

Effect of Chemical Modification on the Performance of a Biomaterial

Improved blood compatibility
Reduced (or increase) Tissue adhesion
Improve lubricating
Increase or decrease wettability of the surfaces
Add biologically active substances to the surface layers
Alter the protein adsorption characteristics
Protect the device from the body or vice-versa
Act as rate limiting membrane

Graft polymers have a number of important and unique advantages for use in application involving the interfacing of the synthetic materials and living systems. By optimizing the graft methodologies the mechanical properties of the grafts can be made to closely resemble those of the unreacted trunk polymer originally selected to have appropriate modulus and durability for the proposed application. The surface properties of the graft polymer, on the other hand, influenced by the graft and can be engineered to produce the desired response in contact with the biological environment.

Hydrophilic vinyl monomers, due to their desirable factors, have been grafted to the backbone of the polyurethane using methods involving free radicals initiation (Imber et al, 1974; Karyla & Whitman, 1967), gamma radiation (Jansen & Ellinghorst,

1979) and anionic techniques (Hoffman & Kraft, 1972; Beachell et al, 1969). One of the most widely used methods for chemical modification of polyurethanes is grafting vinyl monomers by gamma radiation (Abidi et al & Fischer et al, Hoffman et al, 1983; Stannett, 1990). Jansen & Ellinghorst (1979) grafted series of hydrophilic monomers like hydroxyethyl methacrylate, acrylamide etc. onto polyurethane and studied for their mechanical properties and blood compatibility. These authors are successful in showing increased albumin adsorption with the extent of grafting, a sign of better blood compatibility. Boffa et al, 1977 and Hunter et al, 1983 have shown improved compatibility by grafting N-vinylpyrrollidone. Egboh et al (1984) has studied the grafting of NVP onto polyurethane, in a view to widen the applicability of polyurethanes.

1.7 Biostability:

The stability of a medical device in the biological environment is equally important as that of other aspects like blood compatibility. The subject of biostability of polyurethanes has been addressed widely. Hydrolysis is an important degradation mechanism in the biological environment. Schollenger & Steward (1971) have studied the hydrolytic stability of different polyurethanes. They found that polyether polyurethane is considerably stable. Another route of biodegradation is associated with enzymes. Liptova et al (1970) has investigated the influence of enzymes in destabilizing polyurethanes. Hung et al (1995) studied the influence of enzymes in destabilizing polyurethanes.

In general, most of the studies indicate the susceptibility to degradation depends on the structural features of the materials. It seems that by optimizing structural parameters and variables long term biostability could be realized.

1.8 Aim and Scope of the Work:

The main objective of the present study was to develop a synthetic semipermeable membrane, which could be used as immunoisolation matrix for encapsulation of pancreatic islet cells. The polyurethanes have been widely used for biomedical applications, as an implant and extracorporeal applications, but it has not been extensively explored for immunoisolation purpose. Hence, the present study aims at synthesis of polyurethane membranes and their characterization using various physicochemical and biological assays, to identify these polyurethane membranes as potential immunoisolation matrices. Surface modification of the polyurethane by radiation grafting of vinyl monomers has been attempted to elucidate some of the mechanisms governing islet cell viability. On surface modification by radiation grafting using vinyl monomers, it was expected to improve permeation and biocompatibility towards islet cells in addition to having the desired physicochemical characteristics.

The blood compatibility studies of the promising synthesised polyurethane as well as grafted PU membranes will be carried out for the possible use of these membranes as a blood contacting device.

The stability of the promising synthesised polyurethane as well as grafted PU membranes will also be carried out for undertsanding the biostability of these membranes, as the biohybrid pancreas may be considered for arteriovenous (AV) shunt.

The scope of this work is limited to the synthesis and characterisation of permselective PU and grafted PU membranes for the possible use as immunoisolation matrices. The candidate permselective PU and grafted PU membranes will be evaluated for cytotoxicity and biocompatibility with islet cells in *invitro* conditions. It would be attempted to correlate the physicochemical characteristics of the membranes with mechanisms governing islet cell viability. Preliminary assessment of the blood compatibility of candidate materials as well as enzymatic degradation of the materials would also be studied invitro for an insight into blood contacting character and long term stability of the materials. The scope of the study is hence limited to the identification of the possible candidate materials for immunoisolation of islet cells.

CHAPTER 2
MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Materials and their purification:

The diisocyanates used in the synthesis of polyurethanes was toluene diisocyanate (TDI) (Aldrich, USA). Polytetramethylene glycol (PTMG-mol. wt. 2000)(Aldrich, USA), Polypropylene glycol (PPG- mol.wt.2000) (Aldrich, USA) was used as soft segment. 1,4 butanediol (S.D Fine Chemicals, India) was used as a chain extender. Dibutyl tin dilaurate (Fluka) was used as a catalyst. Diisocyanate was used as received. However, all the polyols and chain extender were dried prior to use.

Solvents:

Dimethyl formamide (DMF) was from SD Fine Chemicals, India. The solvent was dried using anhydrous aluminium oxide (SRL, India) before use.

Monomers:

2-Hydroxyethyl methacrylate (HEMA), N-Vinyl pyrollidone (NVP), Ethylhexyl acrylate (EHA) all from Merck, Germany. Ethylene glycol dimethacrylate (EGDMA) (Fluka, Switzerland) were used for grafting onto the polyurethane. All the monomers were purified by vacuum distillation in presence of cuprous chloride. The middle portion of the distillates was collected and stored at 4°C. The ungrafted as well as the grafted sheets were then cleaned using an ultrasonic cleaner and used for the in vitro studies.

2.1 Synthesis:

2.1.1 Polyurethanes:

The polyurethanes were synthesised by the prepolymer method as reported (George & Nair, 1999). In this method, the polymer was formed in two stages. Required quantity of toluene diisocyanate (TDI) was taken in a three necked flask fitted with water condenser, stirring and nitrogen purge. Stoichiometric amount of polyol (PTMG/ PPG) was added slowly under stirring followed by the addition of DMF. Dibutyl tin dilaurate (0.01wt. %) was added as a catalyst alongwith PTMG and the temperature raised to 60°C. NCO/ OH ratio was maintained at 1.1. The composition of polyurethane synthesised is given in Table 2.I.

Table 2.I

Composition of Polyurethane synthesis

Sample code	Composition	Molar ratio	Hard Segment Percentage
PU-1	TDI-PPG-BD	1.05-0.35-0.60	25
PU-2	TDI-PPG-BD	1.05-0.25-0.70	32
PU-3	TDI-PPG-BD	1.05-0.19-0.76	40
PU-4	TDI-PTMG-BD	1.05-0.35-0.60	25
PU-5	TDI-PTMG-BD	1.05-0.25-0.70	32
PU-6	TDI-PTMG-BD	1.05-0.19-0.76	40

The reaction was carried out under nitrogen atmosphere with constant stirring and heating for an hour. After cooling the prepolymer to about 40°C, 1,4 butanediol

was added dropwise and stirred for 30 minutes, simultaneously raising the temperature to 60°C. After completion of the reaction which was noted by an increase in viscosity, the contents of the flask were poured in polyethylene molds after degassing. Curing was carried out for 48 hours at 60°C. After complete curing, the polyurethane films were removed from the molds and extracted for further studies. By changing the molar ratios of TDI/ Polyol / chain extender, polyurethane with varying percentage of hard segment were prepared. The scheme for the synthesis of polyurethanes is given in eq. (1.1) and eq. (1.2) given in Section1.5. The polyurethane cured was dissolved in DMF, casted into a film and cured at 60°C for getting homogeneous films. The recast films were then extracted with hexane and ethanol: water (20:80) to remove all polar and nonpolar unreacted constituents. The films were dried before characterisation. The scheme of synthesis is depicted in Fig. 2.1.

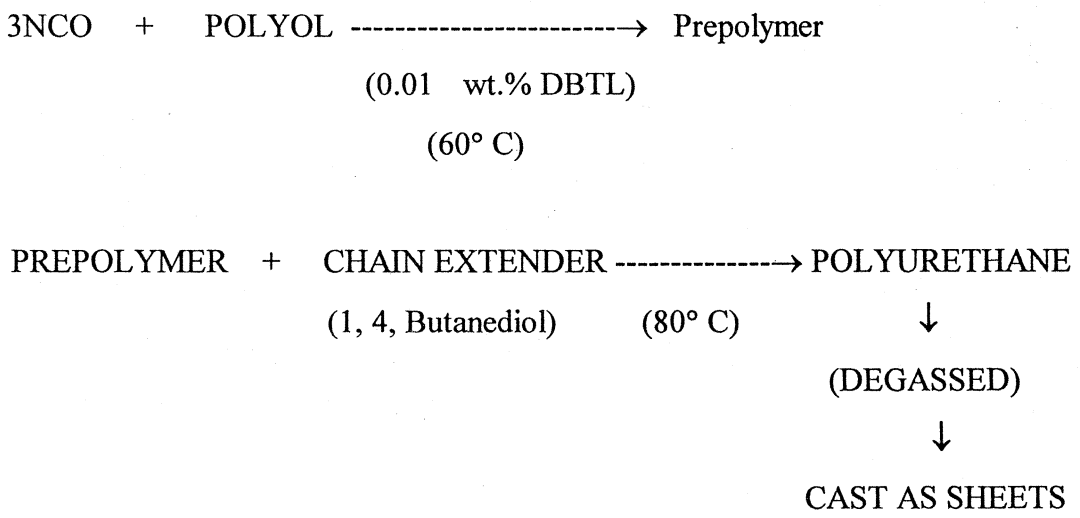


Fig. 2.1 : Flow chart of the synthesis of linear segmented polyurethane

2.1.2 Graft copolymers of polyurethane:

Radiation grafting method was used to modify the surface of polyurethanes with various entities. Gamma radiation has been used extensively to create free radical sites on polymer chains. These radicals can either initiate the polymerisation of a second monomer or recombine with growing radicals yielding the expected grafts. Chapiro (1962 & 1977) has discussed several specific procedures.

The direct radiation grafting which has been followed widely (Nguyen & Chapiro, 1975 & 1973, Chapiro & Lamothi, 1983) was used to prepare the grafts. In this procedure, the polymer strips were swelled in the monomer followed by γ-irradiation. As the radicals were formed on the polymer, they initiate the polymerization of the monomer. Grafts were grown from these sites with the simultaneous formation of the homopolymer. Polyurethane samples with composition TDI / PTMG / BD and having 25 % hard segment was used for grafting. Grafting of vinyl monomers such as hydroxyethylmethacrylate (HEMA), N-Vinylpyrrolidone (NVP) and ethyl hexyl acrylate (EHA) onto polyurethane was achieved by γ-irradiation. The crosslinker, 2wt% ethylene glycol dimethacrylate (EGDMA) was used. 0.005 M Cu^{+} was incorporated to prevent homopolymerization of the monomer. Cleaned polymer pieces were immersed in the monomer for varied time periods. The swollen films were pressed slightly between the folds of filter paper to remove adhered monomers and immediately subjected to γ- irradiation from Co^{60} source (Panoramic batch irradiator, BARC Bombay) under a blanket of nitrogen. The dose rate was 0.5 Mrads for 3 hrs. 10-15 % grafting yields were obtained. The composition of grafted polyurethane membranes is given in Table 2.II. The flow chart of the synthesis of surface grafted polyurethanes is given in Fig. 2.2.

Isolation of the grafts:

Poly (urethane-g-HEMA): The polymer strips after irradiation was extracted with distilled water for removing ungrafted polyHEMA and unreacted monomer. The graft found as an insoluble residue was washed several times in methanol and vacuum dried.

Poly (urethane-g-N-Vinyl pyrrolidone): The NVP grafted polyurethane strips were extracted with water several times and then vacuum dried.

Table 2.II

Composition of ungrafted and grafted membranes

Sample Code	Composition	Remarks
PU-4	TDI-PTMG-BD	Ungrafted polyurethane with 25% Hard segment
G-2	PU-HEMA-EGDMA	HEMA grafted PU using EGDMA as crosslinker
G-4	PU-NVP-EGDMA	NVP grafted PU using EGDMA as crosslinker
G-6	PU-EHA-EGDMA	EHA grafted PU using EGDMA as crosslinker

Poly (urethane-g-EHA): The EHA grafted polyurethane strips were extracted with toluene and then vacuum dried.

SURFACE GRAFTED POLYURETHANES

CURED POLYURETHANE SHEETS

↓

SWELLING IN

↓

HEMA / NVP / EHA + 2Wt % EGDMA

↓ **Gamma irradiation in N_2 atm**
0.5Mrads for 3 hours

EXTRACTION AS FOR POLYURETHANE

Fig. 2.2 : Flow chart of the synthesis of surface grafted polyurethane

Estimation of the graft yield (% grafting):

% Grafting was determined using the relationship

$$\% \text{ Grafting} = (W - Wo / Wo) \times 100 \quad \text{---------- eq. (2.1)}$$

Where W is the weight of the graft polymers and Wo is the initial weight

2.3 Physicochemical Characterisation:

2.3.1 Infrared Spectroscopy:

The attenuated total internal reflection (ATR) spectra of the materials were recorded using NICOLET IMPACT (Model 410, USA) Spectrophotometer. The surface spectrum (FTIR-ATR) of the films was recorded using FTIR Spectrophotometer having a baseline ATR accessory (Nicolet). Films were pressed

against ZnSe crystal provided with the ATR cell of the FTIR (Nicolet) Spectrophotometer. The spectra were scanned from 4000 cm^{-1} to 400 cm^{-1}. The results are discussed in sections 3.1.2.1 and 3.2.1.

2.3.2 Scanning electron microscopy (SEM):

The surface morphology of the samples was examined using SEM. The samples (0.5 x 0.5 cm^2) were placed on double-sided adhesive tape fixed on aluminium stubs with the non-embossed surface on the top. The samples were sputter - coated with gold using an ion sputter in vacuum (Hitachi, Model E_{101}, Japan) and examined in the microscope (Hitachi, Model S $_{2400}$, and Japan). The results are discussed in sections 3.1.2.2 and 3.2.2.

2.3.3 Mechanical Properties:

The mechanical properties of the synthesised films were carried out using Instron (model 1193) Universal testing machine as per ASTM D-882. The crosshead speed was 100mm/min. (10 x 1 x 0.1 cm) wide strips were cut from the sheets. At least 6 samples were tested in each case and s.d. of the values estimated. The results are discussed in section 3.1.2.3 and 3.2.3.

2.3.4 Thermal Analysis:

The glass transition temperature was evaluated using a TA instrument DSC 2920 with TA 4000 controller. Appro. 10 mg of finely cut sample were crimped in an aluminium pan and heated in the temperature range of -50°C to 200°C. The samples were cooled and reheated upto 250°C. The shift in the baseline of the second heating

run is noted as the glass transition temperature (Tg). To obtain subambient temperature range, a refrigerated cooling system (RCS) accessory was used. The glass transition temperature is observed as the shift in baseline of the second scan. Thermogravimetric analysis was carried out by TA Instrument model SDT 2960 simultaneous DTA-TGA was used. 10-20 mg of the samples were heated from 30°C to 600°C at a heating rate of 10°C under a dynamic nitrogen atmosphere. The initial decomposition temperature, 50% decomposition temperature, final decomposition temperature were evaluated from the thermogram. The results of the thermal analysis are discussed in sections 3.1.2.4 and 3.2.4.

2.3.5 Wide angle X- ray diffraction (WAXD) studies:

WAXD studies were performed in an X-ray diffractometer (Model D 5005) Siemens; employing Nickel filtered CuKα radiation. Samples were cut in 2 cm (diameter) size, put in the sample holder and scanned from 2θ = 10 ° to 40° at 40 KV and 30 mA. The results are discussed in section 3.1.2.6 and 3.2.6.

2.3.6 Optical microscopy:

Optical microscopy was used for getting the birefringes pattern of the samples. For viewing the samples crossed polarizers were incorporated. Samples were observed under light microscope NIKON 'OPTIPHOT' with a polariser. Photographs were taken with an FDX-35 camera at 600 X magnification. The results are discussed in sections 3.1.2.5 and 3.2.5.

2.3.7 Contact angle measurement:

A measure of the surface free energy &/or surface wettability of solid materials is most readily and simply obtained by measuring the contact angle of the diagnostic liquid on the solid surface. Underwater solid-air contact angle and solid-octane contact angle were measured by the captive air bubble technique of Hamilton 1972, 1974) using a Ramie' and Hart goniometer (Model 100, USA).

Captive air-in water and octane-in-water contact angles were determined after incubating the samples in double distilled water for 24 hours. The films were then placed on glass slides and fastened on both ends using teflon tapes. The slides were then placed face down in a Perspex trough containing double distilled water. The air and octane droplets were introduced from a micrometer syringe having a bend needle, beneath the sample. The air/water and octane/ water contact angles were then measured. A minimum of 10 readings were taken and averaged. The interfacial free energy of the surface was calculated from the air and octane contact angles using the equations and assumptions of Andrade et al (1979). The results are discussed in sections 3.1.2.7 and 3.2.7.

2.3.8 Permeation studies:

Permeation of glucose, insulin, albumin and immunoglobulins (IgG) were studied using a diffusion cell. The diffusion cells used were side-by-side cells consisting of donor and receptor half-cells. Amounts of known concentrations (5 ml) of the solution containing the solute of interest (glucose, 16.5 mmol/l; insulin, 8g/dl; albumin, 8 g/dl; immunoglobulin, 8 g/dl) were placed in the donor compartment. The pure solvent was kept in the receptor compartment. The whole setup was placed in an orbital-shaking incubator at a rpm of 100 and temperature of 37°C. Periodically the

contents of the receptor cell was removed and replaced by fresh solvent. The aliquots removed was analysed using UV-VIS Spectrophotometer using an o-toluidene method (Caraway, 1976) for glucose at 630 nm, Lowry's method (Lowry et al, 1951) at 640 nm for albumin, insulin and IgG.

The solute permeability coefficient P was determined using the following equation (2.2):

$$\ln (2Co/ Ct - 1) = 2Apt / Vl \quad \text{---------- eq. (2.2)}$$

In this expression, Co is the initial concentration of the donor cell, Ct is the solute concentration in the receptor cell at time t, V is the volume of each half cell, l is the swollen membrane thickness, and A is the effective area of permeation. A plot of Vl/2A ln (2 Co/ Ct -1) versus t yielded a slope from which the value of permeability coefficient was calculated (Stringer & Peppas, 1996) in each case. The results are discussed in section 3.1.2.8 and 3.2.8.

2.2 Characterisation of the grafted polyurethane membranes:

The instrumental techniques (IR, SEM, Mechanical properties, Contact angle and Permeation Studies) mentioned above in section 2.3 were employed for analysing the physicochemical characteristics of the grafted membranes.

In vitro studies

2.5 Biocompatibility studies:

As discussed section 1.5.1, polyurethanes are an interesting family of materials with broad possibility for processibility, superior physical and mechanical properties and biocompatibility that made them so appealing for biomedical applications. For long term use as a medical device for immunoisolation purposes, it is very important to analyse the biocompatibility of the synthesised and surface grafted polyurethanes. Hence, this section deals with the biocompatibility assessment and their suitability with pancreatic islets *in vitro*, using various standard assays. The various assays used were as follows:

i) Cytotoxicity assay using fibroblast cells(L929, NIH 3T3 cells or VERO cells)
ii) Cytotoxicity testing by MTT assay and NR assay
iii) Image analysis for analysing the morphology of the islets
iv) Islet insulin release studies

(i) and (ii) assays are employed for determining the general response of the material for use in medical device and (iii) and (iv) assays for specific response of the material towards the islet cells.

Biocompatibilities were done only with the sterilised samples.

Sterilisation by Gamma irradiation:

The samples were gamma sterilised using a Panoramic Batch Irradiator (PANBIT), using a Co^{60} source for 2.5 Mrads at a dose rate of 0.4 -0.49 Mrads / hr.

2.5.1 Cytotoxicity assay:

Assessment of the cytotoxicity is one of the tests employed for determining the biological response of the material for use in medical devices. Cytotoxicity studies were carried out using fibroblast cells (L929, NIH 3T3 or VERO cells) and mouse islet cells.

Protocol for testing using L929 cells:

The direct contact evaluation of the materials for medical devices (ASTM F 813-83) was used to assess the cytotoxicity of the material using L929 cells. Cytotoxicity assay was done with the films (1 x 1 cm^2) using direct contact of the materials with a monolayer of mouse fibroblast cells (L929).

L929 cells were subcultured from stock culture (National Centre for Cell Sciences, Pune) by trypsinization and seeded onto multiwell tissue culture plates. Cells were fed with Dulbecco's Minimum Essential Medium (DMEM), supplemented with bovine serum and incubated at 37°C in 5% CO_2 atmosphere when the cells attained a monolayer; the material was kept in contact with the cells. After 24 hrs. contact, the morphology of the cells was assessed using a phase contrast inverted microscope (Leica, WILD MPS 32, Germany) **in** comparison with the negative (cells on tissue culture plate without the material) and positive control (a toxic material like Cu wire placed in contact with the cells).

2.5.2 Cytotoxicity testing by MTT assay using NIH 3T3 cells / VERO cells:

MTT assay was performed as per standard protocol (Doyle et al, 1995) to assess their viability. Briefly, sterile membrane pieces of uniform weight (100 mg each) were incubated in 10 ml of DMEM at 37°C for 15 days. This extract containing the membrane leach out products was filtered through 0.22 μ filter and used for testing the effect of leaching products on the cells. NIH 3T3/ VERO cells were obtained from ATCC, USA, seeded on 96 well plates and incubated at 37°C in 5% CO_2, 95% air. These cells were then exposed to varying concentrations of membrane extracts for 24 hrs.

2.5.3 Islet isolation and biocompatibility testing:

Biocompatibility testing with islet cells was done only with those samples which showed promising results by MTT assay using fibroblast cells (NIH 3T3 or VERO cells).

Islets were isolated following the protocol of Shewade et al (1999). Briefly, BALB/c mice of 8 weeks were sacrificed by cervical dislocation, pancreas dissected, minced and incubated in 1 mg/ml collagenase P (Boehrringer Mannheim, Germany) on a shaker platform for 20 minutes. Freshly isolated islets were then cultured in RPMI 1640 (Gibco BRL, NY, USA) medium with 10 % FCS for 48 hrs. Islets (n = 30) were handpicked and cultured on the hydrogel coated plates (35 mm petri dishes, Nunclon, Denmark) and their morphology observed under phase contrast microscope for 5 days. Viability of islets cultured on membranes was determined by trypan blue dye exclusion test, using 0.4 % (w/v) trypan blue after 3 days. Islets staining blue were scored dead while viable islets did not take up the dye.

2.5.4 Image analysis:

Islet morphometry studies were carried out on a sophisticated image analysis system (Kontron Elektronik GmbH, Munchen, Germany) connected to a Ziess (Axioplan 2) microscope. Islets after 3 days in culture were observed, images captured with a VarioCam PCO CCD imaging camera and processed to obtain binary images. Binary images were taken in further computations, using Kontron image analysis software (KS400 ver. 2.0). Area and diameter of islets cultured on tissue culture polystyrene (TCPS) control and on membranes were measured.

2.5.5 Islet insulin release determination:

Islets were cultured in presence of membranes in RPMI 1640 with 10 % FCS for 3 days. Islets were then hand-picked and placed in 1 ml of Krebs Ringer bicarbonate buffer (pH 7.4) with 1 mg/ ml bovine serum albumin (Sigma Chemicals co., St. Louis, USA) and 10 mM HEPES (now referred as KRBH) supplemented with 5.5 mM glucose. The plates were incubated at 37°C in 5% CO_2 atmosphere for 1 hr. Islets were also challenged with KRBH containing 16.0 mM glucose and incubated for 1 hr. At the end of incubation supernatant was collected and immunoreactive insulin was assayed by RIA kit (Diagnostic Products Corporation, L.A., USA).

2.5.6 Statistical analysis:

Results are expressed as mean ± s.e.m for normally distributed data. Differences between groups were tested by t-test or Mann Whitney test as appropriate. Computations were performed using Sigma -Stat statistical package (Jandel Scientific, version 4.0 for Windows 95, SPSS Inc., Chicago, and USA).

2.6 Blood compatibility studies:

Protocol on Preliminary screening of Materials for Blood Compatibility:

The method for the preliminary screening of materials was selected from ISO 10993(1992). Sterilised samples (γ- irradiated) were taken for the blood compatibility studies. Only the promising PU and grafted PU were assessed for blood compatibility.

Samples (triplicates) were transferred into sterile petri plates. Samples were hydrated for 5 min using PBS. After aspiration of PBS, whole blood collected in ACD (1.25ml) for each sample was added. Blood sample (0.25 ml) was taken after 1 min to get initial analysis values. The samples were incubated with blood for 1 hr at 37 °C, with agitation at 70 rpm. Blood samples were collected for analysis at the end of 1 hr. Simultaneously, empty wells were treated with blood under the same conditions as in the case of material. Samples were collected for analysis to negate the effects of polystyrene on blood. Plasma was separated by centrifugation to analyse LDH and plasma Hb. Materials were rinsed thoroughly with PBS and representative sample was fixed with 2% glutaraldehyde and dehydrated with graded concentrations of alcohol for SEM analysis. Representative material after rinsing was stained with Leishman's stain and viewed under light microscope (Lieca), for assessing cell adhesion.

2.6 Biodegradation studies:

The polyurethane with hard segments 25% and 32% (PU-4 and PU-5) and the surface modified polyurethanes (G-2, G-4 and G-6) which were found better in biological assays above were assessed for biostability by enzymatically degradation studies.

The polyurethane (PU) with 25 % and 32% hard segments (PU-4 and PU-5) and the surface modified polyurethane with HEMA, NVP and EHA as grafting monomers were used for biodegradation studies. For degradation: Trypsin from Bovine Pancreas (Sigma Chemicals Company), Bromalein from pineapple stem (Sigma Chemicals Company), Tris (Hydroxy methyl) methylamine (Qualigens Fine Chemicals) and tri-Sodium Citrate (Qualigens Fine Chemicals) and Papain from papaya latex and HEPES buffer was used as given in Appendix A.

2.7.1 Treatment with enzymes:

The stability of polyurethanes in enzymes was studied by immersing PU films of size 8 x 1 cm^2 using enzymes (Trypsin, Bromalein and Papain) with appropriate buffers-100 BAEE units/ ml Trypsin, tris buffer (pH 8.1 - buffer in which trypsin was dissolved), 100 units/ ml Bromalein and citrate buffer (pH 6.0 - buffer in which bromalein was dissolved) and Papain was dissolved in HEPES buffer. The composition of the buffers and enzymes are given in Appendix A (A-5) for 1 month. After 1 month the polymer pieces were removed, cleaned with distilled water and dried. The change in properties was determined.

2.7.2 Weight Change Measurements:

After determined interval of time the films were taken out of from the solutions, washed extensively in distilled water, dried and weighed. The extent of degradation was expressed as the percentage weight change of the films before and after treatments.

2.7.3 Infra red Analysis:

Immediately before and after treatments, specimens were analysed by ATR-FTIR spectroscopy using a Nicolet Impact 410 FTIR spectrophotometer as discussed earlier.

2.7.4 Mechanical testing of films:

Mechanical properties of the samples after treatments was done using Instron UTM 1193 according to the ASTM specification D 882 (1981). The stress and percentage strain of the treated samples was compared with the untreated samples.

CHAPTER 3

RESULT AND DISCUSSION

RESULT & DISCUSSION

3.1 Synthesis and characterisation of linear segmented polyurethanes:

Among the diversified materials used in biomedical applications, polyurethane possesses an unparalleled position due to its unique properties arising from the structural features but it has not been much explored for immunoisolation of islet cells. Polyurethanes enjoy outstanding versatility owing to their range of monomers, polymerization reactions, processing, structures, properties and applications. This versatility has contributed greatly to our understanding of polymer structure relations in general and to the production of high performance elastomers. In the broadening scope of artificial organs, polyurethanes also have been incorporated into designs for an artificial pancreas. The utilization of polyurethane to encapsulate or support Islets of Langerhans is being approached. Zondervan et al (1992) crosslinked an aliphatic polyurethane with dicumyl peroxide in order to retard degradation of the polymer following implantation. Results with encapsulated Islets of Langerhans showed a good insulin response to glucose, although the speed of response was slower than that of free cells. Robert et al (1993) had reported on nonporous polyurethane membranes (consisting of aromatic diisocyanate alkyl diamine hard and soft segments of polyalkylene oxide) that are readily permeable to both glucose and insulin but impermeable to immunoglobulins.

3.1.1 Synthesis of Polyurethanes:

Polyether based polyurethane shows good biocompatibility and formulation versatility. Products ranging from soft, flexible elastomers to hard rigid plastics can be easily produced from readily available raw materials. Polyurethanes are elastomeric products of organic isocyanates, medium molecular weight polyol and low molecular

weight chain extenders formed in the condensation polymerization reaction. They are composed of short, alternating blocks of hard and soft segments. The soft segment is typically a polyether, polyester or polyalkyl diol with a molecular weight between 500 and 5000. The hard segment is a normally an aromatic diisocyanate that has been polymerized by low molecular weight diol and diamine, termed as a chain extender, to produce an oligomeric aromatic urethane or urethane-urea segment of molecular weight between 300 and 3000. PU elastomers typically exhibit a 2-phase microstructure. This phase segregation results in the superior physical and mechanical properties of these materials. Polyurethanes are usually prepared by a two step process. First the prepolymer is formed from a mixture of polyol and diisocyanates, followed by the addition of a chain extender. General reactions of these types are given in equation 3.1 and equation 3.2.

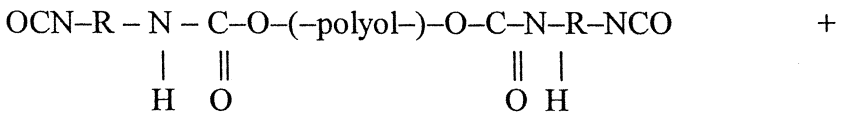
m OCN – R – NCO + HO–(– polyol–) –OH ⟶

Diisocyanate **Polyol**

OCN–R – N – C–O–(–polyol–)–O–C–N–R–NCO -----eq.(3.1)
| ‖ ‖ |
H O O H

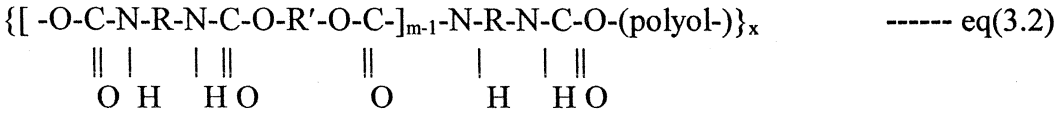
Prepolymer

The prepolymer thus formed is normally a thick, viscous liquid or low melting solid. The prepolymer is then converted into the final high molecular weight polymer by further reaction with a diol chain extender. The chain extension reaction is shown below eq. (3.2)



prepolymer

Chain Extender



------ eq(3.2)

Polyurethane

General equation for polyurethane synthesis

These polymers generally form linear primary chains due to covalent bonding with both soft and hard segments. Due to high degree of hydrogen bonding, paracrystalline regions are formed. The individual chains are long and due to extensive H-bonding the polymer appears to be crosslinked. This crosslinking can be reversed by the addition of heat or solvent. This is the reason behind their extensive use as thermoplastic elastomers.

The reaction of the isocyanate with the polyol produces the soft segment and that of the isocyanate with the chain extender gives the hard segment. The general characteristics of linear segmented polyurethane elastomers are given below:

i) The hard rigid isocyanate segments separate into glassy or semicrystalline domains.

ii) The hard domains are dispersed at low to moderate hard segment content in the amorphous or semicrystalline matrix of the macroglycol soft segments.

iii) The hard domains act as multifunctional cross-linking sites and as reinforcing fillers, thus resulting in materials which possess both high modulus and elastomeric behaviour.

iv) For the segregation into domains, the driving force is provided by the chemical incompatibility of the hard and soft segments.

In this study, polyurethanes with varying percentage of hard segments was synthesised using the standard 2-step procedure with the molar ratios mentioned in Table 2.I. Two types of polyols viz. Polypropylene glycol (PPG) and Polytetramethylene glycol (PTMG) were used alongwith toluene diisocyanate (TDI) and 1,4 butanediol (BD) for the synthesis. Initially, the diisocyanate and polyol were reacted together in a three-necked flask in stoichiometric amounts to form an intermediate prepolymer as shown in equation- (3.1). The NCO/ OH ratio was maintained to 1.1. The prepolymer having excess unreacted NCO was then converted into high molecular weight polymer by further reacting with a chain extender as in equation- (3.2). The reaction was catalysed by 0.01 wt.% dibutyl tin dilaurate.

3.1.2 Characterisation of linear segmented polyurethanes:

3.1.2.1 Infrared Spectroscopy:

IR spectroscopic method is one of the widely employed techniques to understand the morphological features of polyurethanes particularly in terms of hydrogen bonding (Brunett et al, 1982 & Seymour et al, 1970). Surface Infrared Spectroscopy couples the analytical method of IR spectroscopy with the physical phenomenon of total internal reflection to enable the molecular vibrations within the surface regions of materials to be studied.

The characteristic peaks of polyurethanes such as 3307, 2960, 1733, 156, 1536, 1598, 1221 cm^{-1} were obtained for the synthesised polyurethane, confirming that the synthesised polymers were polyurethanes. A representative IR spectrum is shown in Fig 3.1. Table 3.I gives the IR peak assignments of polyurethane. The - NH peak centred at around 3300 cm^{-1} appears to be an indication of high degree of hydrogen bonding. The free - NH peak around 3460 cm^{-1} appears as an extremely weak shoulder. The carbonyl absorption region shows 2 peaks, one at 1730 cm^{-1} of free -C = O groups and another at 1702 cm^{-1} indicative of the bonded - C = O groups.

Table 3.I

Major IR peaks for polyurethanes

Peak (cm^{-1})	Assignments
3307	NH str.
2960	CH_2, CH_3 str.
1733	Amide I, C = O str.
1536	Amide II, NH def.
1376	C-CH_3, sym., def.
1598	Phenyl
1221	-C-O polyether

Hydrogen bonding in polyurethane results from the presence of the -NH group which is the proton donor and the carbonyl or ether oxygen groups, which are hydrogen bond acceptors. The hydrogen bond acceptor may be either the hard segment (the carbonyl of the urethane group) or the soft segment (ether oxygen). Relative amounts of hydrogen bonds determine the morphology of the polyurethanes (Sung & Schneider, 1978; Bonart et al, 1980; Sung et al, 1982). The extent of hydrogen bonding in the synthesised polyurethane (Lelah & Cooper, 1986) was estimated from the ratio of the absorbances of the peaks at 1703 and 1733 cm^{-1}, as shown in equation 3.1.

Hydrogen Bonding Index , R = A_{1703} / A_{1733} ----------eq.(3.1)

Fig 3.2(a) and (b) shows the relationship of hydrogen bonds for different hard segment content for the PTMG system (x) and PPG (•) respectively. The hydrogen bonding increase with hard segment content for the PTMG system. However, in the PPG system, instead of increasing trends for the hydrogen bond with hard segment content, we also observed that the hydrogen bonding for the 40 % hard segment decreased as depicted in Fig. 3.2b. These trends of hydrogen bonding in the synthesised polyurethanes have been correlated with the permeation of model compounds such as glucose, insulin and higher molecular weight proteins as discussed in Section 3.1.2.8. Briefly, the permeation constants of smaller molecules like glucose (mol.wt. 180) was found to be affected by the hydrogen bonds trends of the PUs, whereas the permeation of the higher molecular weight proteins such as insulin (mol.wt. 6000) and also having coiled structures are found to be unaffected by the hydrogen bonding trends in polyurethanes.

In the case of insulin, the permeation constant is found to be independent of the hydrogen bonding. It seems, therefore, that the permeation of glucose with a molecular

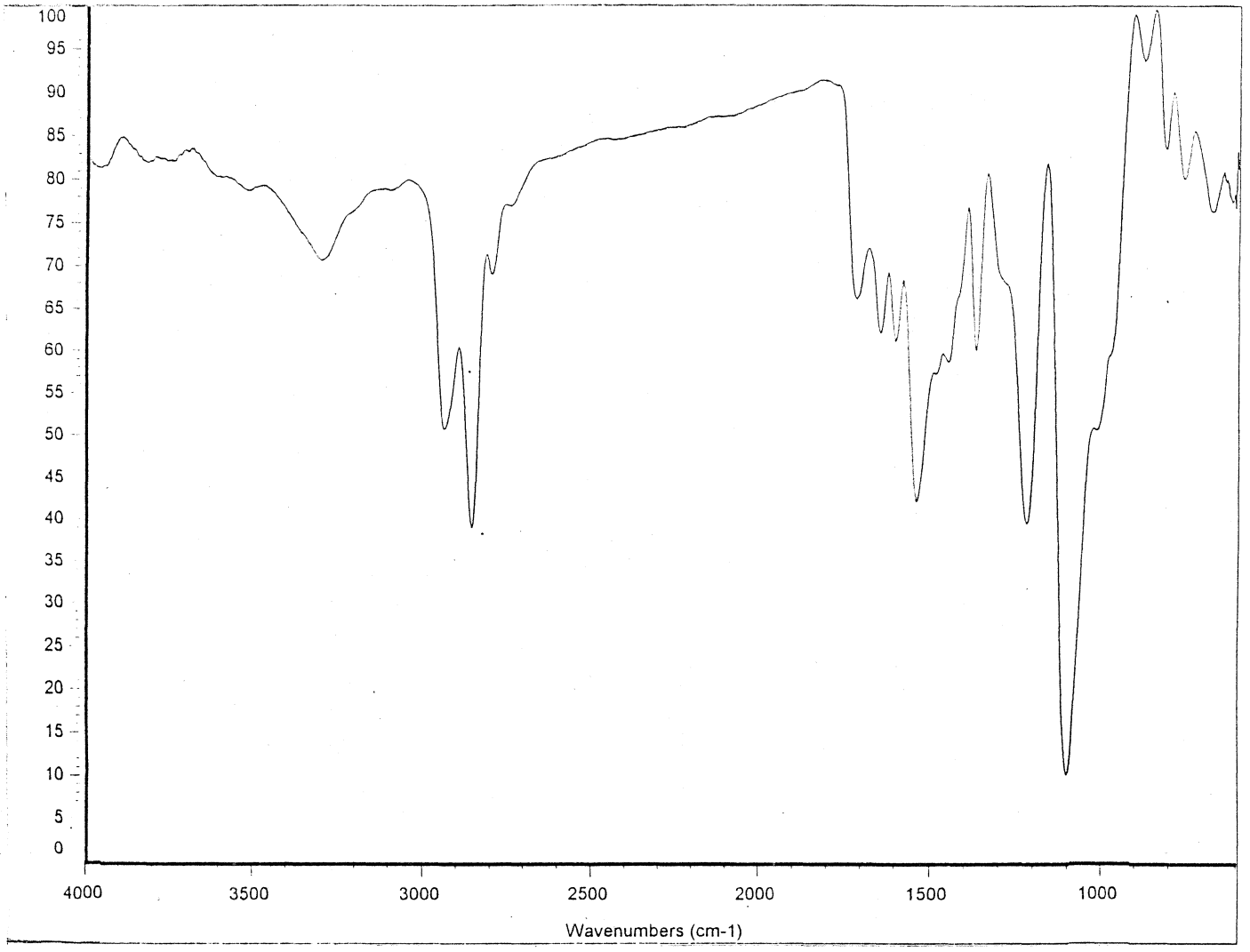


Fig. 3.1 Representative Infrared Spectrum of polyurethane

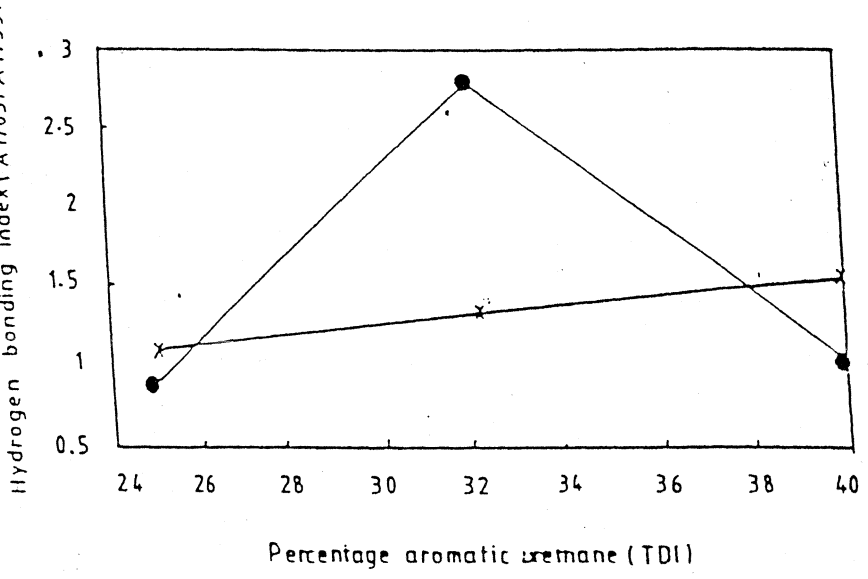


Fig. 3.2 The relationship of hydrogen bonds for different hard segments **(•)** PPG system **(x)** PTMG system

weight of 180 (being a small molecule) is affected by hydrogen bonding of polyurethanes, but the permeation of molecules such as insulin with a molecular weight of 6000 and a coiled structure will not affected by hydrogen bonding in the matrix.

3.1.2.2 Scanning electron microscopy (SEM):

SEM helps to study morphology structure of polymer and to assess the gross uniformity of the sample. To a large extent, the surface features affect the interaction of the polymers with biological molecules. An analysis of the surface features of PU is important because they help to detect the presence of any phase separation in the system. A representative SEM picture is shown in Figure 3.3. The surface shows no phase separation and a smooth nonporous membrane at all compositions.

3.1.2.3 Mechanical properties:

Mechanical properties are the totality of properties determining the response of materials to external mechanical influences, manifested in the ability of the materials to develop reversible and irreversible deformation and to resist failure. The mechanical properties of polyurethanes are dependent on a number of factors that include the type and interaction of hard and soft segments, which results in higher tensile strength (Sreenivasan, 1993). Molecular orientation in polyurethane is also important from theoretical and practical viewpoints and contributes to the better understanding of the structure -property relationships. When mechanical forces are applied on polyurethane, change in the orientation and mobility within the segmental domains can occur (Smith, 1974).

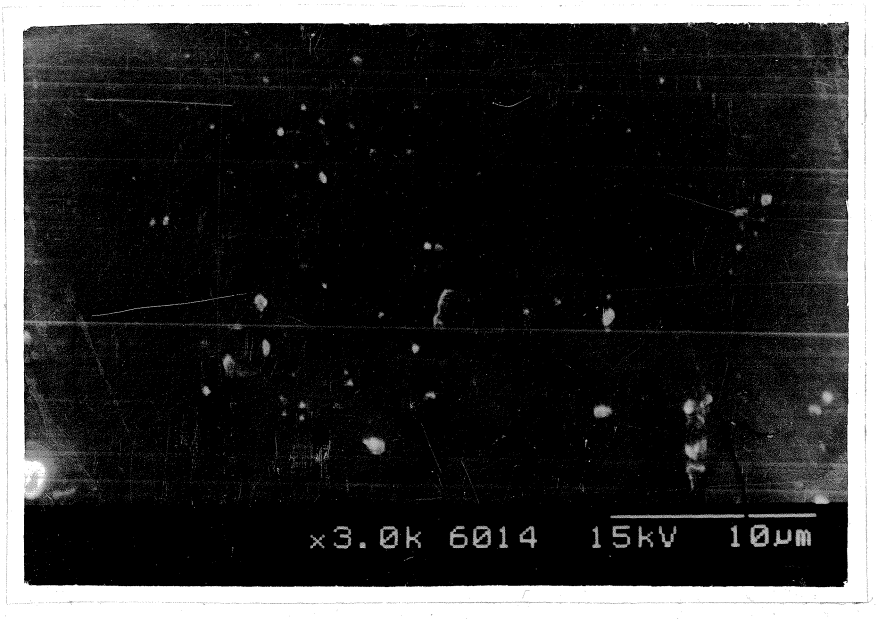


Fig. 3.3 Representative scanning electron micrograph of polyurethane

According to Allegrezza et al (1974), segmented orientation in polyurethane passes through three stages as hard segment content increases, and that at low hard segment content, very little equilibrium orientation is retained in either the hard or soft segments. In general, the hard segments acts as multifunctional crosslinking sites and as reinforcing fillers, thus enhancing the mechanical properties.

The mechanical properties of different polyurethanes with varying percentages of hard segments are given in Table 3.II. Polyurethanes prepared with PTMG as soft segment shows overall good mechanical properties than the polyurethanes prepared with PPG as soft segment. We observe that the value of stress at break for PU-2 and PU-3 (in the PPG system) is less than that for PU-1, whereas in the PTMG system, the stress of PU-4 and PU-6 is less than PU-5. This was contrary to expectation and is believed to be due to local crystallinity effects with the increase in hard segment content. It is possible that the soft segment may be under strained conditions and contributes to higher tensile stress and elongation (as in PU-5, PTMG based polyurethane with 32% hard segment).

Table 3.II

Mechanical properties of synthesised polyurethane membranes

Sample code	Stress at Peak (MPa)	Stress at Break (MPa)	% Strain at Break (%)	% Strain at maximum load
PPG System				
PU-1	6 ± 0.96	6 ± 0.94	443 ± 127.7	421 ± 118
PU-2	2 ± 0.11	2 ± 0.11	193 ± 54	193 ± 54
PU-3	2 ± 0.13	1 ± 0.13	59 ± 12.7	56 ± 12.5
PTMG System				
PU-4	2 ± 0.04	2 ± 0.04	898 ± 25	895 ± 23
PU-5	16 ± 3.3	16 ± 3.3	785 ± 25.8	785 ± 25.8
PU-6	5 ± 0.42	5 ± 0.44	204 ± 26.5	201 ± 26.2

3.1.2.4 Thermal analysis:

The term thermal analysis applies to a family of techniques that monitor primarily physical properties as a function of temperature or time at fixed temperature. Whenever a material undergoes a change in physical state, such as melting or transition from one crystalline form to another, or whenever it reacts chemically, heat is absorbed or liberated. In other words, thermal analysis comprises of a group of techniques in which a physical property of a substance is measured as a function of temperature while the substance is subjected to a controlled temperature program. Temperature and energy of transitions, weight loss, dimensional change and viscoelastic properties may be measured by techniques of thermal analysis.

Differential scanning calorimetry (DSC):

Thermal transitions in polymers are readily measured using DSC. The main quantities that are determined from a DSC curve are the temperature of the beginning and the end of the thermal event, the temperature of the peak maximum, the amount of the material involved in the transition, or the heat of transformation (Runt & Harrison, 1980). Within these general headings fall the measurement of glass transition temperature, crystallinity, purity, rate of reaction, rate of crystallization, and rate of decomposition, and the analysis of active material. Polymers are known to undergo phase changes from glassy to rubbery state as the temperature is increased. As the melt is cooled, a temperature eventually is reached at which all the long range segmental motions cease. This temperature which is indicative of the abrupt cessation of large scale polymer backbone motion is called the glass transition temperature or Tg. Rubbery polymers become stiff and brittle below the Tg. Most polymer physical properties such as brittleness, modulus and heat capacity undergo a marked change at Tg.

On the molecular scale, the glass transition temperature signals the beginning of large-scale molecular motion. Below Tg, the molecules are confined to vibrate in a localised lattice, although there is no large-scale molecular order (Seymour et al, 1973), Aitken & Jeff (1977)). The Tg varies widely with the polymer structure. For polymer, which can crystallize to any extent, there also exists a melting temperature, Tm, due to the melting of the crystalline phase.

In DSC, the sample and a reference material are subjected to a closely controlled programmed temperature variation. In case of a transition in the sample, thermal energy is added or subtracted from the sample or reference container in order to keep both at the same temperature. By varying the temperature, transitions can be observed in the DSC thermogram corresponding to the morphology. In DSC, the samples were intially heated from -50° C to 150° C and annealed. Annealing is given to induce phase mixing in PUs. In polyether urethanes, hard and soft segments that comprise the backbone segregate in the solid state into hard and soft domains and hence produce 2-phase morphology. On heating, the extended soft segments contract or relax, causing additional hard segments to be pulled out of the hard domains and phase mixing will occur. Subsequent rapid cooling creates a thermodynamic driving force for the 2 phases to separate again but the high viscosity causes demixing to occur slowly and a new equilibrium state is achieved. Soft segment Tg is sensitive to soft segment domain purity, so this method is used in DSC measurement. After first heating and annealing, a second heat is given upto 200 °C. The shift in the baseline of the second heating run is noted as the glass transition temperature. Generally, PU shows two Tg, one occurring at low temperature, which is the Tg of soft segments and other occurring at higher temperature, which is the Tg of hard segment.

Various thermal studies on PUs were done earlier (Schneider et al, 1975) showing the nature of TDI-PTMO- based PUs. The 2,6-TDI samples, having crystalline hard domains, which restrict phase mixing, exhibit a soft segment Tg that is relatively independent of hard segment content. The 2,4-TDI systems, on the other hand, give soft segment Tg values which increase with increasing hard segment content, indicative of considerable phase mixing with the amorphous 2,4-TDI based hard domains. In addition, Sung et al (1980) have investigated PUs based on TDI hard segments, ethylene diamine (ED) and butanediol (BD) chain extenders and PTMO or polybutylene adipate soft segments. They found that the extent of phase segregation improved significantly when ED was used as a chain extender instead of BD.

DSC thermograms of the polyurethanes with different percentage of hard segments are shown in Figure 3.4. The glass transition temperature (Tg) of the soft segment is observed to appear at -40 °C to 15 °C as concluded from the shift in baseline. The shift in baseline between 100 °C to 150° C indicates hard segment Tg of PU. The Tgs of the various compositions are shown in Table 3.III(i). The thermogram also shows endothermic peaks. The first endotherm centred around 40 °C to 75 °C is attributed to the disruption of domains with limited short-range order. The endothermic peak around 120-190°C, as observed in PU-2, PU-3 and PU-6 has been assigned to the dissociation of domains having long range order and the endotherm above 200°C is attributed to the disruption / melting of the microcrystallites. On critical analysis of the DSC curves for the PPG and PTMG systems, the soft segment Tg in the PPG system is more or less the same but in the PTMG system along with the increase in hard segment, the soft segment Tg increased marginally. PPG has more free volume than PTMG because of the $-CH_3$ branching in PPG while PTMG molecule is linear. So, the PPG system shows a Tg at lower temperature while the PTMG system shows Tg at a higher temperature. At 15°C, the PTMG system shows a sharp peak at Tg. It was suspected to be due to the

Table 3.III (i)

Tgs of the synthesised polyurethane membranes

Sample code	Tg of soft segment (°C)	Tg of hard segment(°C)
PU-1	-12.3	100.1
PU-2	-12.5	100.2
PU-3	-12.2	-
PU-4	13.0	120.0
PU-5	13.1	-
PU-6	15.0	100.1

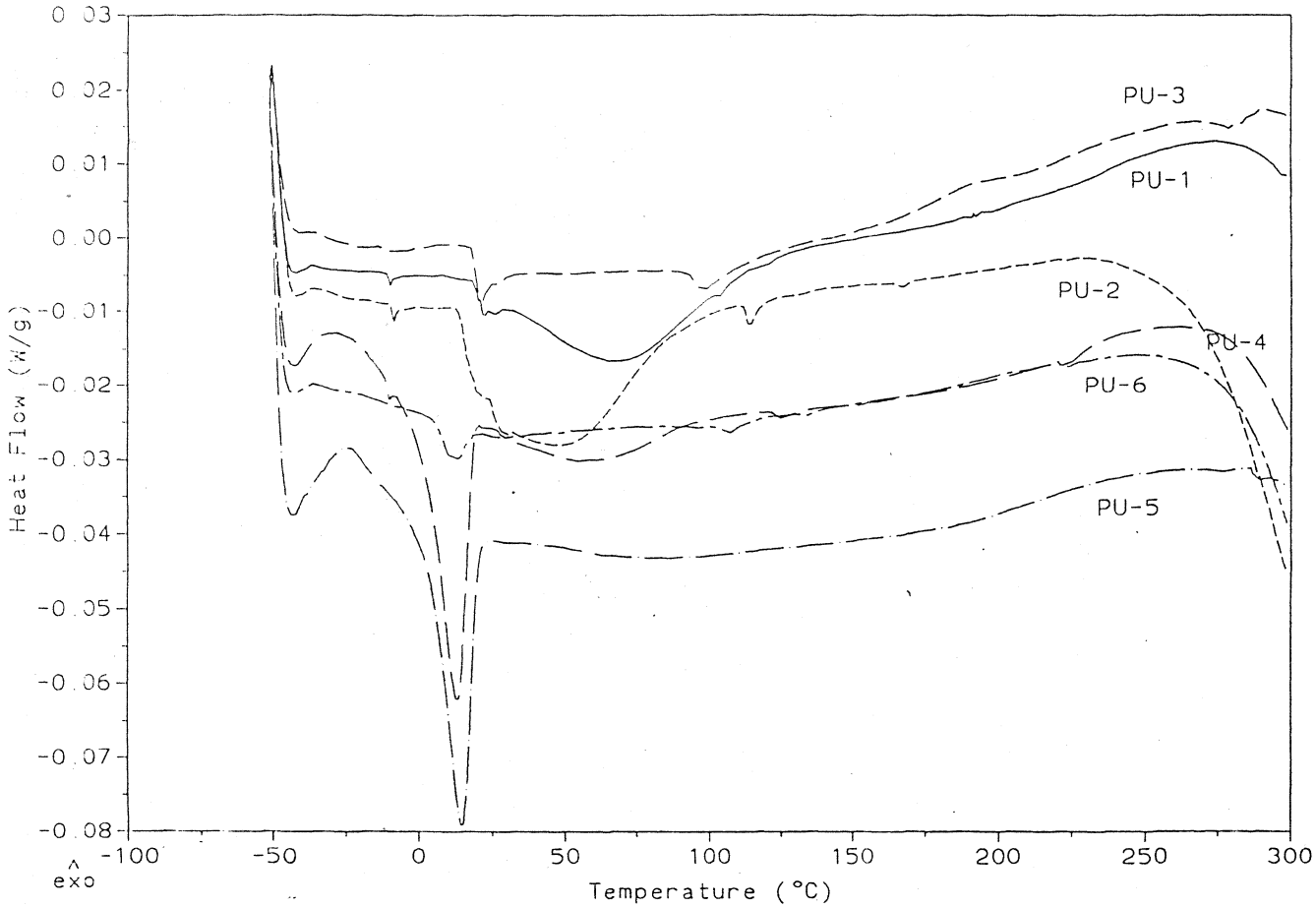


Fig. 3.4 DSC curves of polyurethane **(a)** PU-1(PPG-25%) **(b)** PU-2 (PPG-32%) **(c)** PU-3 (PPG-40%) **(d)** PU-4(PTMG-25%) **(e)** PU-5(PTMG-32%) (f) PU-6 (PTMG-40%).

semicrystalline nature of PTMG as compared to PPG. The further confirmation of the semicrystallinity of PTMG based PUs was done by polarised microscopic analysis and wide angle X-ray diffraction studies as discussed later in the sections 3.1.2.5 and 3.1.2.6 respectively.

Thermogravimetric analysis (TGA):

The technique of TGA involves change in weight of the material under examination as the temperature is increased at a predetermined and preferably at a linear rate. Typical applications include the assessment of thermal stability and Decomposition and some information on sequence distribution in copolymer and composition of filled polymers.

Fig 3.5 shows the representative thermogram of PU with 25% hard segment of the PTMG system (PU-4). The DTG curve of PU shows two distinct stages of decomposition. The first stage of decomposition corresponds to urethane bond breaking and occurs at 313 °C (Fig 3.5). Stage II is the polyol decomposition, which occurs at 433 °C. The thermal decomposition of a representative PU (PTMG based PU with 25% hard segment) shows the initial decomposition at 285.90 °C, 50% decomposition at 425.47 °C and final decomposition at 453.97 °C, as observed in Fig 3.5. The TG curves of the synthesised PUs with varying percentages of hard segment, is shown in Fig3.6.

Thermal Stability of Polyurethanes:

A measure of thermal stability of polyurethane can be ascertained from the temperature of 50% decomposition. The thermal decomposition temperatures are

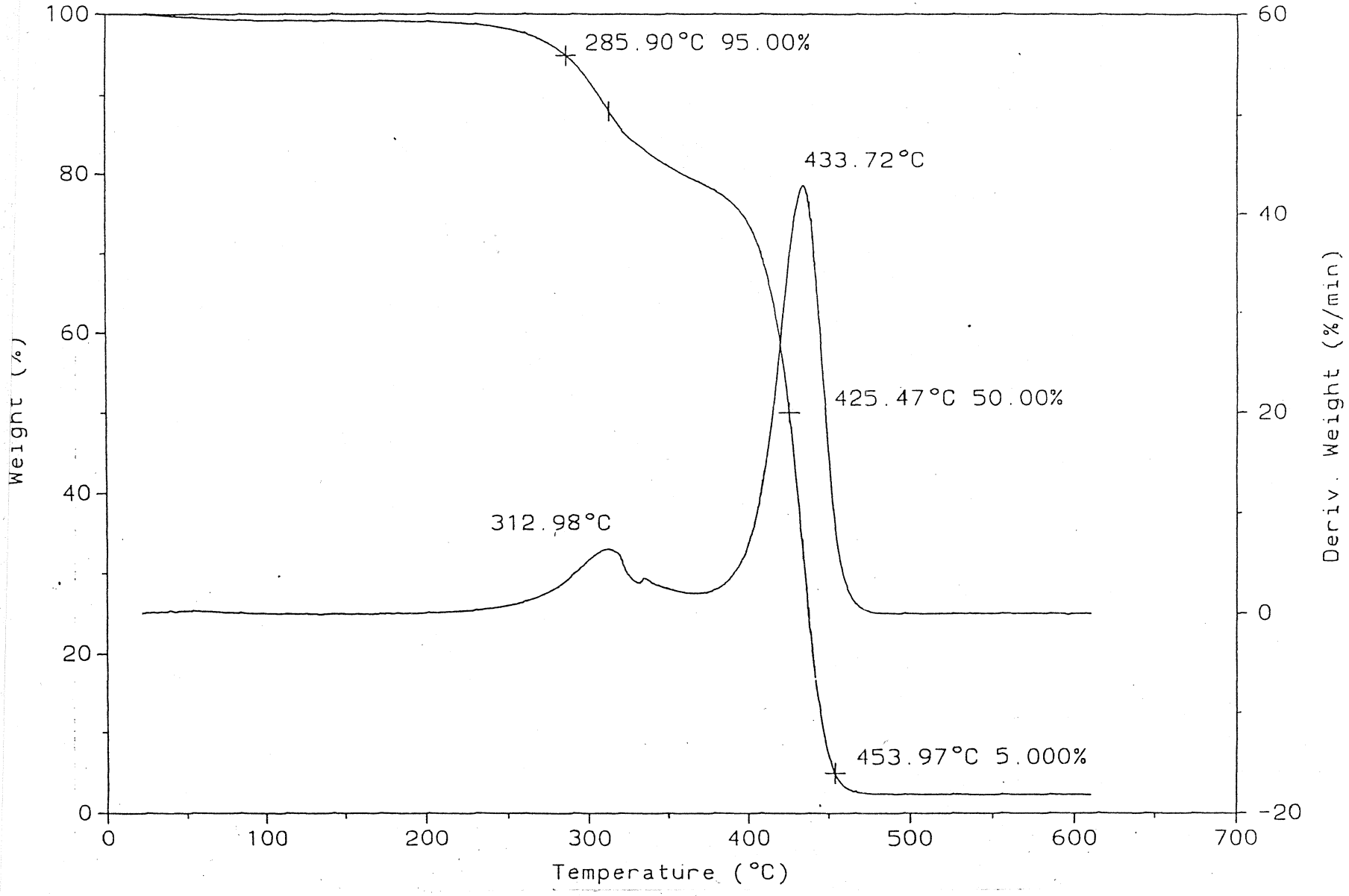


Fig. 3.5 Representative thermogravimetric analysis of polyurethane

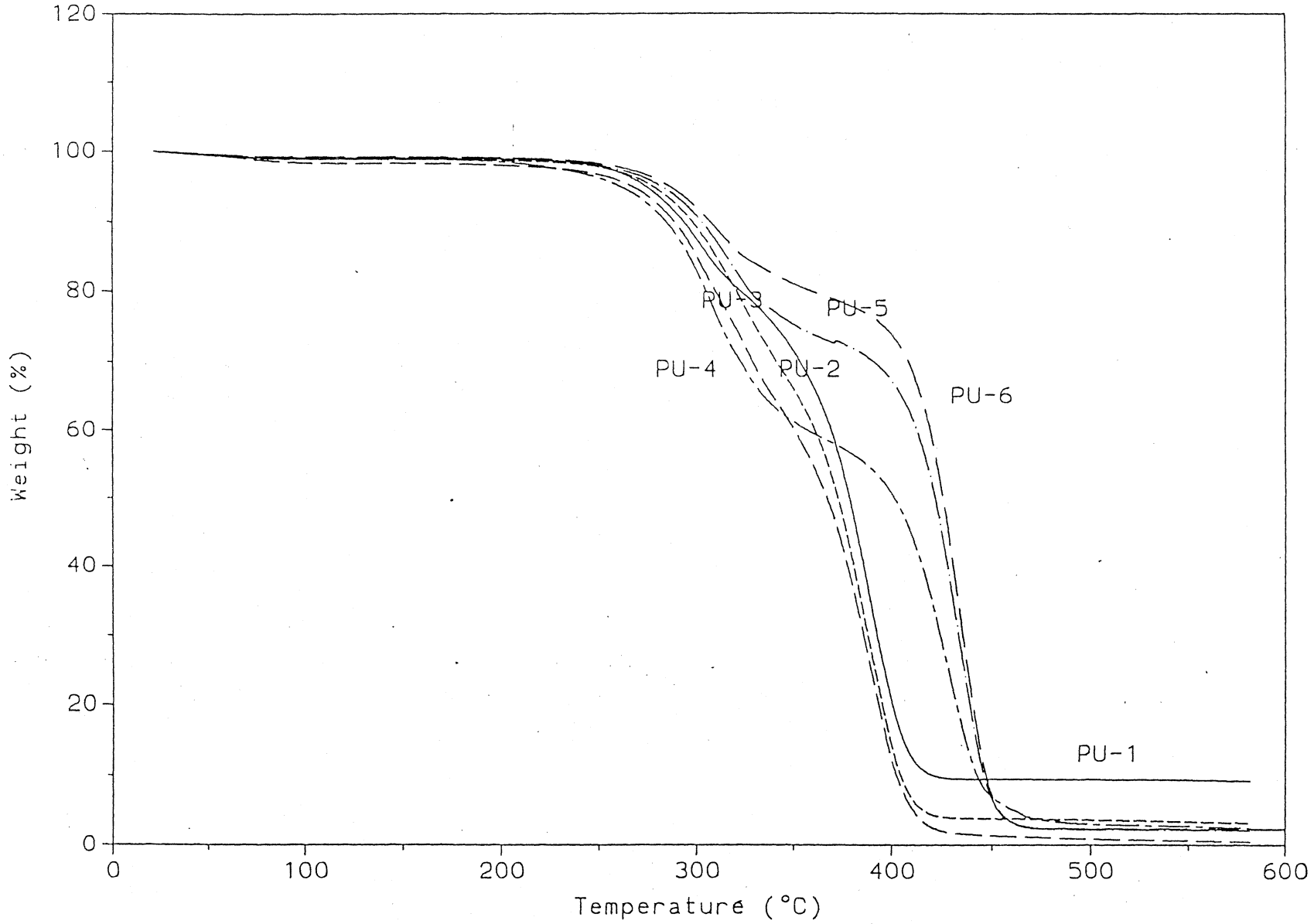


Fig. 3.6 TG curves of polyurethanes **(a)** PU-1(PPG-25%) **(b)** PU-2 (PPG- 32%) **(c)** PU-3 (PPG-40%) **(d)** PU-4(PTMG-25%) **(e)** PU-5(PTMG-32%)

given in Table 3.III(ii). Decomposition temperature for initial degradation, at 50% weight loss and the final decomposition of polyurethanes are indicated. Accordingly as observed from Table 3.III(ii), among the PPG and the PTMG system, the latter is found to be more thermally stable than the former, PU-4 is more thermally stable than the remaining polyurethanes as it has higher value of temperature at 50 % decomposition. The decomposition is completed at the final temperature, Tf that is also given in Table 3.III(ii).

Table 3.III(ii)

Thermogravimetric data of the synthesised polyurethanes

Sample code	%Hard segment	Initial decomposition temperature (Ti) (°C)	50% decomposition temperature (°C)	Final decomposition temperature (°C)
PU-1	25	273.78	378.60	581.89
PU-2	32	277.38	372.67	415.86
PU-3	40	266.77	366.93	410.91
PU-4	25	285.90	425.47	453.97
PU-5	32	281.65	421.53	453.30
PU-6	40	260.73	401.45	461.22

3.1.2.5 Wide angle X-ray diffraction studies:

Fig 3.7 shows the WAXD curves of PU (a) PPG (b) PTMG. The WAXD curves conclusively indicate that PTMG (PU-4), in particularly shows the crystalline peak in contrast to the PPG. However, an intense sharp peak centred around 28° to 30° in PTMG (PU-4 and PU-5) with 25% and 32% hard segments respectively, suggests that some crystallinity exist in this composition. But, this peak is not so prominent in PU-6. This is also reflected in the DSC scans of the PTMG systems, PU-4 and PU-5 with 25% and 32% hard segments, as shown in Fig. 3.4, where the soft segment Tg is depicted as a sharp and intense peak. However, in PU-6 the intensity of

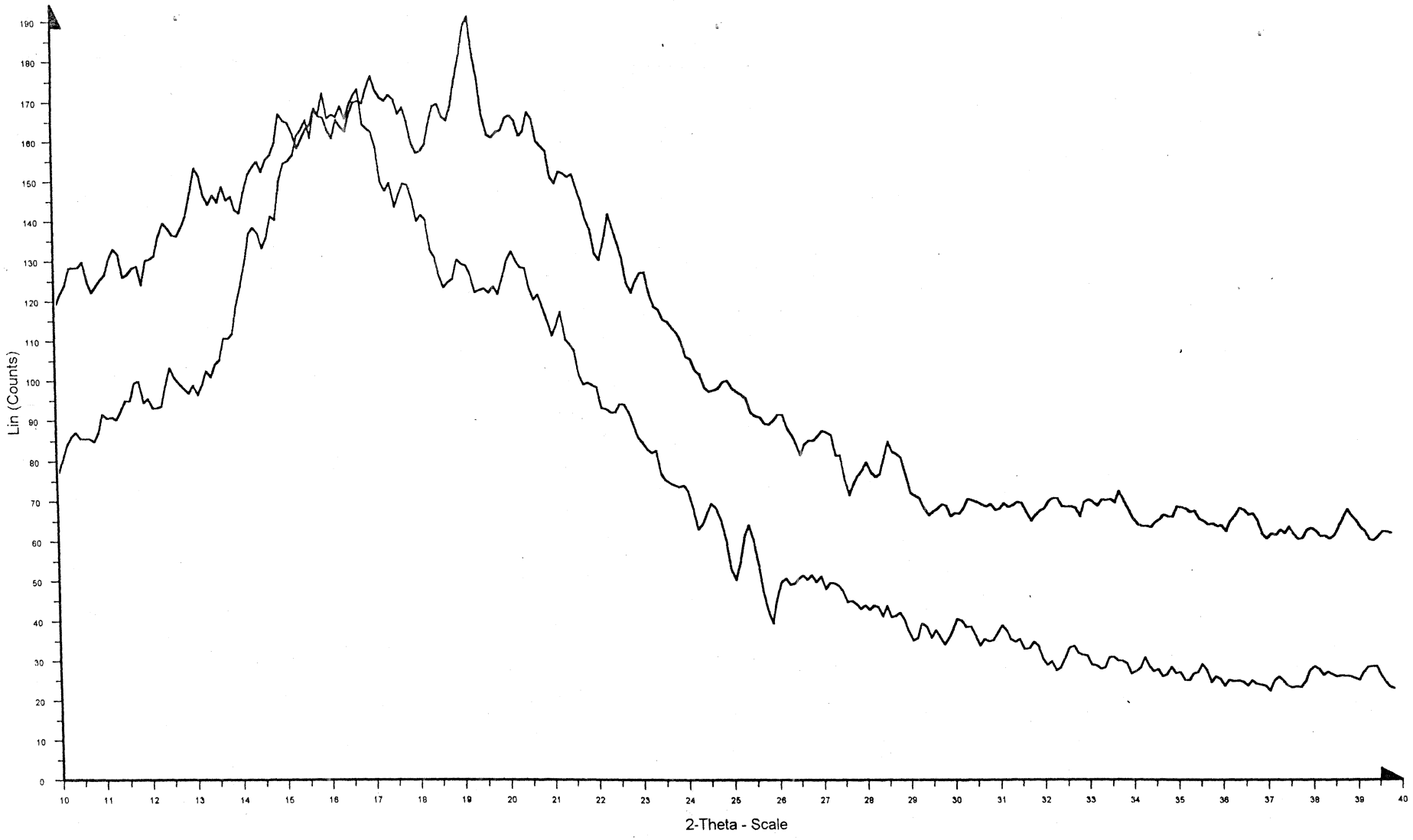


Fig. 3.7 Representative WAXD curves of polyurethanes (a) PPG system (i) PU –1 (ii) PU-2

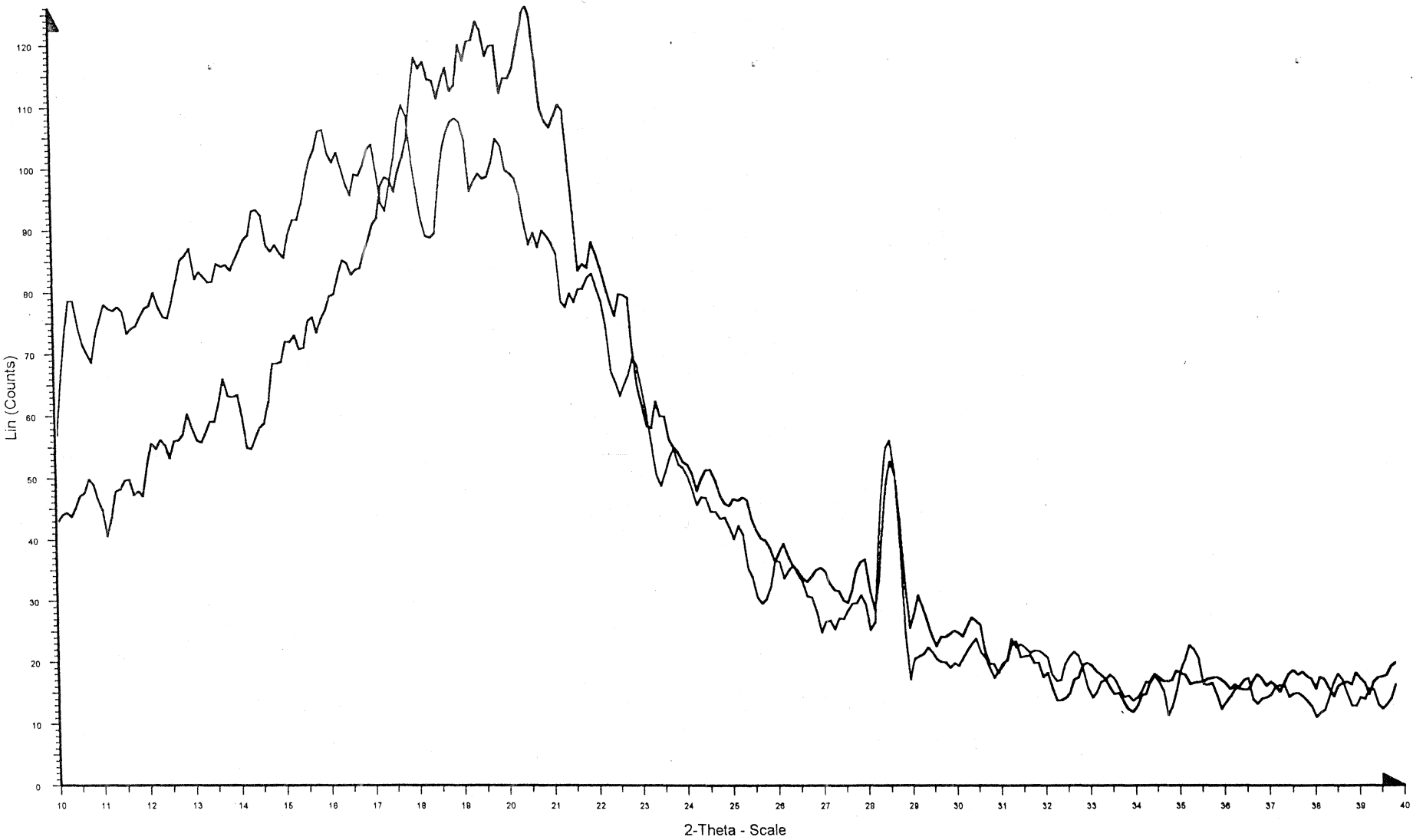


Fig. 3.7 Representative WAXD curves of polyurethanes (b) PTMG system (i) PU –4 (ii) PU-5

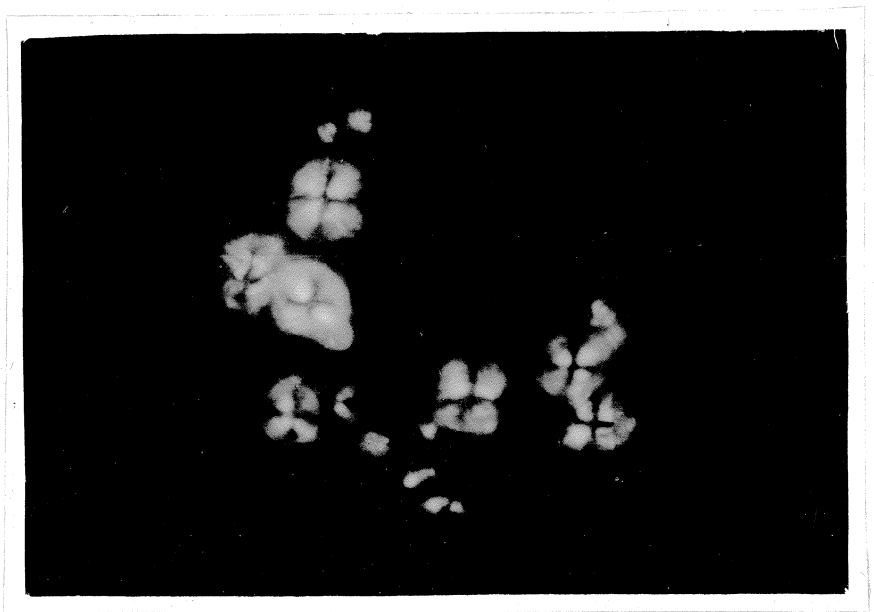


Fig. 3.8 Optical polarised micrograph of polyurethane (PTMG system) PU-4 (Mag.600 X)

the peak had decreased which was also seen in WAXD analysis. In PUs, the crystallinity arises mainly due to the orientation of soft segment and with the increase in the hard segment content, the soft segment content is expected to decrease resulting in the decreased ability for crystallization. Hence, it can be explained that with the increase in hard segment, the soft segment crystallinity decreases. However in PPG system, an intense halo, centred around 19-20 ° shows short range ordering in the polymers which in fact reflected in DSC scans also at around 10 °C, as depicted in Fig 3.4. As seen from Fig. 3.4 showing the DSC curves of the polyurethanes with varying percentages of hard segment, the PTMG system shows a sharp peak at 15 °C, which is absent in PPG based PUs. Hence by polarising microscopic analysis and WAXD studies it is confirmed that this peak is due to the semicrystalline nature of PTMG as compared to PPG.

3.1.2.6 Polarized microscopy:

Optical photomicrograph of the polymer samples recorded using crossed polarizers is shown in Fig 3.8. Spherulite-like structure is seen in the PTMG system. By close examination it can be seen that the structure are not of maltese type. Spherulite -like star shaped structures can be seen in the PTMG system (PU-4, in particular) which may be due to some sort of crystallinity. It may also be due to the birefringes arising from the differences in polarizibilities of hard and soft segments. These structures are however not seen in the polyurethanes synthesised using PPG as glycol, indicating the amorphous nature of PPG system.

3.1.2.7 Contact angle studies:

Adsorption and segregation depend on surface chemistry as well as on surface free energy. Determination of contact angles (advancing, receeding, or critical) by use

of goniometer is a convenient and simple method of measuring the surface free energies of the polymeric samples. Determination of contact angle in an aqueous environment is an important consideration in the determination of solid surface energies in systems of biological interest. Since biomaterials for blood contacting applications, interfere with blood, which has a surface tension close to that of water, measurements of contact angle of polymers in the water phase is desirable. The contact angle of polymers was related to surface wettability and hydrophilicity of polymers. Contact angle decrease with increase in surface wettability and hydrophilicity. Contact angles of the polyurethanes were determined using captive air bubble technique (Wang & Cooper, 1983). A decrease of the surface air -water contact angle (θ_{air}) and increase of the octane water contact angle (ϕ_{octane}) is suggestive of the hydrophilicity (Hamilton, 1974) in a system. According to Table 3.IV, the polyurethane with hard segment of 25% (PU-1) with air of 42.75 ° is more hydrophilic in the PPG series .The polyurethane with 25% hard segment (PU-4) is also found to be most hydrophilic in the PTMG series. Between the PPG and PTMG, the polyurethane prepared using PTMG, as glycol is more hydrophilic and this may be due to the hydrophilicity of PTMG polyol as compared to PPG polyol.

Interfacial surface free energy:

The characterisation of the gel-water interface is considered, particularly with respect to obtaining the interfacial free energy between the polymer and water, γ_{sw} utilising contact angle data. Air in water and octane in water, contact angle data for the PU were used to estimate γ_{sv}^{d}, γ_{sv}^{p}, γ_{sv}, γ_{sw} and γ_{wv} for the polymer water interface as a function of bulk water fraction in the polymer. γ is the interfacial free energy, related to solid-liquid γ_{sv}, liquid-vapour γ_{wv} and solid-liquid γ_{sw} interfaces. The minimum interfacial free energy hypothesis states that if the polymer-water interface has a very low interfacial free energy, then protein adsorption should be very low and highly reversible. According to Young's equation, $\gamma_{sv} - \gamma_{sw} = \gamma_{wv} \cos\theta$, where θ is the

equlibrium contact angle at the three phase junction. According to Hamilton's method (Hamilton, 1974),

$$I_{sw} \cong 50.5 (1 - \cos \theta)$$

$$\gamma_{sv}^{p} \cong \frac{I_{sw}^{2}}{4 \times 50.5}; \qquad \gamma_{sv}^{p} \cong \frac{(\gamma_{sv} - \gamma_{sw}) - I_{sw} + 72.1}{9.3}$$

Where I_{sw} is the non-dispersive interactions at the solid-water interface, θ is the solid-octane contact angle.

$$\gamma_{sv} = \gamma_{sv}^{d} + \gamma_{sv}^{p}$$

Then the interfacial surface free energy of solid liquid interface is,

$$\gamma_{sw} = \gamma_{sv} - (\gamma_{sv} - \gamma_{sw}) \qquad \text{------eq. (3.2)}$$

Interfacial surface free energies (γ_{sw}) of samples were calculated using θ_{air} and ϕ_{octane}. The interfacial free energy is related to blood compatibility of polymers. As the interfacial surface free energy approaches zero, the polymer becomes more blood compatible. According to Table 3.IV, the interfacial surface free energy of PU-4 is very low, which may be an indication of more blood compatibility.

Table 3.IV

Surface Parameters for synthesised Polyurethanes

Sample code	Air-water Contact angle θ_{air} (°)	Octane-water contact angle ϕ_{octane}(°)	γ_{sw} (interfacial Free energy between solid and water)
PU-1	43 ± 1	142 ± 1	1.3
PU-2	47 ± 1	133 ± 1	1.8
PU-3	52 ± 1	125 ± 1	2.7
PU-4	29 ± 1	136 ± 1	1.2
PU-5	49 ± 1	131 ± 1	2.3
PU-6	46 ± 1	99 ± 1	13.5

3.1.2.8 Permeation studies:

Membrane permeabilities were measured using a two-chamber dialysis cell. The experimental procedure is discussed in detail in section 2.3.8 and permeation coefficient was determined as in eq- (2.2). We had chosen model compounds such as glucose, insulin, albumin and immunoglobulin. The results of the permeability studies through PU membranes are summarised in Table 3.V (b). Robert et al (1993) had reported on nonporous polyurethane membranes (consisting of aromatic diisocyanate alkyl diamine hard and soft segments of polyalkylene oxide) that are readily permeable to both glucose and insulin but impermeable to immunoglobulins. Robert et al (1993) have also reported that the permeation constants, P, for glucose as 1.1 x 10^{7} cm^{2}/s, insulin as 0.266 x 10^{7} cm^{2}/s, and albumin as 0.00116 x 10^{7} cm^{2}/s through nonporous polyurethane membranes. Serum dependent cell lines (RAJI and MOPC- 31 C) could be grown and maintained successfully in these nonporous dense polyurethane membranes for at least 2 months. However, the permeation constants of glucose and insulin reported by them are low. In the present study, novel polyurethane consisting of aromatic diisocyanate alkyl diol hard and soft segments of hydrophilic polyalkylene oxide was synthesised. We have obtained higher permeation for glucose and insulin (Table 3.V a and b) through these polyurethanes. In general, the permeation of hydrophilic solutes like glucose should be higher in more hydrophilic systems (in this case, the 25% of PPG, PU-1 and PTMG series, PU-4). However, it is observed that the permeation was also dependent on the hard segment content of the polyurethanes for both PPG and PTMG series. The hydrogen-bonding tendency increased with the hard segment content in the case of PTMG, but the permeation of glucose in this system decreases with increase of hydrogen bonds (Fig.3.9). As stated earlier, the hydrogen bonding in the case of PPG was not uniformly increased except for the 32% hard segment content. Permeation constants for glucose proportionately decreased with increased hydrogen bonding. Although hydrogen bonds are weak, they

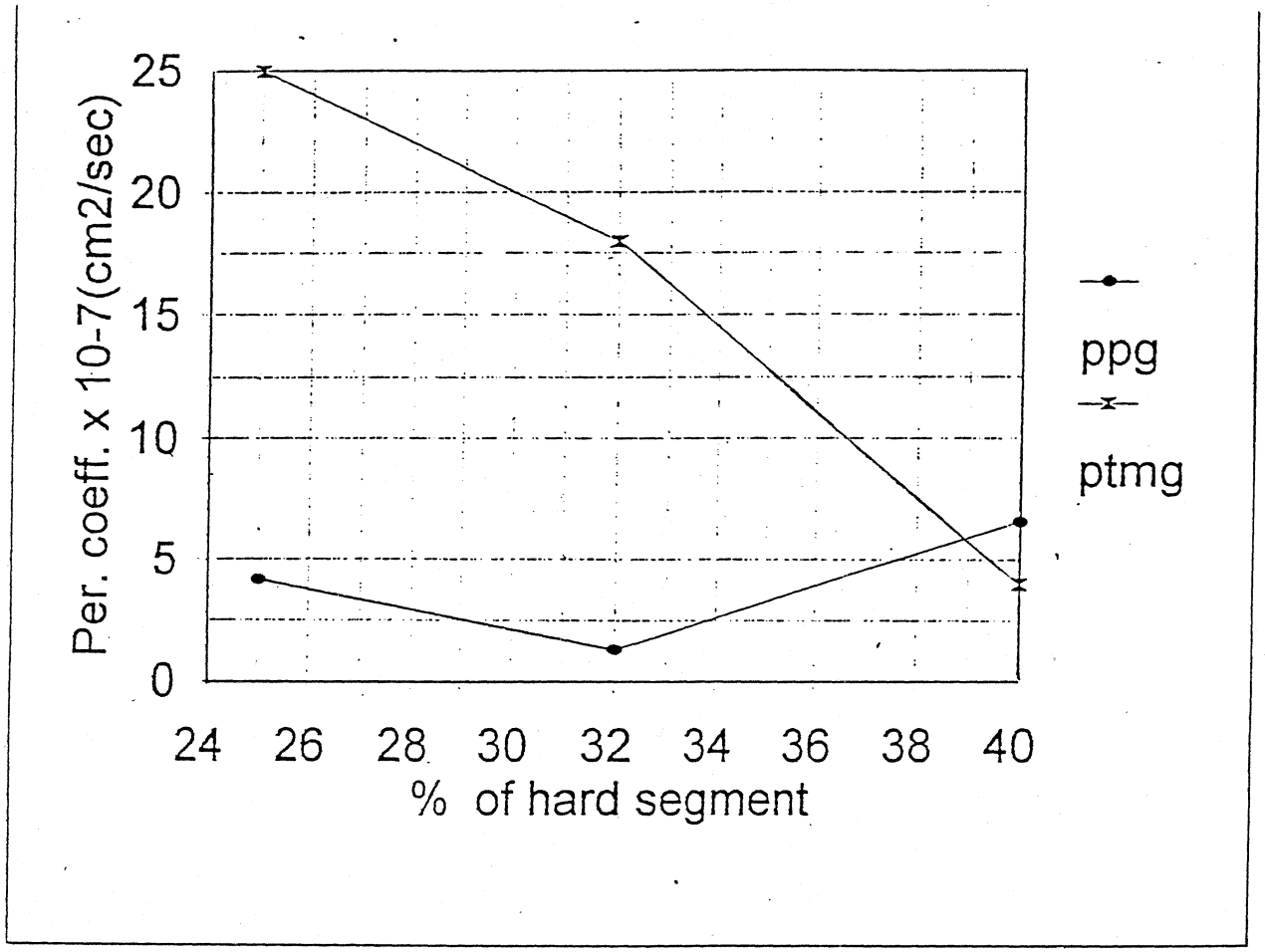
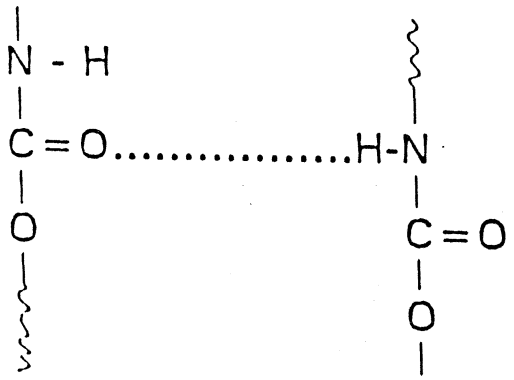


Fig. 3.9 Variation of permeability with percentage of hard segments in polyurethanes **(•)** PPG system **(x)** PTMG system

may form virtual crosslinks that in turn reduce the permeation (George & Nair, 1999). The virtual urea to urea crosslinking is shown below:



VIRTUAL CROSSLINKING
URETHANE TO URETHANE

In the case of insulin, the permeation constant is found to be independent of the hydrogen bonding. The permeation constant for insulin is also higher than that determined by Robert et al (1993). It seems, therefore, that the permeation of glucose with a molecular weight of 180 (being a small molecule) is affected by hydrogen bonding of polyurethanes, but the permeation of molecules such as insulin with a molecular weight of 6000 and a coiled structure will not affected by hydrogen bonding in the matrix.

The permeation constants for albumin and immunoglobulins were not determined because the concentration of albumin permeated was negligible. Table 3.V(a) gives the actual amount permeated through these membranes in one hour interval in moles per litre. It is evident that a higher amount of glucose can permeate through these membranes. The membranes are also permeable to insulin but impermeable to albumin and immunoglobulins.

Table 3.V (a)

Amount permeated through polyurethane membranes in 1 hour

Sample Code	Glucose (mmol/L)	Insulin (mmol/L)	Albumin (mmol/L)	Immunoglobulin (mmol/L)
PPG system				
PU-1	0.11	0.0021	0.00010	0
PU-2	0.10	0.00082	0.0035	-
PU-3	0.15	0.00082	0.0000821	-
PTMG System				
PU-4	0.8	0.002	0.0000328	0
PU-5	0.10	0.0015	0.000073	-
PU-6	0.09	0.0030	0.0020	-

Table 3.V (b)

Permeation Coefficient for Glucose and Insulin

Sample Code	Glucose[a]	Insulin[a]
PPG system		
PU-1	4.2	1.8
PU-2	1.3	0.4
PU-3	6.6	3.8
PTMG System		
PU-4	25	0.28
PU-5	18	2.3
PU-6	4	1.6

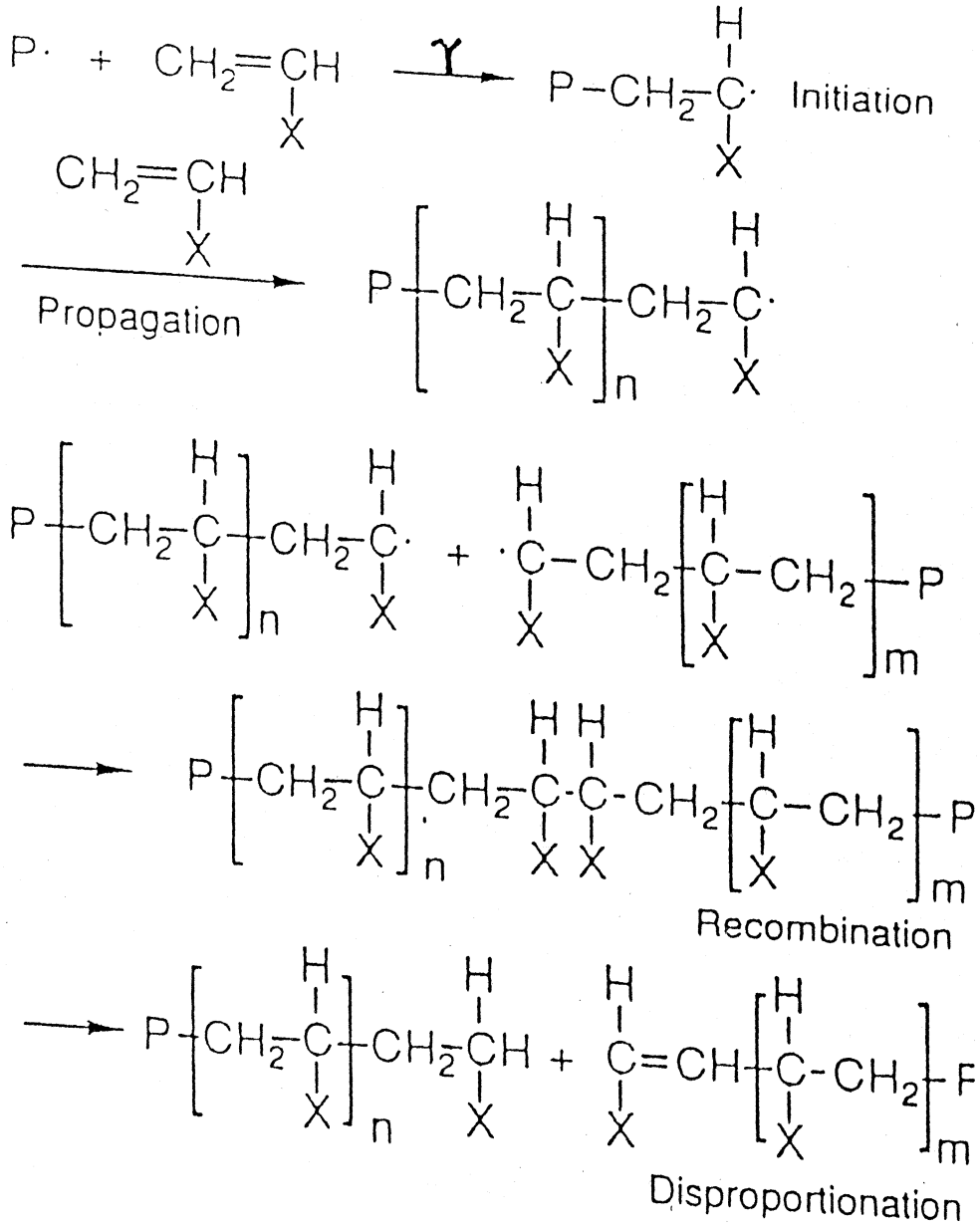
[a] Values are permeability x 10^{7} (cm^2/sec)

Hence, the synthesised polyurethane membranes have adequate physicochemical properties, permeation properties in particular. Permeation analysis shows that higher amount of glucose can permeate through these membranes. The membranes are also permeable to insulin but impermeable to albumin and immunoglobulins. Further cell contact nature of these membranes is discussed in chapter 3.3 and 3.4 for their use as immunoisolation barriers for islet cell encapsulation.

Section 3.2

3.2 Characterization of surface modified polyurethane membranes:

Surface modification of polymers is one of the methods to develop materials having appropriate surface properties and bulk properties. Such synthetic approaches potentially can maintain a polymer's desirable bulk properties and at the same time provide new, different interfacial properties. The most important advantage of surface modification strategy is the possibility of designing separately the substrate and the surface i.e. the substrate with suitable physical and mechanical properties for its targeted applications and the surface with improved biocompatibility as well as physiological activity. One of the easiest ways to surface modify the polyurethane, as suggested by several researchers (Boretos et al, 1968, 1967 & Chavapil, 1979) is by grafting other entities. Grafting of various hydrophilic and hydrophobic monomers onto segmented polyurethane membranes offers an alternative technique for improving the biocompatibility properties of these materials while maintaining some of the properties of the backbone polymer and the side chain graft polymer. In order to achieve this, various vinyl monomers such as N- Vinyl pyrrolidone (NVP), Hydroxyethyl methacrylate (HEMA) and ethyl hexyl acrylate (EHA) had been grafted on the synthesized polyurethane membrane. The scheme for a typical polymerisation of a vinyl polymer is given in Fig. 3.10.



Where P denotes the polymer,

$CH_2 = \underset{\displaystyle X}{\underset{|}{CH}}$ denotes typical vinyl/acrylic monomer

Fig. 3.10 Typical polymerisation of a vinyl/acrylic monomers

With an ultimate aim of having possible better materials for immunoisolation of islets, an effort has been made to surface modify the synthesized polyurethane membrane (with 25 % hard segment content), which was found to be one of the promising material for islet cell immunoisolation, by radiation grafting.

3.1.1 Synthesis of surface grafted polyurethanes:

Grafting of vinyl monomers such as hydroxyethylmethacrylate (HEMA), N-Vinylpyrrolidone (NVP) and ethyl hexyl acrylate (EHA) onto polyurethane was achieved by γ-irradiation. The crosslinker, 2wt% ethylene glycol dimethacrylate (EGDMA) was used. 0.005 M Cu^{+} was incorporated to prevent homopolymerization of the monomer. Cleaned polymer pieces were immersed in a mixture of monomer, crosslinker (EGDMA) and Cu^{+} for varied time periods. The swollen films were pressed slightly between the folds of filter paper to remove adhered monomers and immediately subjected to γ- irradiation from Co^{60} source (Panoramic batch irradiator, BARC Bombay) under a blanket of nitrogen. The dose rate was 0.5 Mrads for 3 hrs. The materials were extracted extensively with distilled water (PU-g-HEMA and PU-g-NVP) or toluene(PU-g-EHA), after the grafting procedure to remove the ungrafted chains.

As discussed earlier in section 2.2.2 (eq. 2.1), the grafting yield was determined from:

$$\% \text{ Grafting} = (W - Wo / Wo) \times 100 \quad \text{---------- eq. (2.1)}$$

Where W is the weight of the graft polymers and Wo is the initial weight

10-15 % grafting yields were obtained. The composition of grafted polyurethane membranes is given in Table 2.II. The flow chart of the synthesis of surface grafted polyurethanes is given in Fig. 2.2. A detailed discussion of the grafting procedure is mentioned in chapter 2.

The physicochemical characterization of these modified grafted membranes is discussed in the following sections. The techniques for characterizing these membranes were the same as that of the synthesized polyurethane membranes as discussed in the earlier section 3.1.

3.2.2 Physicochemical characterization of grafted polyurethanes:

3.2.2.1 IR Studies :

i) Poly (urethane-g-HEMA): Typical IR spectrum of poly (urethane-g-HEMA) is shown in Fig.3.11. The spectrum shows peaks centered around 3500 cm^{-1} and 900 cm^{-1}, characteristics of grafted poly (HEMA). The -C=O absorption peak of polyurethane appears around 1722 cm^{-1}.

ii) Poly (urethane-g-NVP): The IR spectrum shows peak centered around 1670 cm^{-1} arising from the -C=O stretching mode of P (VP) branches, further grafting of NVP onto polyurethanes. The peak at 1722 cm^{-1} is due to the -C = O groups of polyurethane. Typical IR spectrum shown in Fig. 3.12.

iii) Poly (urethane-g-EHA): The peaks centered at 1400 cm^{-1} could be assigned to the grafted EHA chains. The carbonyl peak of poly (EHA) chains however merges with the carbonyl absorption peak of polyurethane (1722 cm^{-1}). The broadening of the peak in the CO- absorption region is possibly a manifestation of these two peaks. Fig. 3.13 shows the typical IR spectrum of the poly (urethane-g-EHA).

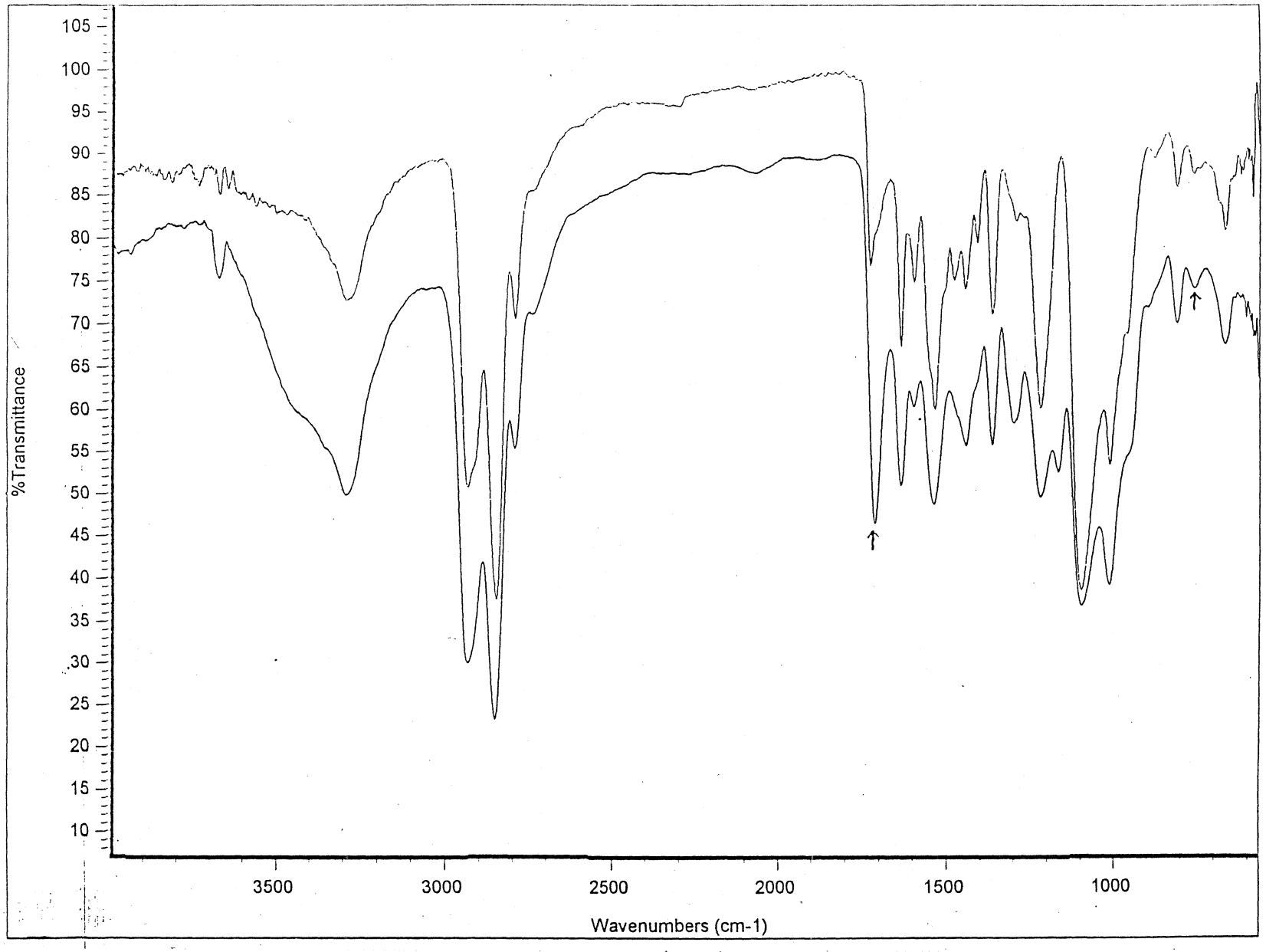
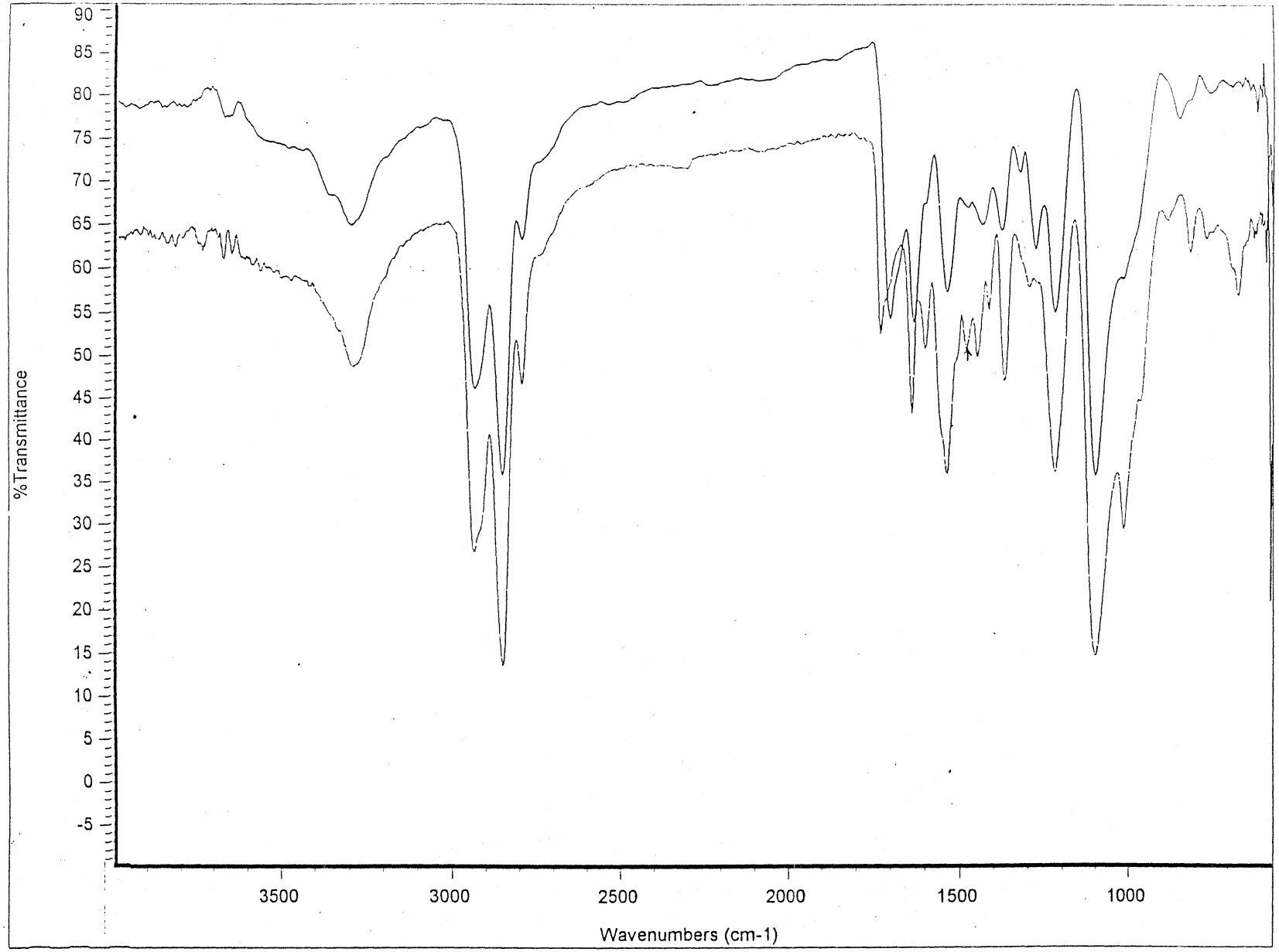


Fig. 3.11 Infrared spectrum of PU - g - HEMA


%Transmittance
90
85
80
75
70
65
60
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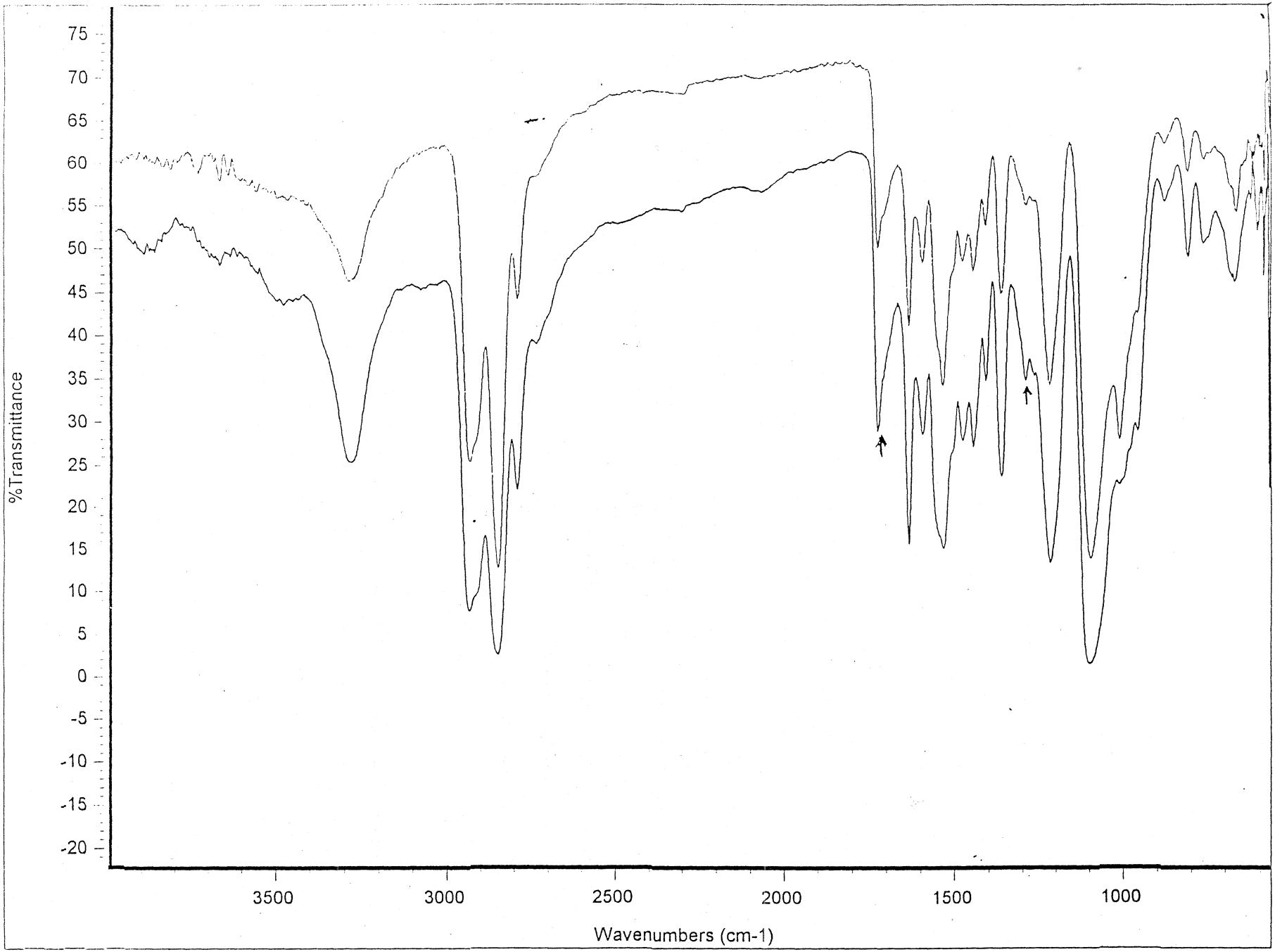
Wavenumbers (cm-1)


75
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10
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0
-5
-10
-15
-20
%Transmittance
3500
3000
2500
2000
1500
1000
Wavenumbers (cm-1)

3.2.2.2 Scanning Electron Microscopy (SEM) :

SEM photographs of the grafted polyurethanes membranes are shown in Fig.3.14. While the ungrafted membrane, as shown in Fig. 3.3, appeared to have a smooth surface. On grafting the PU membrane, the surface gets altered and seems that the grafted species is localised.

3.2.2.3 Mechanical Properties :

Table 3.VI summarises the ultimate stress strain parameters of the grafted polyurethane membranes. Compared with the ungrafted polyurethane, the grafted membranes have lower values of stress and strain. The ultimate strain is considerably reduced in the grafts. The flexibility of polyurethane, as is known, arises due to the soft segments. The reduction in strain with grafting reflects the confinement of grafting to the soft segment. The reduction in ultimate stress and strain, as reported by Jansen and Ellinghorst, 1985, may be a manifestation of the morphological changes induced by grafting. The probabilities of soft segment orientation along the direction of stress and stress induced soft segment crystallinity can be considered as additional factors in deciding the ultimate mechanical parameters (Agarwal et al).

The grafting of the monomer chains, particularly to the soft segments, is certainly affected by these factors resulting in a gross reduction of the mechanical properties. The grafting process also affects the interaction among the domains and hence effecting the mechanical properties.

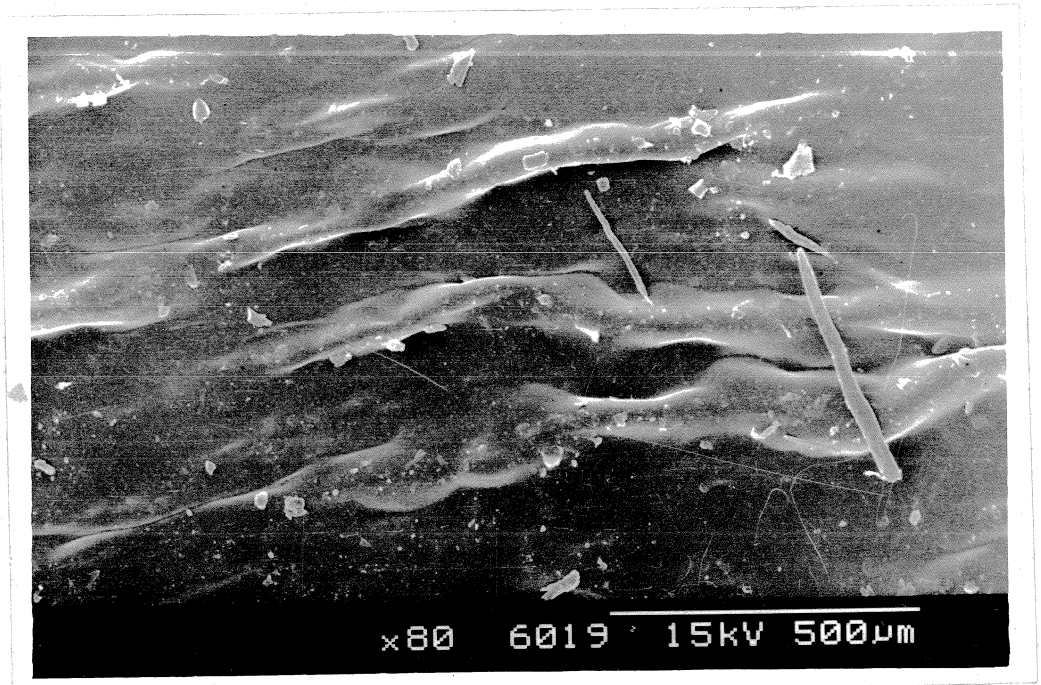

Fig. 3.14 Representative SEM of grafted polyurethane (a) PU-g-HEMA

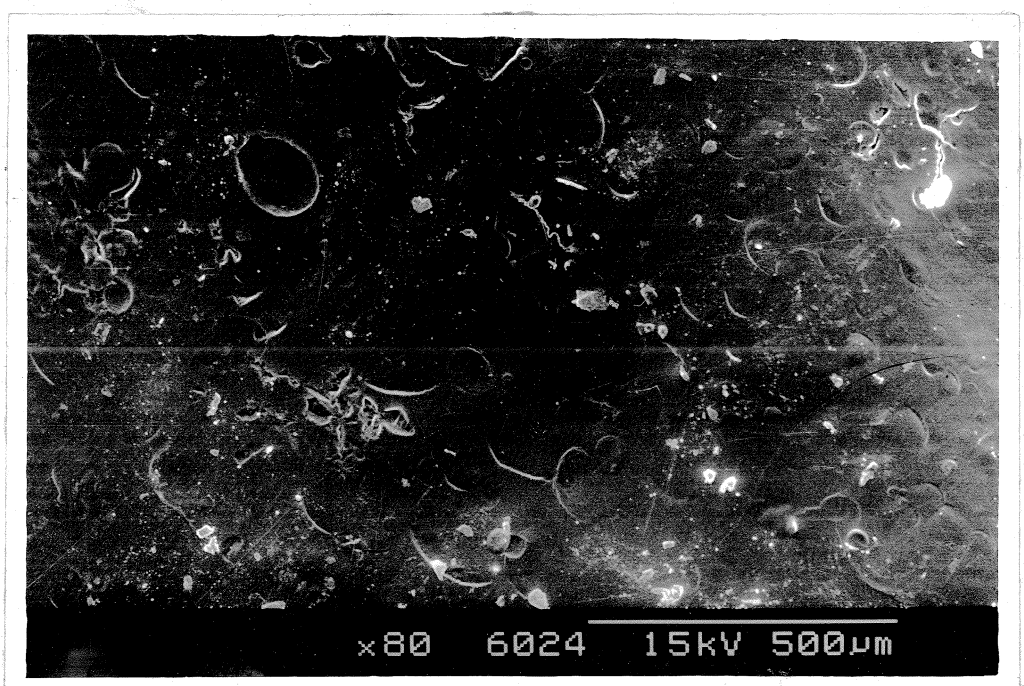

Fig. 3.14 Representative SEM of grafted polyurethane (b) PU-g-NVP

Table 3.VI

Mechanical Properties of the ungrafted and grafted polyurethanes

Sample code	Composition	Stress at Break (Mpa)	% Strain at maximum load
PU-4	TDI-PTMG-BD (25%)	2 ± 0.03	895 ± 32
G-2	PU-g-HEMA	2 ± 0.25	675 ± 22
G-4	PU-g-NVP	1 ± 0.03	674 ± 25
G-6	PU-g-EHA	2 ± 0.03	700 ± 28

3.2.2.4 Thermal Properties :

3.2.2.4.1 Differential Scanning Calorimetry :

The differential scanning calorimetry analysis of the grafted polyurethanes show similar thermal behaviour as in the bare polyurethane. However apart from the glass transition temperature of soft segment and hard segment and crystalline peak at 15 °C of the PTMG system and other endotherms of polyurethane as discussed earlier in Section 3.1.2.4, the DSC thermogram of grafted polyurethanes shows additional endotherms of the grafted species.

Poly (urethane-g-HEMA) membrane (G-2) shows additional endotherm at 91.48 °C, which can be assigned due to the grafted HEMA. Fig 3.15 (a) shows the DSC curves of ungrafted membrane, PU-4 and PU-g-HEMA (G-2). The peak due to HEMA is further confirmed by the DSC thermograms of polymerised HEMA alone which also shows a broad endotherm at around 90 °C, as depicted in Fig 3.15(b). The Poly (urethane-g-NVP) membrane (G-4) shows an endotherm at around 100 °C, which may be due to grafted NVP molecules. Fig 3.16(a) shows the DSC curves of ungrafted membrane, PU-4 and grafted membrane PU-g-NVP (G-4). This is further

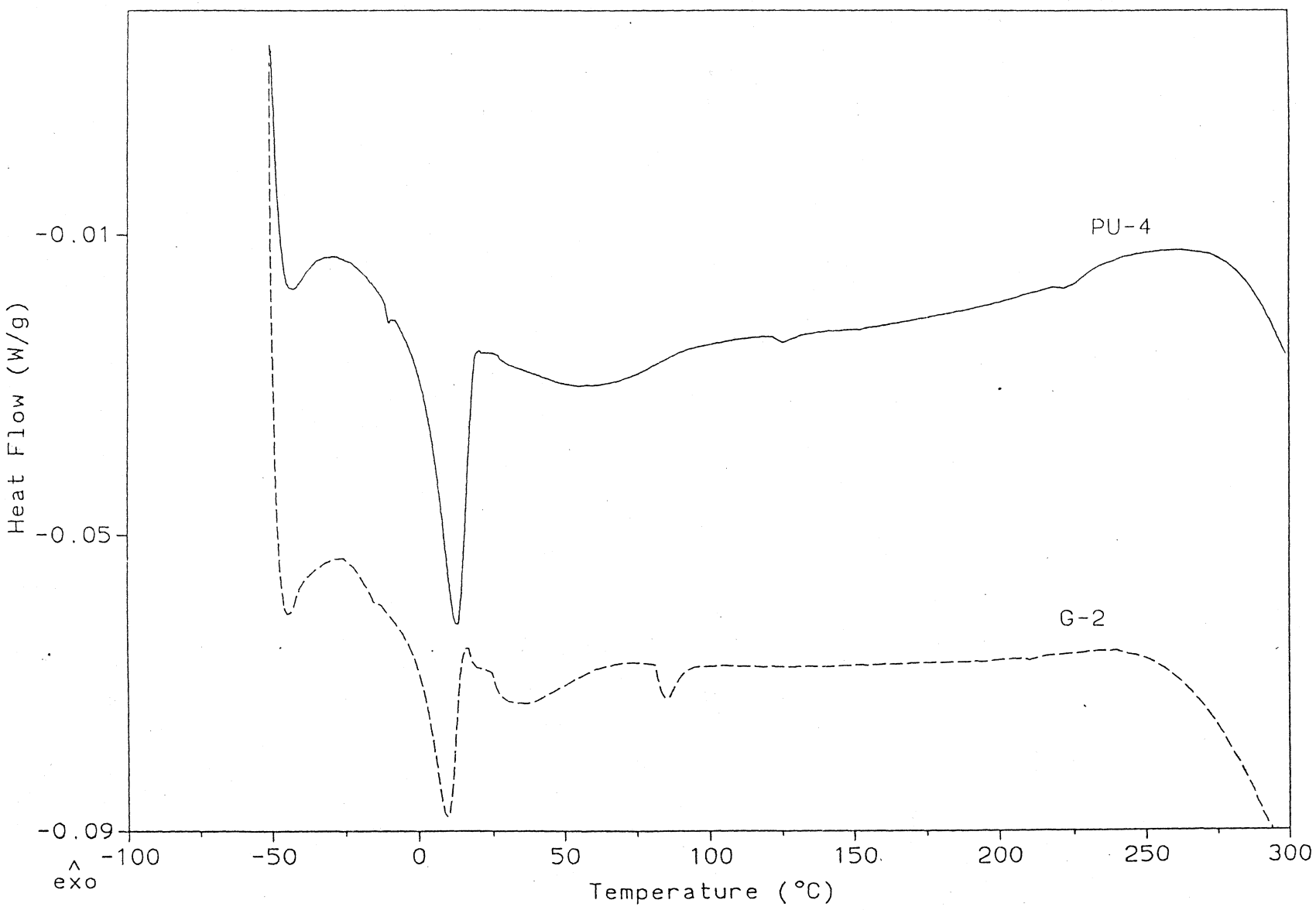

Fig. 3.15 (a) DSC curves of PU - 4, PU - g - HEMA

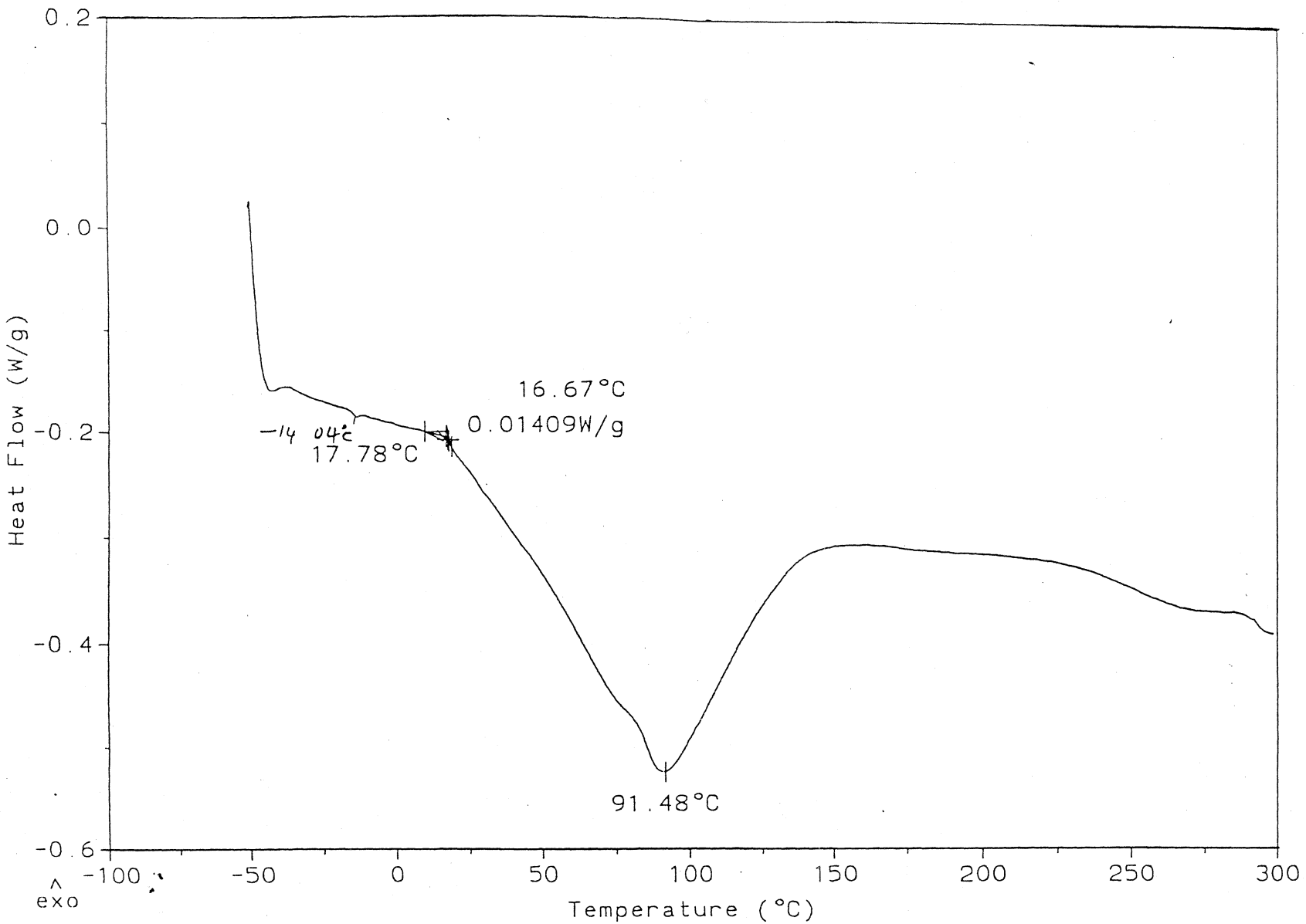

Fig. 3.15 (b) DSC curve of polymerised HEMA

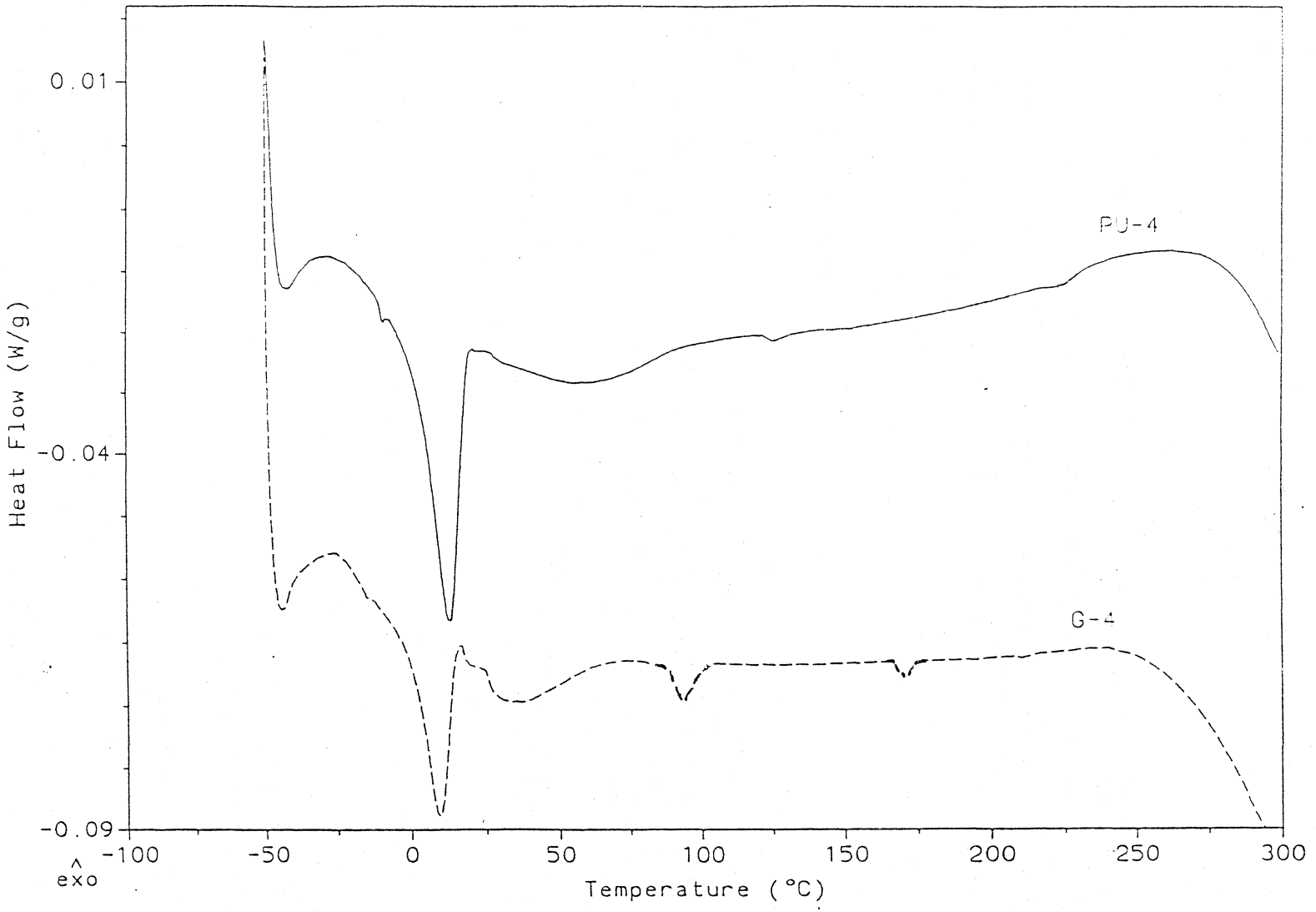

Fig. 3.16 (a) DSC curves of PU - 4, PU - g - NVP

confirmed by the DSC thermograms of polymerised NVP, alone which also shows a broad endotherm at around 100 °C, as depicted in Fig 3.16(b).Poly (urethane-g-EHA) membrane (G-6) shows an endotherm at around 25°C. Fig. 3.17 (a) shows the DSC curves of ungrafted membrane, PU-4 and grafted membrane PU-g-EHA (G-6). This is further confirmed by the DSC thermogram of polymerised EHA, alone which also shows an endotherm at around 25 °C (Fig. 3.17b).

Besides these in all the thermogram of the grafted membranes, the sharp endotherm at around -15 °C that is due to the soft segment crystallinity of PTMG is observed, though the intensity of the peak has reduced with grafting. The grafting process might have reduced the crystalline nature of the soft segment, tending it more towards the amorphous nature.

3.2.2.4.2 Thermogravimetric Analysis:

The derivative thermogram shows 2 peaks at around 325 °C and 425 °C corresponding to the urethane bond breaking and the polyol decomposition respectively. The thermogravimetric analysis shows a gradual weight loss. Table 3.VII shows decomposition temperature for initial degradation, at 50% weight loss and final decomposition of the ungrafted and the grafted polyurethane membranes.

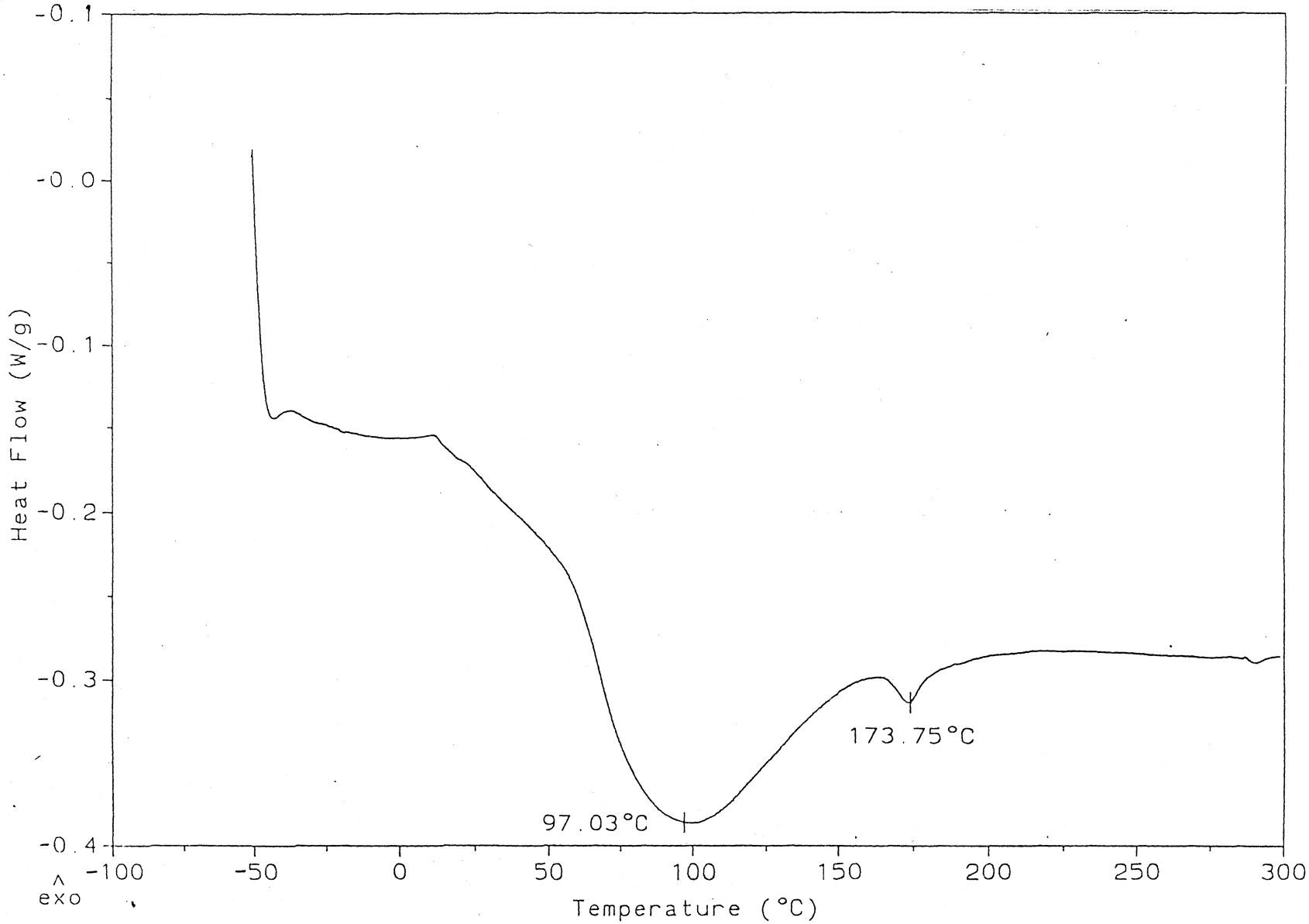

Fig. 3.16 (b) DSC curve of polymerised NVP

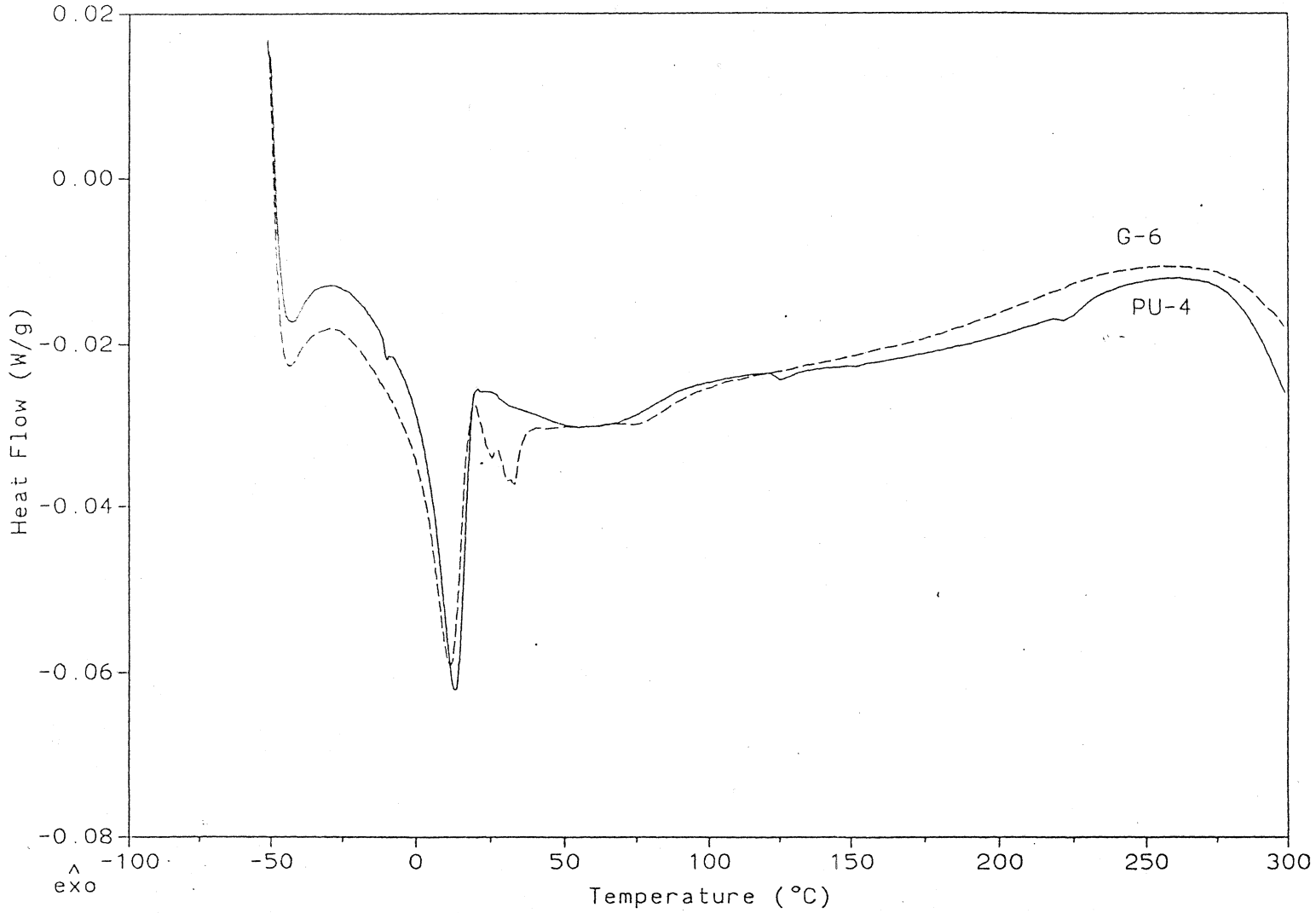

Fig. 3.17 (a) DSC curves of PU - 4, PU - g - EHA

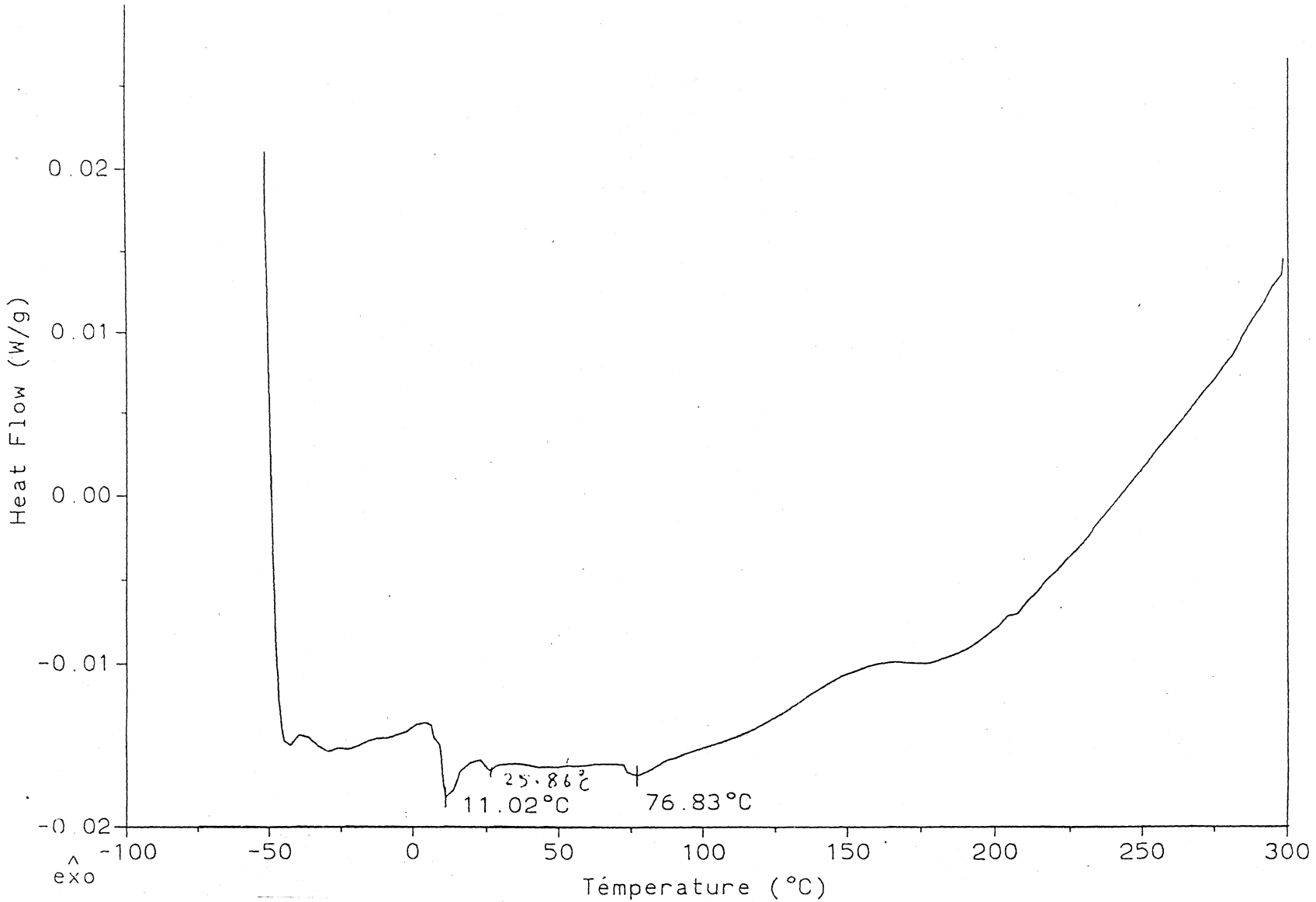

Fig. 3.17 (b) DSC curve of polymerised EHA

Table 3.VII

Thermogravimetric decomposition temperatures of ungrafted and grafted polyurethane

Sample code	Initial decomposition temperature(Ti) (°C)	50% decomposition temperature (°C)	Final decomposition temperature (Tf) (°C)
PU-4	285.90	425.47	453.97
G-2	273.92	413.76	437.15
G-4	273.01	413.58	438.83
G-6	274.38	411.18	443.61

Since the thermal stability of the material can be ascertained from the temperature of 50% decomposition. According to Table 3.VII, it is evident that by grafting of these monomers on PU, the thermal stability of these materials is not much affected. Figures 3.18 shows the TG curves of PU-4, PU-g-HEMA (G-2), Fig 3.19 shows the TG curves of PU-4, PU-g-NVP (G-4) and Fig. 3.20 shows the of TG curves of PU-4, PU-g-NVP (G-6) respectively.

3.2.5 Wide Angle X-ray Diffraction (WAXD):

The representative WAXD curves of the grafted (PU-g-HEMA) and the ungrafted samples as depicted in Fig 3.21, shows a peak centred at around 28° to 30° as that shown in the ungrafted sample. However, the intensity of the peaks had reduced slightly. This observation confirms that some sort of crystallinity is retained in the grafted samples. Similar behaviour was observed for other grafted materials.

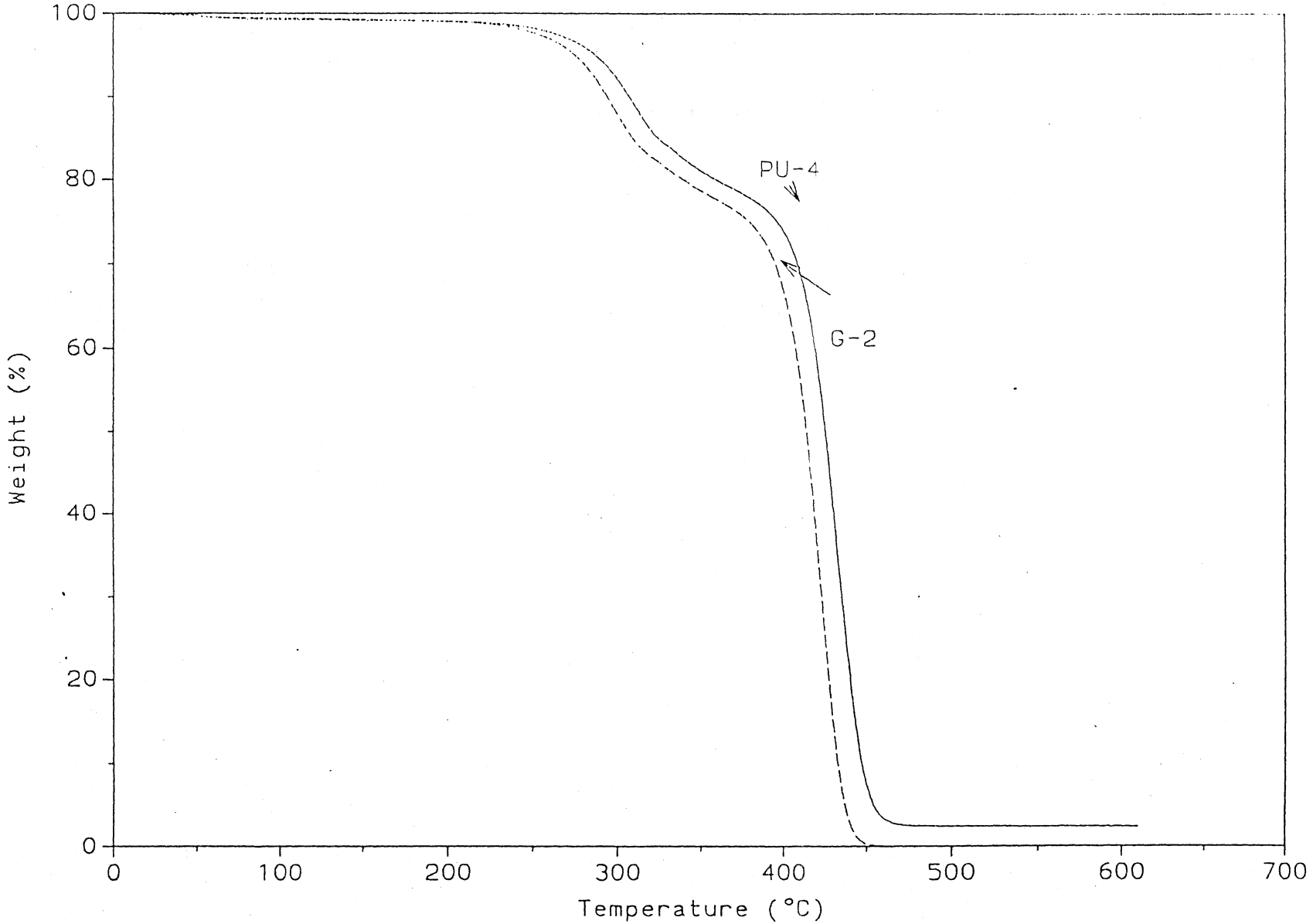

Fig. 3.18 TG curves of PU - 4, PU - g - HEMA

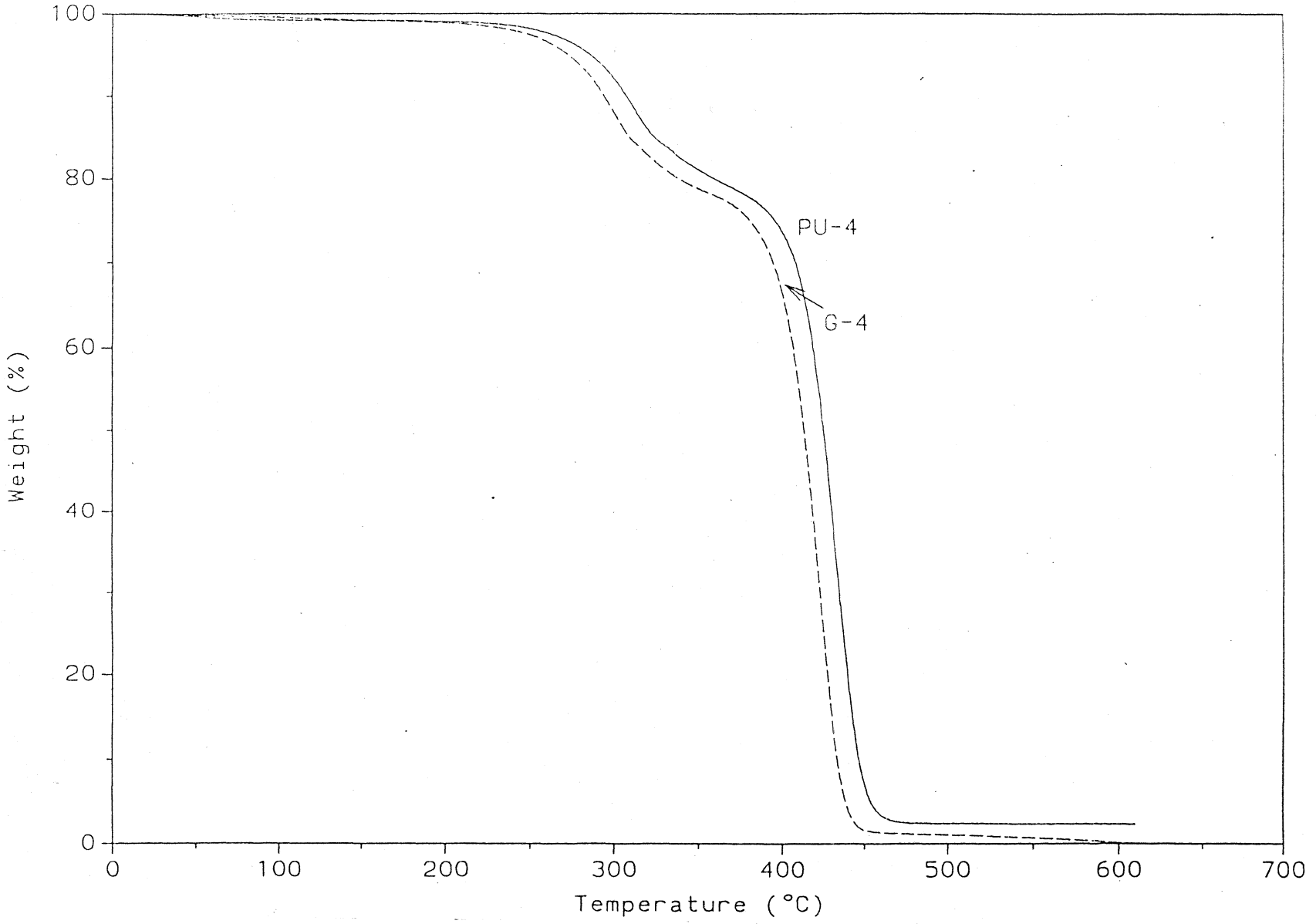

Fig. 3.19 TG curves of PU - 4, PU - g - NVP

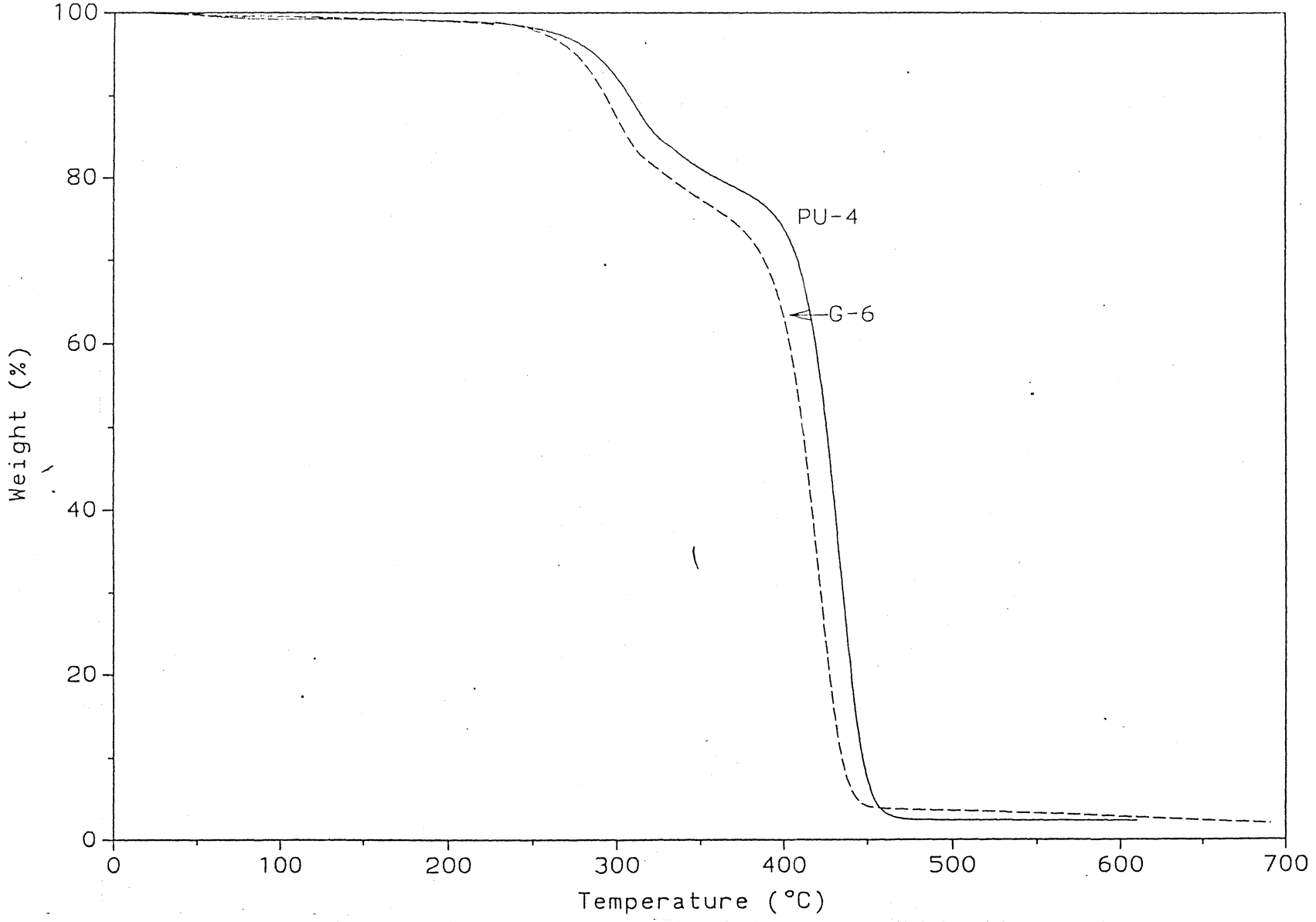

Fig. 3.20 TG curves of PU - 4, PU - g - EHA

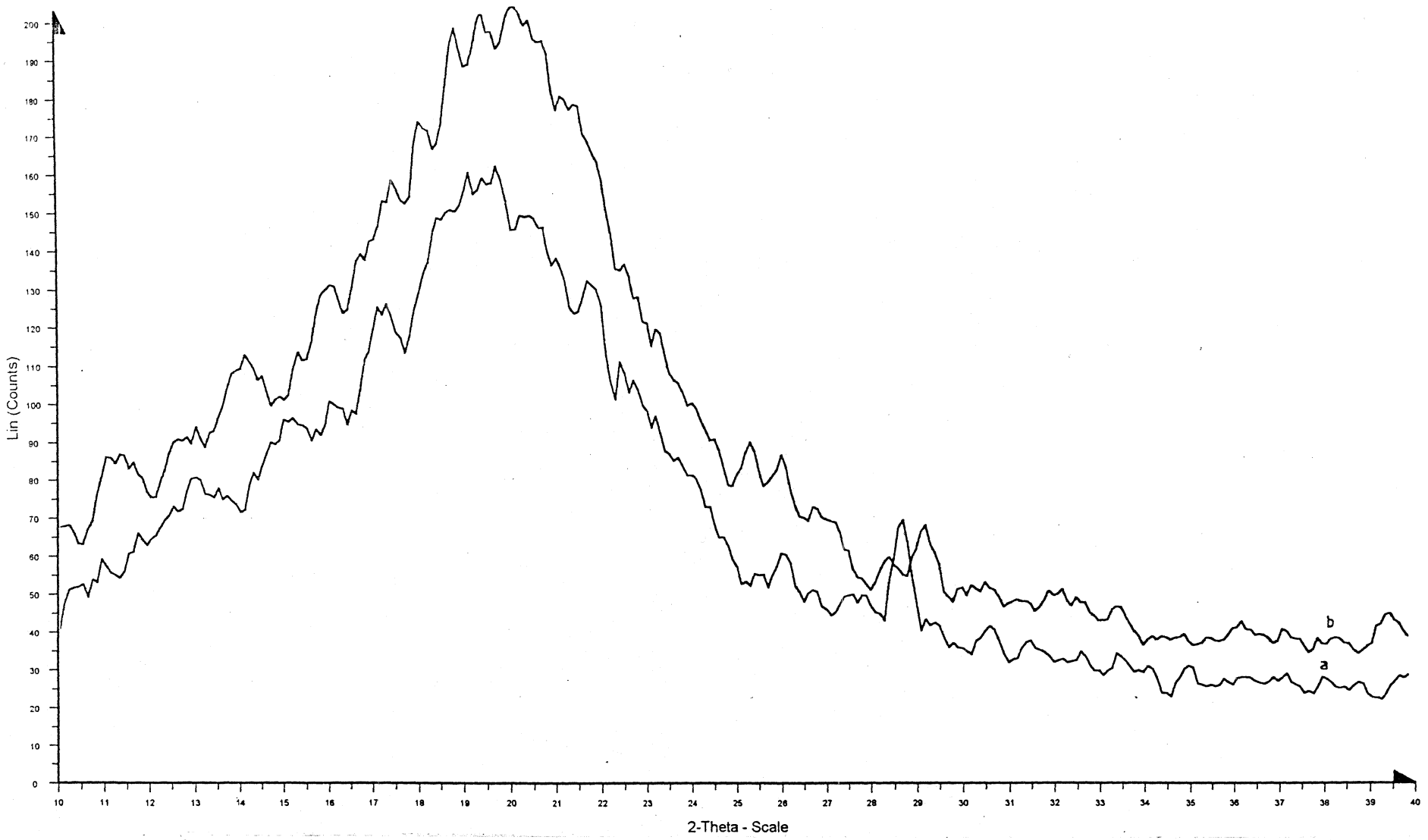

Fig. 3.21 Representative WAXD curves of **(a)** ungrafted and **(b)** grafted polyurethane

3.2.6 Polarised Microscopy:

Optical microscopic studies of the grafted samples were carried out as mentioned earlier in the section 2.3.4. Spherulite-like structures could be seen as in the grafted samples also, but the number had reduced as compared to ungrafted membrane shown in Fig. 3.8 suggesting that with grafting the material is tending towards amorphous nature. Fig 3.22 shows the optical polarised photomicrograph of the grafted membrane.

3.2.7 Contact Angle studies:

A decrease of the surface air-water contact angle (θ_{air}) is suggestive of the hydrophilicity in the system. Several aspects have appeared correlating the surface morphology of polyurethane and blood compatibility (Goldberg et al, and Gogolewski & Galletti, 1984). Hydrophilic surfaces are believed to be more blood compatible (Bruck, 1972), although it has also been suggested that a good hydrophilic/ hydrophobic balance is required for optimal blood compatibility (Ratner et al, 1979). The decrease in the air-water contact angle (θ_{air}) indicates the enhanced hydrophilicity of the graft (Table 3.VIII) or in other words contact angle values further suggest the grafting of relatively hydrophilic monomers HEMA and NVP, though with poly (urethane-g-HEMA), the value has not decreased much from the ungrafted membrane (PU-4). This may be due to the nonuniformity of the monomers grafted on the surface. But the air - water contact angle of the grafted membrane with EHA as monomer has increased indicating the relatively hydrophobic nature of poly (urethane-g-EHA). The poly (urethane-g-NVP) is relatively more hydrophilic, since NVP is more hydrophilic among the monomers used in the present studies.

Fig. 3.22 Optical polarised photomicrograph of the grafted polyurethane

Table 3.VIII

Surface parameters of grafted and ungrafted polyurethanes

Sample code	θ_{air} (°)	ϕ_{octane}(°)	γ_{sw}
PU-4	42 ± 1	138 ± 2	1.16
G-2	43 ± 1.25	136 ± 1.5	1.6
G-4	28 ± 1.5	160 ± 1	0.3
G-6	57 ± 1	123 ± 1.5	3.72

Interfacial surface free energies (γ_{sw}) of samples were calculated using θ_{air} and ϕ_{octane}. The interfacial free energy is related to blood compatibility of polymers. As the interfacial surface free energy approaches zero, the polymer becomes more blood compatible.

According to Table 3.VIII, the ungrafted membrane PU-4 has a interfacial energy of 1.16, PU-g-HEMA(G-2) shows a value of abut 1.6 which is slightly higher than PU-4, PU-g-NVP shows a value of 0.3 which is lesser than the ungrafted membrane, while PU-g-EHA shows a value of about 3.72 which is higher than PU-4. These variations in the values indicates that the surface features is altered with grafting various entities on polyurethanes, and it mainly depends on the type of the entities used for grafting.

3.2.8 Permeation studies:

The grafted membranes shows better permeation to glucose and insulin as compared to the ungrafted membrane as is evident from the values of the permeation coefficient given in Table 3.IXb. It is observed that the permeation coefficient of glucose and insulin for ungrafted polyurethane is substantially less than the grafted

polyurethane using various monomers. The poly (urethane-g-EHA) membrane, G-6 shows higher values for glucose and insulin (Table 3.IXb). It may be due to the higher free volume in ethylhexylacrylate. Table 3.IX (a) shows the actual amount permeated in 1 hour. It is observed that the poly (urethane-g-NVP) and poly (urethane-g-EHA) shows same values while poly (urethane-g-HEMA) shows almost similar value to ungrafted polyurethane for glucose permeation. Also, the amount of insulin permeated in 1 hour through the grafted polyurethane is more than that of the ungrafted polyurethane. Fig 3.23 and 3.24 shows the plot between concentration of glucose and insulin permeated versus time. In both these figures, the amount of glucose and insulin permeated through the grafted membranes is substantially more than that of the ungrafted membrane. PU-g-EHA showing maximum permeation for both glucose and insulin followed by PU-g-NVP and then PU-g-HEMA grafted membranes.

The grafted membranes are almost impermeable to albumin and immunoglobulins, so the permeation coefficients were not determined for these.

Table 3.IX (a)

The actual amount of glucose and insulin permeated in one hour in mmol/l

Sample code	Glucose(mmol/l)	Insulin(mmol/l)	Albumin(mmol/l)	Immunoglobulin (mmol/l)
PU-4	0.8	0.002	0.0001	0
G-2	0.9	0.00763	0	-
G-4	2.2	0.0021	0	-
G-6	2.2	0.0220	0	-

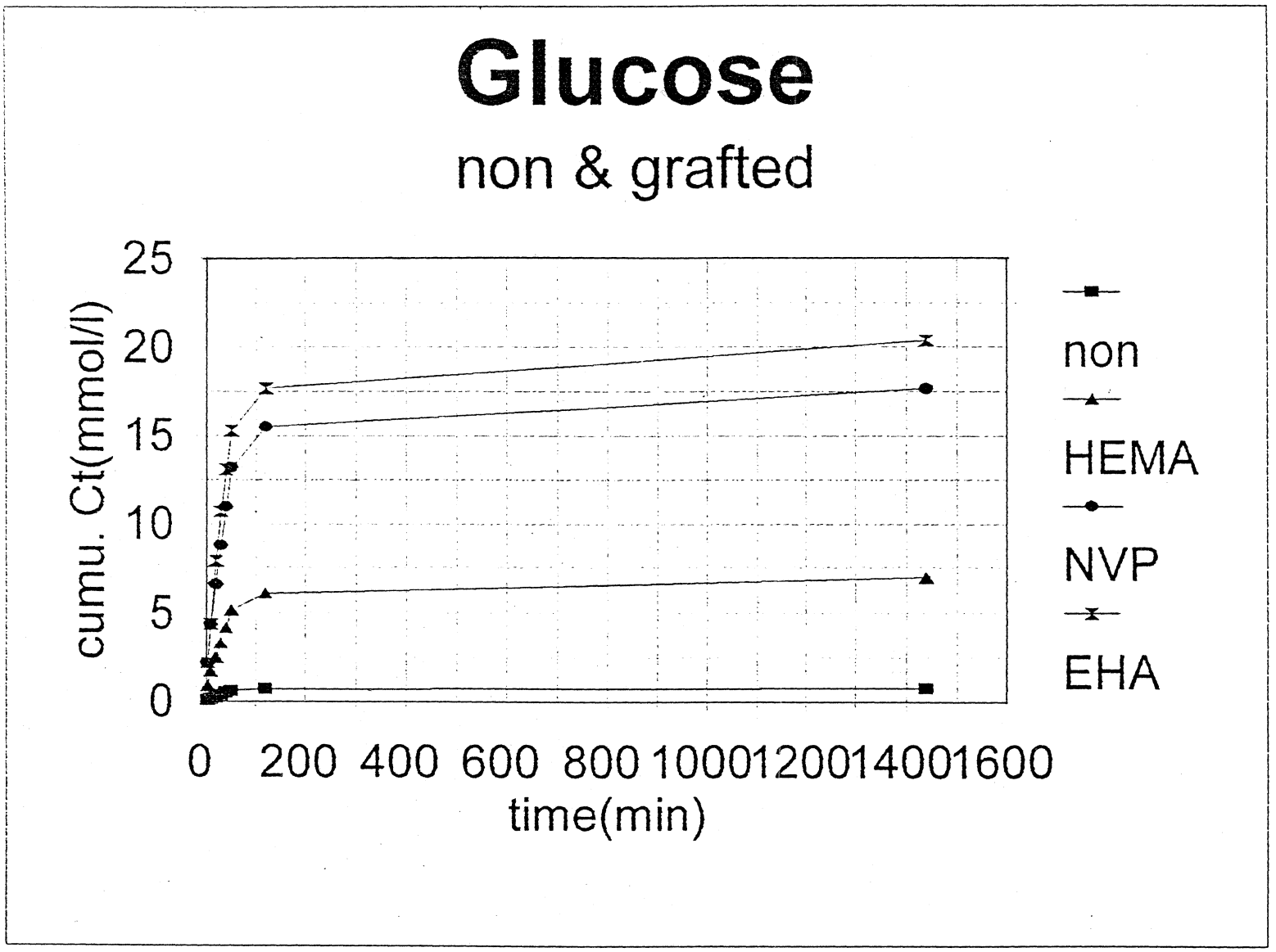

Fig. 3.23 Glucose permeation through ungrafted and grafted polyurethane Membranes

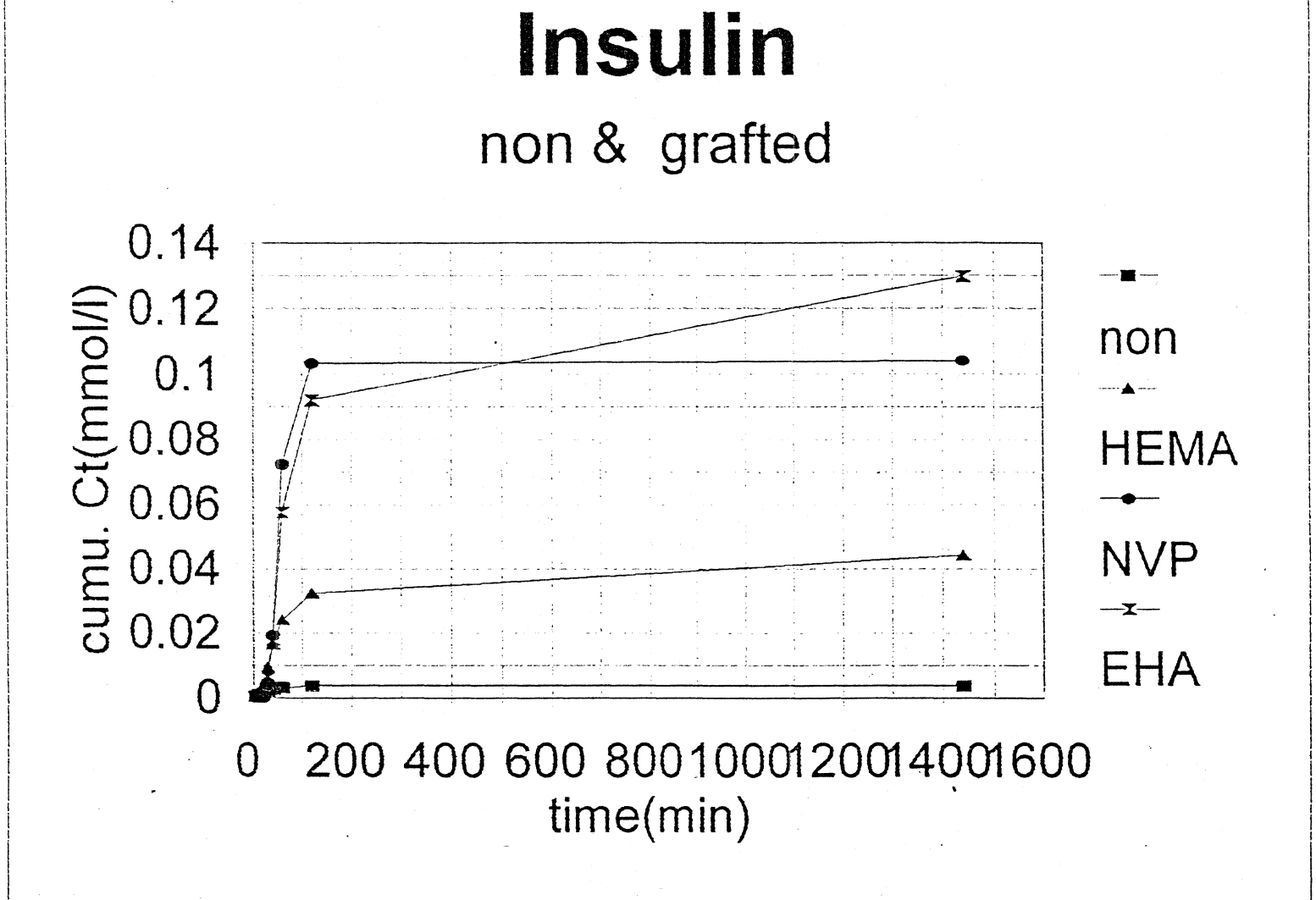

Fig. 3.24 Insulin permeation through ungrafted and grafted polyurethane

Table 3.IX (b)

Permeation coefficient for glucose and insulin through grafted and ungrafted membrane

Sample code	Glucose(p x 10^{7} cm^2/sec)	Insulin (p x 10^{7} cm^2/sec)
PU-4	25	0.28
G-2	25	0.9
G-4	26	4.8
G-6	28	7.6

From the various physicochemical characterisation studies, it is evident that though grafting of various monomer results in decrease in mechanical properties but other properties can be improved substantially. The surface modification of polyurethane by grafting different monomers using γ-irradiation is one of the easiest and better techniques for immunoisolation purposes. The membrane grafted with ethylhexyl acrylate and N- vinyl pyrrolidone showed better surface and permeation properties. Cell contact nature of these membranes is discussed in section 3.3 for their use as candidates for immunoisolation purposes for islet cell encapsulation.

Section 3.3

In vitro biocompatibility studies of the synthesized & surface modified polyurethane membranes:

3.3.1 Background:

Biocompatibility is defined as the ability of a material to perform with an appropriate host response in a specific application (Williams, 1987). There are three reasons for biocompatibility testing:

i) To identify any adverse reactions that may lead to failure or which may contribute to unacceptable clinical outcome.

ii) To determine if devices constructed of new materials or processes function as intended under simulated use conditions.

iii) To advance new technology

Biocompatibility of a material is a two-fold process, one which is concerned with the compatibility with the tissues and other more rigid one being compatibility with blood. Considering the first aspect, the following are the different types of unfavourable responses:

i) Sorption of cellular constituents from tissue leading to change of physical, chemical and mechanical properties of materials.

ii) Leaching of ingredients from the implant.

iii) Sorption of vital cellular constituents such as antigens, hormones, antibodies and other circulating drugs.

iv) Material-tissue contact leading to scar tissue and immobolization of the implant.

v) Physical and solid state carcinogenesis.

While coming to the latter, the compatibility of a material with blood or blood compatibility can be defined as the property of a material or device that permits it to function in contact with blood without inducing adverse reactions. Unfortunately, this simple definition offers little insight into what a blood compatible material is. More useful definitions become increasingly complex. This is because of the complex mechanisms involved when a foriegn material comes in contact with the blood. When blood contacts a foriegn surface, proteins immediately adsorb on the surface. Depending on the type of proteins adsorbed and their distribution on the surface, multiple responses may be elicited. These responses include the initiation of coagulation, contact and complement cascades as well as cellular adhesion and activation, either by direct contact with the surface or indirectly with the products of these protein pathways (Mason et al, 1983 and Mohammad, 1988). The adsorption of plasma proteins such as fibrinogen, fibronectin, and immunoglobulins can promote platelet and leukocytes adhesion and activation, which can lead to thrombus formation. The adsorption of factor XII, the Hageman factor, and its activation by high molecular weight kininogen and prekallikrein initiates the intrinsic coagulation pathway that results in the formation of procoagulant thrombin and subsequent polymerization of fibrinogen to fibrin. The binding of complement protein C3b to a surface may initiate complement activation and subsequent release of inflammatory mediators (Kazatchkine et al, 1988 and Chenoweth (1987). All these processes are interrelated and can ultimately culminate in thrombus formation, indicating that the surface is not blood compatible (Szycher, 1983 & Hiyashi et al, 1990).

Structure-Property (biocompatibility) relationship in polyurethane:

Bulk and surface properties of PUs are governed by the molecular architecture, which influences the biocompatibility. Extensive research works have been carried out to explore the structure-property relationship of PU for medical applications.

The polyol plays an important role in determining biocompatibility. Lemm, 1975 found the PPG based PUs are more thrombogenic than the corresponding PEG counterpart. Lelah et al, 1986 found that PEG based PU is more thrombogenic than the corresponding PPG and PTMG based materials. Takahara et al, 1985 have indicated that PPG (Mn 1000), PTMG (Mn 2000) and PEG (Mn 600) based PUs is more compatible than the other PUs of their corresponding polyol series. Lyman et al, 1972 synthesized PU based on diphenylmethane diisocyanate (MDI), PPG (Mn = 425, 710, 1025 & 2025) and ethylene diamine/hexamethylene diamine.

Tissue culture experiments using fibroblastic cells indicated the normal growth on the surface of their PPG (Mn 710) based PU when compared with the others. They also made similar studies on segmented copolyurethane with bony hamster kidney cell culture in vitro (Lyman, 1971). Brash et al, 1985 found that the fibroblastic cell growth on the polyester urethanes (estane) is slighly lesser and the platelet adhesion is higher than on the PPG or PTMG based polyetherurethanes.

Phase separation also influences the biocompatibility of PUs. It is governed by the molecular nature of the groups present in the matrix. The surface morphology is often controlled by the microphase separation. Takahara et al, 1985 found that the microphase separation in polyurethaneurea is strongly influenced by the number of

methylene units in the diamine (chain extender). The domain size of the hard segment of this PU increases slightly with the increase in diamine length. The ethylene diamine chain extended polymer is more thromboresistant when compared with the butanediol chain extended PU, due to higher phase segregation of the former (Takahara et al, 1985).

Cooper et al, 1986 synthesised a series of PU with varying types of soft segment and/or hard/soft segment ratio to study the effect of phase separation on biocompatibility. They found that the hard to soft segment ratio in PEG and polydimethyl siloxane (PDMS) based PUs does not influence the blood compatibility. With PTMG and PPG based PUs, material containing relatively large amount of soft segment and possessing large levels of phase separation, causes the formation of relatively large thrombus during blood contact.

The casting solvent has some effect on biocompatibility (Lelah et al; 1986). It would be expected that solvent polarity, volatility and viscosity would be among the factors that influence surface structure and composition on casting. Sevastinos et al, 1988 studied the effect of molding characteristics on the surface structure of PUs and their relationship with blood compatibility. The cleaning and extraction of PU also determine the biocompatibility of PUs.

Protein adsorption is an important initial event taking place at material-blood/tissue interface. Several mechanisms were suggested for the protein adsorption on the PU surface (Matsuda et al, 1977, Pitt &Cooper, 1986 and Stuoo et al, 1977).

As discussed section 1.5.1, polyurethanes are an interesting family of materials with broad possibility for processibility, superior physical and mechanical properties

and biocompatibility that made them so appealing for biomedical applications. For long term use as a medical device for immunoisolation purposes, it is very important to analyse the biocompatibility of the synthesised and surface grafted polyurethanes. We have chosen synthesized polyurethanes, using PTMG and PPG as the polyols (composition given in Table 2.I), because of their suitable physicochemical and permeation properties as reported section 3.2. This section deals with the biocompatibility assessment and their suitability with pancreatic islets *in vitro*, using various standard assays. As dicussed in section 2.5, the various assays used were as follows:

i) Cytotoxicity assay using fibroblast cells(L929, NIH 3T3 cells or VERO cells)
ii) Cytotoxicity testing by MTT assay and NR assay
iii) Image analysis for analysing the morphology of the islets
iv) Islet insulin release studies

As discussed earlier in section 2.5, (I) and (ii) assays are employed for determining the general response of the material for use in medical device and (iii) and (iv) assays for specific response of the material towards the islet cells.

3.3.1 Interaction of synthesised PU membranes with fibroblast cells and islet cells:

3.3.2.1 Cytotoxicity determination by direct contact method (ASTM F 813-83):

The cell culture methods take advantage of the sensitivity of cells cultivated in vitro towards toxic compounds. Standard cell culture tests were developed for the biocompatibility evaluation of biomedical devices. One of the most sensitive toxicity testing protocols is based on the direct contact of the sample with the cell culture.

Assessments of the cytotoxicity of the membranes were carried according to ASTM method (section 2.5.1). Briefly, membranes were placed in direct contact with the monolayer of fibroblast cells for 24 hrs. The fibroblasts are spindle shaped cells. The cells were evaluated for the general morphology, vacuolization, detachment, cell lysis and degeneration when in contact with the materials.

Comparison of these properties of the cells before and after direct contact with the samples is the basis for the evaluation of a possible toxic effect of the samples. Samples showing greater than 60% cell death were considered to be toxic. Figure 3.25a shows the photomicrograph of the monolayer of fibroblast cells in tissue culture plate (control). Figure 3.25 d shows the corresponding photomicrograph of fibroblast cells in contact with one of our material, PU-2, showing the area of interface between the materials and cells. The cells in Fig 3.25d, lost their characteristic shape and become rounded showing cell death indicating that PU-2 is cytotoxic.

Membranes PU-1, PU-4, PU-5 and PU-6 (compositions given in Table 2.I), did not induce deleterious effects such as detachment, degenerative and lysis with L929 fibroblast when placed directly on the monolayer of cells as shown in Figures 3.25 a, b and c respectively. However, membranes PU-2 (Fig. 3.25 d) and PU-3 induced toxic effects on cells and were thus excluded from further testing.

3.3.2.2 Cytotoxicity determination by MTT assay:

The MTT assay (Mosmann 1983) is a sensitive, quantative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazon product which is insoluble in water. The amount of formazon produced is directly proportional to the cell number in

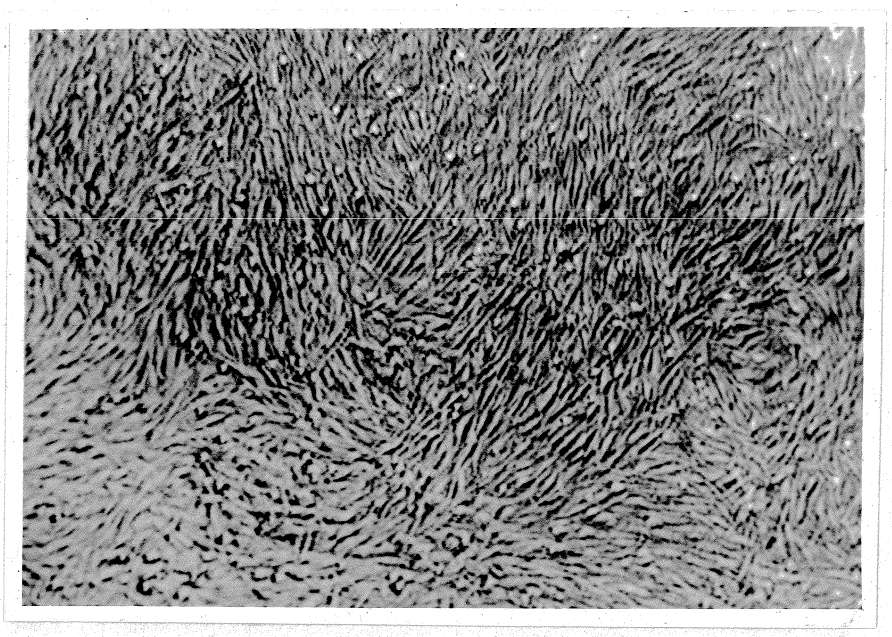

Fig. 3.25 Photomicrographs showing direct contact testing using L929 cells (Magnification X 100) **(a)** Cells after direct contact with the control material

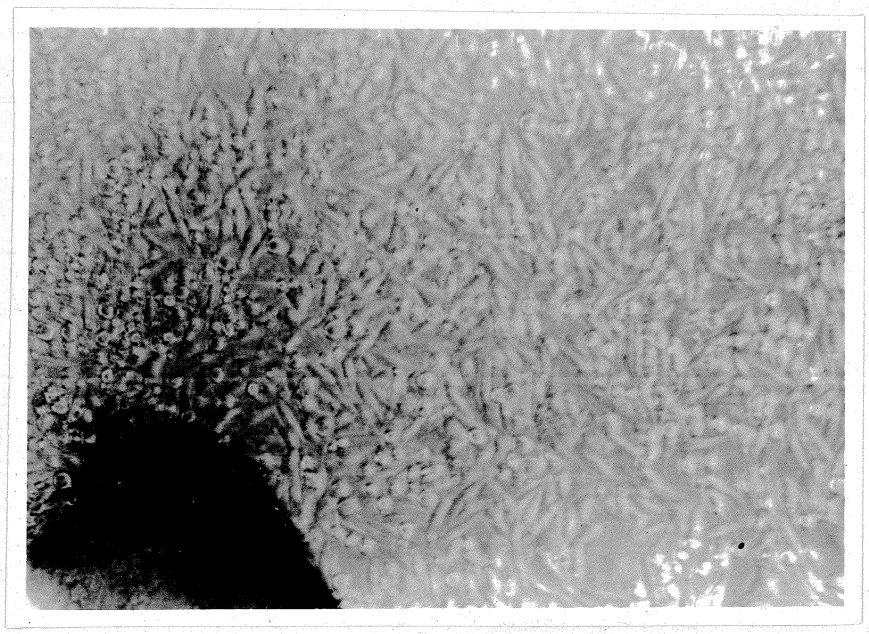

Fig. 3.25 Photomicrographs showing direct contact testing using L929 cells (Magnification X 100) **(b)** Cells after contact with the membrane PU-1

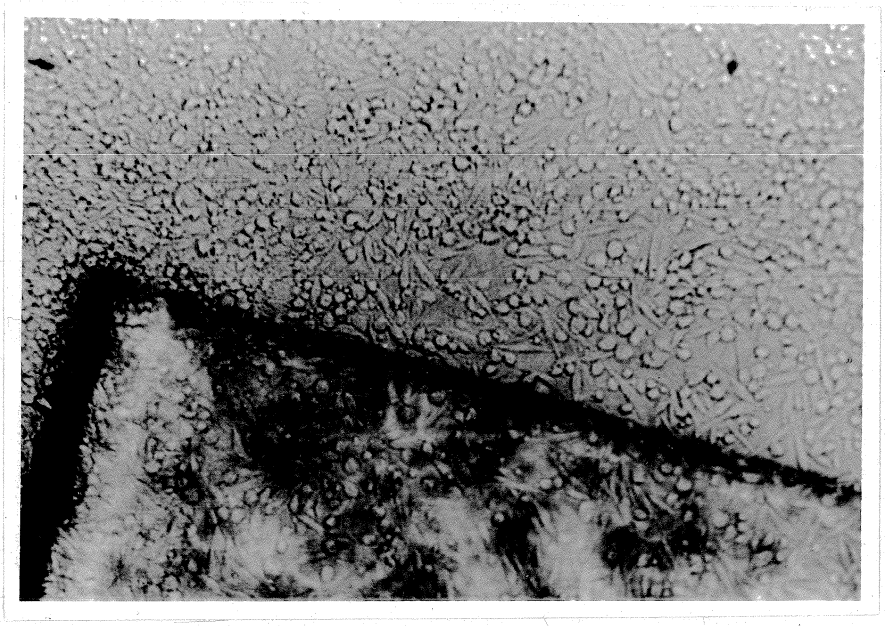

Fig. 3.25 Photomicrographs showing direct contact testing using L929 cells (Magnification X 100) **(c)** Cells after contact with the membrane PU-4

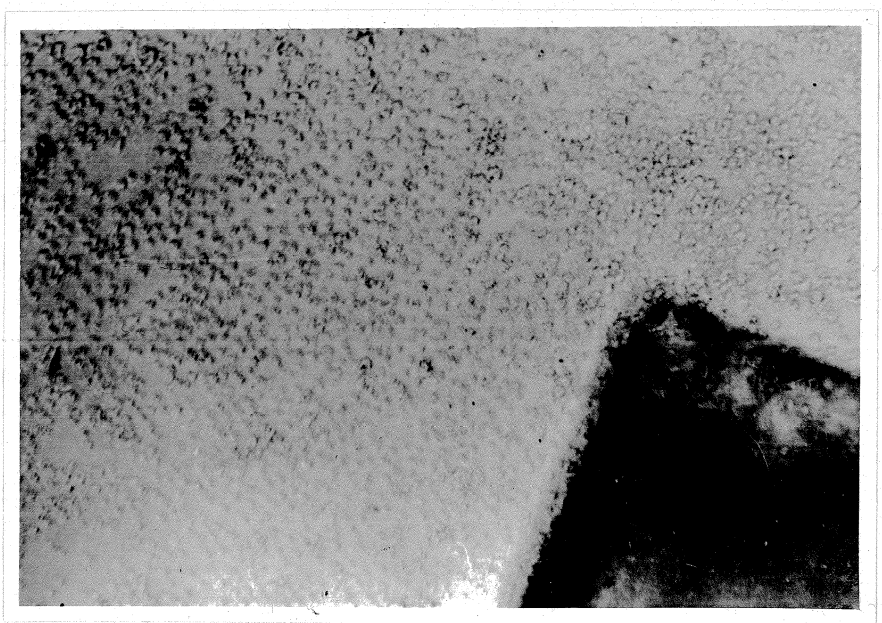

Fig. 3.25 Photomicrographs showing direct contact testing using L929 cells (Magnification X 100) **(d)** Cells after contact with the membrane PU-2

the range of cell lines (Mosmann 1983; Gerlier & Thomasset 1986; Grailer et al. 1988; al-Rubeai & Spier 1989). The results are consistent with those obtained from 3H-' thymidine uptake assays. The MTT assay is more useful in the detection of cells, which are not dividing but are still active. It can, therefore, be used to distinguish between proliferation and cell activation (Gerlier & Thomasset 1986). The technique permits the processing of a large number of samples with a high degree of precision using a multiwell scanning spectrophotometer (microELISA reader).

The great advantage of this type of test is that it is possible to produce results, which can be expressed quantitatively. When the extract is known or can be predicted, it is possible to produce standard curves and identify the level at which the extract is known or can be predicted, it is possible to produce standard curves and identify the level at which the extract is cytotoxic.

MTT assay was performed as per standard protocol mentioned in section 2.5.2. Briefly, sterile membrane pieces of uniform weight were incubated in DMEM at 37° C for 15 days. NIH 3T3 cells cultured on a 96 well plate were then exposed to varying concentrations of the membrane extracts for 24 hrs. and the viability was measured. MTT assay revealed no cytotoxic effects of leach out products from membranes PU-4, PU-5 and PU-6, while the leach outs from membrane PU-1 were proved cytotoxic as depicted in Fig. 3.26. Percentage viability of NIH3T3 cells was found high at the concentrations of PU-4, PU-5 and PU-6 leach outs tested.

3.3.2.3 Compatibility with islets of Langerhans:

Islet isolation and biocompatibility testing was carried out as described in section 2.5.3. Figure 3.27 shows the phase contrast photomicrograph of islets cultured on tissue culture polystyrene (TCPS) plate taken as control. Islets cultured on

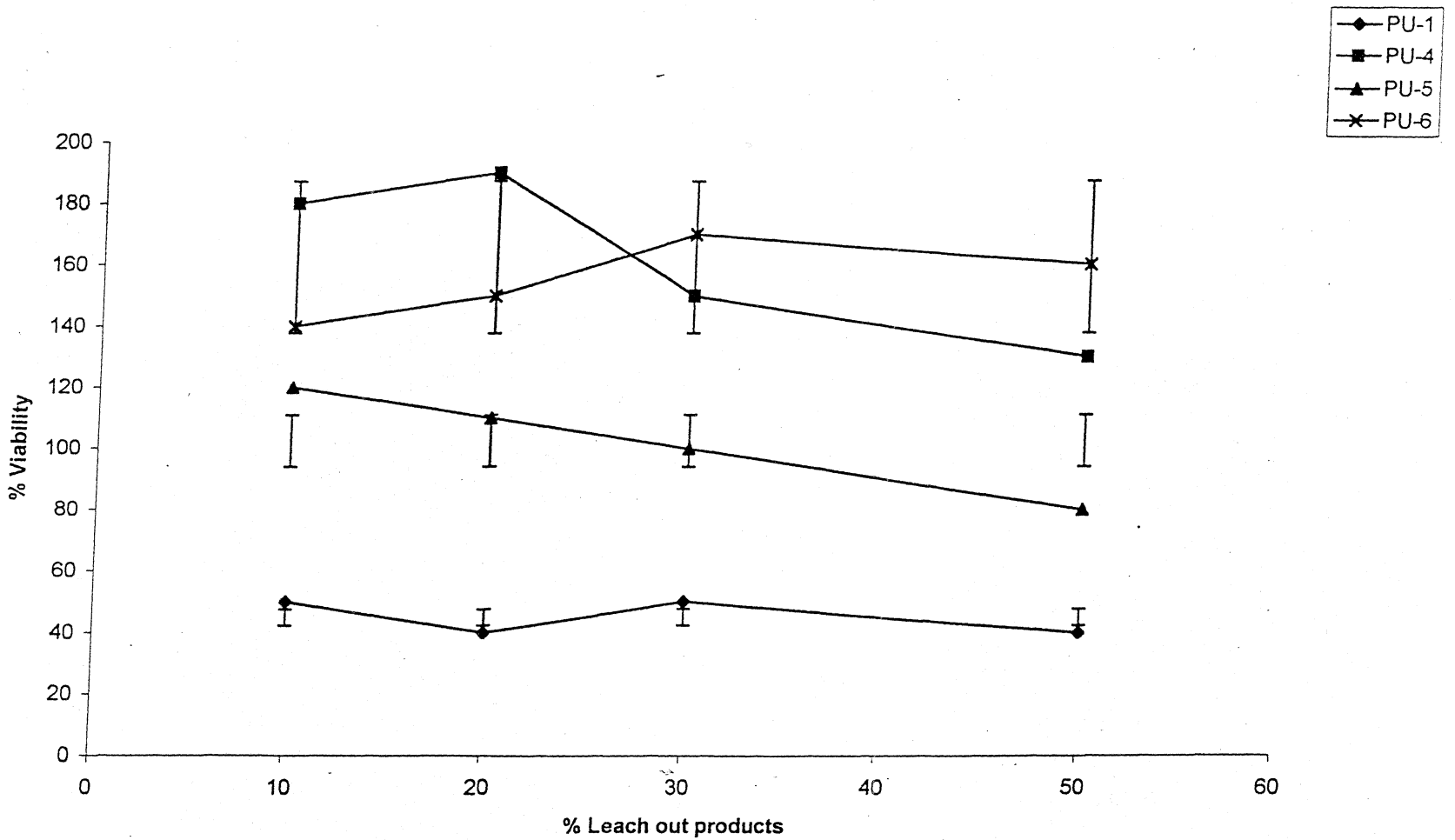

Fig. 3.26 Cytotoxicity evaluation of membrane PU-1, PU-4, PU-5 and PU-6 By MTT assay using NIH 3T3 cells

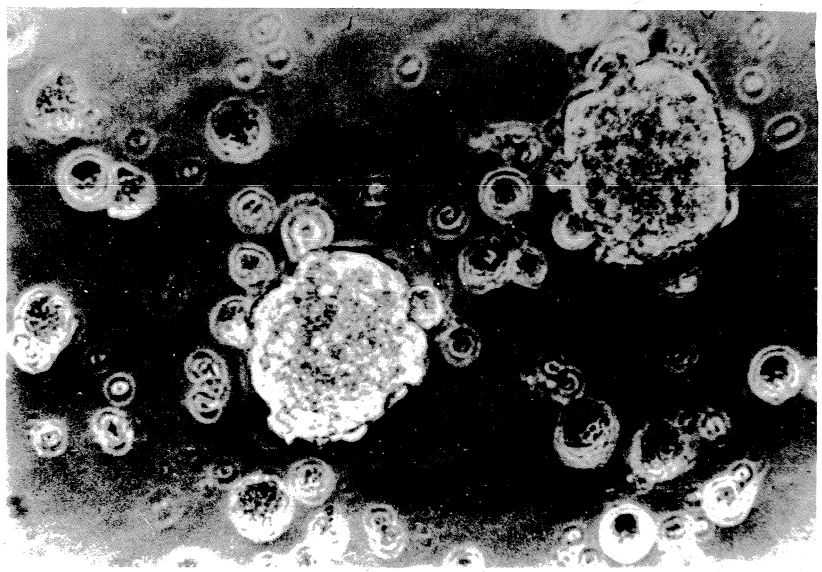

Fig. 3.27 Representative phase contrast photomicrograph showing the morphology of islets in contact with **(a)** TCPS control

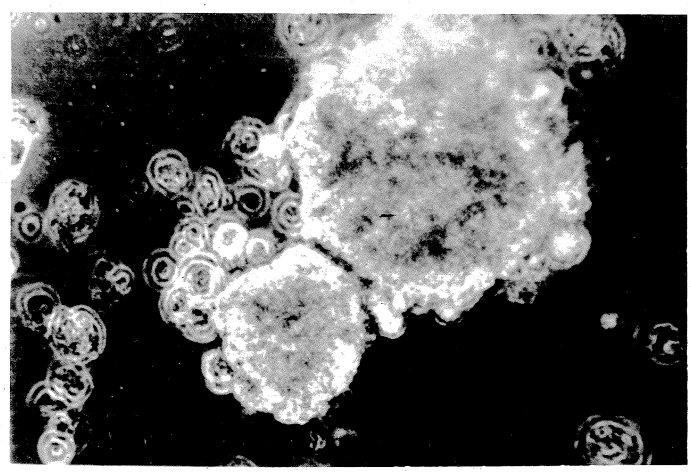

Fig. 3.27 Representative phase contrast photomicrograph showing the morphology of islets in contact with **(b)** Membrane PU-4

membranes PU-4 and PU-5 showed normal morphology and architecture and after 3 days of culture on PU-4 and PU-5 they showed viability, which was high and comparable to control (Fig. 3.27a) ($p > 0.05$). Figure 3.27b shows the photomicrograph of the islets in contact with material PU-4. However, islets that were cultured on membrane PU-6 started disintegrating and their viability was significantly less than islets cultured on TCPS control (Fig.3.27 a). Figure 3.28 shows the viability of islets in contact with TCPS control, PU-4, PU-5 and PU-6.

3.3.2.4 Islet Morphometry:

Morphometry of islets cultured on PU-4 and PU-5, which were found compatible by the assays above, is shown in Table 3.X. These islets show comparable area and diameter to islets cultured on TCPS control ($p > 0.05$). The islets in contact with PTMG based PUs with 25% and 32 % hard segments showed exactly same morphology of the islets as in contact with TCPS control indicating no degeneration or lysis of the cells.

Table 3.X

Islet morphometry studies of islets cultured on PU-4, PU-5 and TCPS control (n=100)

	TCPS (control)	PU-4	PU-5
Area (μm^2)	11779.54 ± 984.2	11673.4 ± 846.1	12352 ± 1048
Diameter (μm)	118.42 ± 3.57	116.4 ± 2.3	110.3 ± 3.6

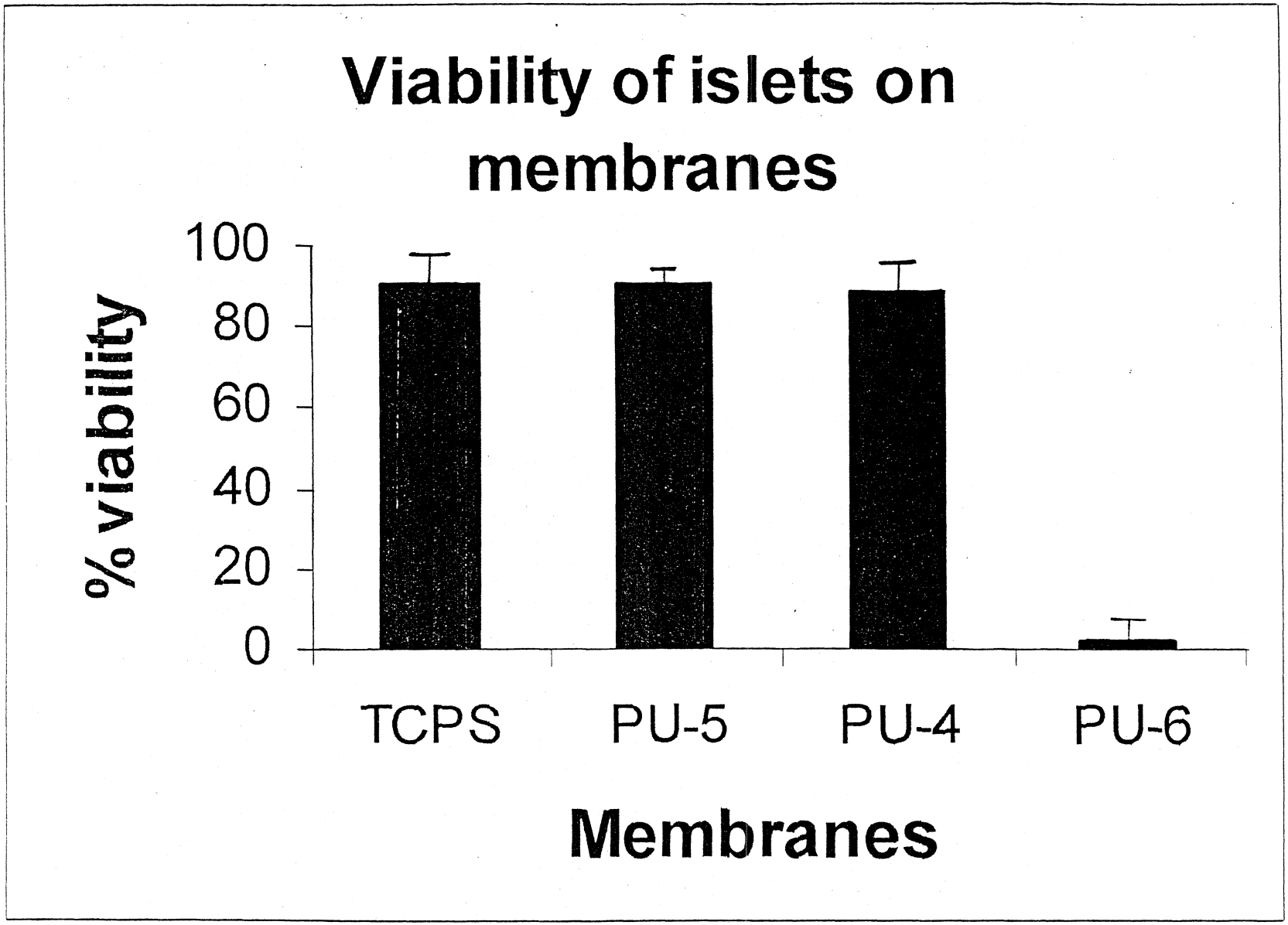

Fig. 3.28 Viability of islets cultured on TCPS control, PU-4, PU-5 and PU-6

3.3.2.5 In vitro islet functionality:

In vitro insulin release from islets on PU-4 and PU-5 is shown in Table 3.XIX. Basal and stimulated insulin release (in 5.5 mM and 16.5 mM glucose) after 3 days of culture was comparable to control islets on TCPS. This shows that PU-4 and PU-5 can respond to the basal and higher glucose challenges, with comparable results as that of the control(TCPS).

Table 3.XI

In vitro functionality assessment of islets cultured on PU-4, PU-5 and TCPS control

	Insulin secreted (IU / 10 islets)	
Membranes	Basal (5.5 mM)	Stimulated (16.6 mM)
TCPS (control)	99.5 ± 4.2	245.6 ± 22.0
PU-4	98.4 ± 3.7	247.1 ± 21.5
PU-5	102 ± 2.1	243 ± 12.4

3.3.2.6 Statistical analysis:

Results are expressed as ± s.e.m. differences between the groups were tested by Student's t test or the Mann Whitney test whichever was appropriate. Computations were performed using a Sigma- Stat statistical package (Jandel scientific, version 2.0 for Windows 95, SPSS Inc. Chicago, USA).

3.3.3 Role of Physicochemical properties of membranes in Biocompatibility:

Among synthesized membranes the polyurethane prepared using PTMG as glycol were seen to be more hydrophilic (Table 3.IV), as decrease in the surface air-water contact angle (θ_{air}) and increase in octane contact angle (ϕ_{octane}) for polymeric materials is suggestive of the hydrophilicity in the system (Hamilton, 1974). Membranes PU-1 to PU-6 were subjected to biocompatibility studies. Membranes PU-1, PU-4, PU-5 and PU-6 did not induce deleterious effects on L929 fibroblast cells on direct contact indicating their better tolerance. However, membranes PU-2 and PU-3 which were seen to induce degenerative effects on L929 cells on contact proved non compatible and thus excluded from further testing as described in section3.3.1.1. According to Fig. 3.25, the percentage viability of NIH3T3 fibroblasts cells (Control viability taken as 100%), on exposure to leach outs, were found to be high for all the PTMG based PUs i.e. PU-4, PU-5 and PU-6 indicating the non-cytotoxicity of these leach outs. Leach outs from PU-1 reduced viability of NIH3T3 cells significantly indicating their toxicity. The results from the MTT assay showed very high rates of cell growth percentages, moreover some materials demonstrated better growth or activity as measured with respect to controls. The MTT assay is an extremely sensitive indicator of cell metabolic activity. In figure 3.25, the increase in viability, in membranes PU-4 and PU-5 for 10% and 20% leach outs, than that of the TCPS control, may be due to the difference in the original number of cells as appoximately 10^7 cells/ ml were obtained for the analysis. Even a minor difference in the count originally could result in the wide difference in the final result as the cell is under the active process of division. The evidently high value of the viability for PU-4 and PU-5 (PU based on PTMG with hard segments25% and 32%) for 30% leach outs and sudden decrease in viability for 50% leach outs may be an artifact. As MTT assay is a

very sensitive test depending on the number of original cells as mentioned earlier. It may be possible that the cells may not have equally stimulated. The exact reason needs to be explored.

Contradictory results about non-toxic nature of PU-1 in direct contact test and toxicity in MTT assay could be because, direct contact test exposes cells to material only for 24 h and this period is insufficient to extract leach outs in medium. While, in MTT test membranes were extracted for 15 days in medium and thus leach outs come in sufficient amount in medium to induce toxicity.

Membranes PU-4, PU-5 and PU-6 were further tested for their suitability with islet system. Membranes PU-4 and PU-5 preserved normal morphology, architecture and viability of islets, which was comparable to islets cultured on TCPS control as described in section 3.3.1.3. This suggests the biocompatibility of PU-4 and PU-5 towards pancreatic islets. Decreased viability of islets on PU-6 could be because of the surface hydrophobicity of PU-6 in comparison with PU-4 and PU-5, suggesting role of surface hydrophilicity as one of the determinants in deciding biocompatibility of membranes towards pancreatic islets (George et al). The surface free energy of PU-4 and PU-5 was also observed to be the least (Table 3.IV). According to the minimal surface free energy hypothesis (Andrade et al, 1973), which has wide acceptance for better blood compatibility, the interfacial surface free energy of the material should be zero. Though it is still unclear whether the least surface free energy hypothesis has any relevance with respect to cytocompatibility of the materials with islets, the present study of cytocompatibility of polyurethane membranes (PU-4, PU-5 and PU-6) does suggest an influence of minimal surface energy parameters. As increase in percentage of hard segment could increase the surface free energy parameters of polyurethane membrane this also can be one of the pivotal factor while designing membranes for

islet immunoisolation.Islets cultured on PU-4 and PU-5 also showed insulin secretion in low and stimulated glucose medium (Table 3.XI). This indicates that the islets have well preserved functionality and can respond to the environmental glucose concentration changes. These polyurethanes were also shown to be permeable to glucose and insulin and impermeable to transplant rejection effectors such as the antibodies and immunocytes, providing complete immunoisolation of the islets (George & Nair, 1999). This permeation property of the PU-4, PU-5 will ensure the fast sensing of environmental glucose by the islets and fast availability of releasing insulin in the blood circulation, maintaining the normoglycemia. Surface free energy of material has been directly correlated to protein adsorption (Van Recum & Von Kooten, 1995). Thus reduced protein adsorption on PU-4, PU-5 would also ensure the maintenance of membrane permeability as well as reduced cellular adhesion (Van Recum & Von Kooten, 1995) on the surface increasing its biocompatibility. The biocompatibility status of PU-4 and PU-5 with its favorable physico-chemical properties makes them ideal candidates in islet immunoisolation matrices.

3.3.4 In vitro biocompatibility studies of surface modified polyurethane membranes in reponse to fibroblast cells and islet cells:

It is the surface of a biomaterial, which first comes into contact with the living body when the biomaterial is implanted. Therefore, the initial response of the living body to the biomaterial may depend to a large extent on its surface properties. Several polymers are used as biomaterials for substituting diseased vital organs (Szycher, 1983 & Ratner, 1989). Among these materials, polyurethanes possess an unparalleled position due to their unique properties arising from their specific structural features (Lelah & Cooper, 1986). One of the easiest ways to modify the properties of polyurethane, as suggested by various researchers, is by grafting other entities (Jansen & Ellinghorst, 1985, Bruck, 1977, Evangelista, 1986 and Jansen & Ellinghorst, 1979). By optimizing the grafting procedure and by choosing appropriate monomers, it is possible to preserve the vital features of the trunk polymer simultaneously modulating the required properties. Chemical modifications of PU by grafting vinyl monomers for improving the blood contacting properties are carried out widely (Jansen & Ellinghorst, 1979; Ratner, 1980; Abidi, 1979; Fischer, 1982; Hunter et al, 1983). To the best of our knowledge, surface modified PU by radiation grafting has not been much explored for islet cell immunoisolation purposes.

The synthesis and characterisation of nonporous PU membranes with differing hard segment content and the suitability of some of these membranes with pancreatic islet cells in vitro has been earlier discussed in chapter 2 & 3. One of the candidate biocompatible membrane (PTMG based PU with 25% hard segment, PU-4), with better physicochemical and permeation characteristics,

was surface modified by radiation grafting using monomers such as hydroxyethyl methacrylate (HEMA), N-vinyl pyrollidone (NVP) and ethyl hexyl acrylate (EHA) and characterised as discussed in section 3.2.

This section deals with the biocompatibility of these surface modified membranes suitability with, special reference to compatibility with pancreatic islets in vitro.

In vitro biocompatibilty studies using islet cells showed that the synthesised nonporous PU membrane (PU-4) was compatible as discussed in section 3.3.1. Islet morphometry and insulin release studies as discussed in sections 3.3.2.4 and 3.3.2.5, suggests that this membrane as candidate for islet cell immunoisolation purposes. This membrane was further grafted using various monomers such as HEMA, NVP and EHA. The composition of the grafted membranes was taken as mentioned in Table 2.II.

3.3.3.1 Cytotoxicity Assessment of surface grafted membranes:

Cytotoxicity assessment of grafted membranes determination was done by MTT assay and NR assay, using VERO cells, to confirm the viability of cells at mitochondrial level as well as lysosomal level.

1. By MTT assay:

A detailed account of MTT assay is discussed in section 3.3.2.2. MTT assay revealed no cytotoxic effects of leach outs from all the grafted membranes. Fig. 3.29 shows the viability of VERO cells as tested by MTT assay. The viability of VERO cells

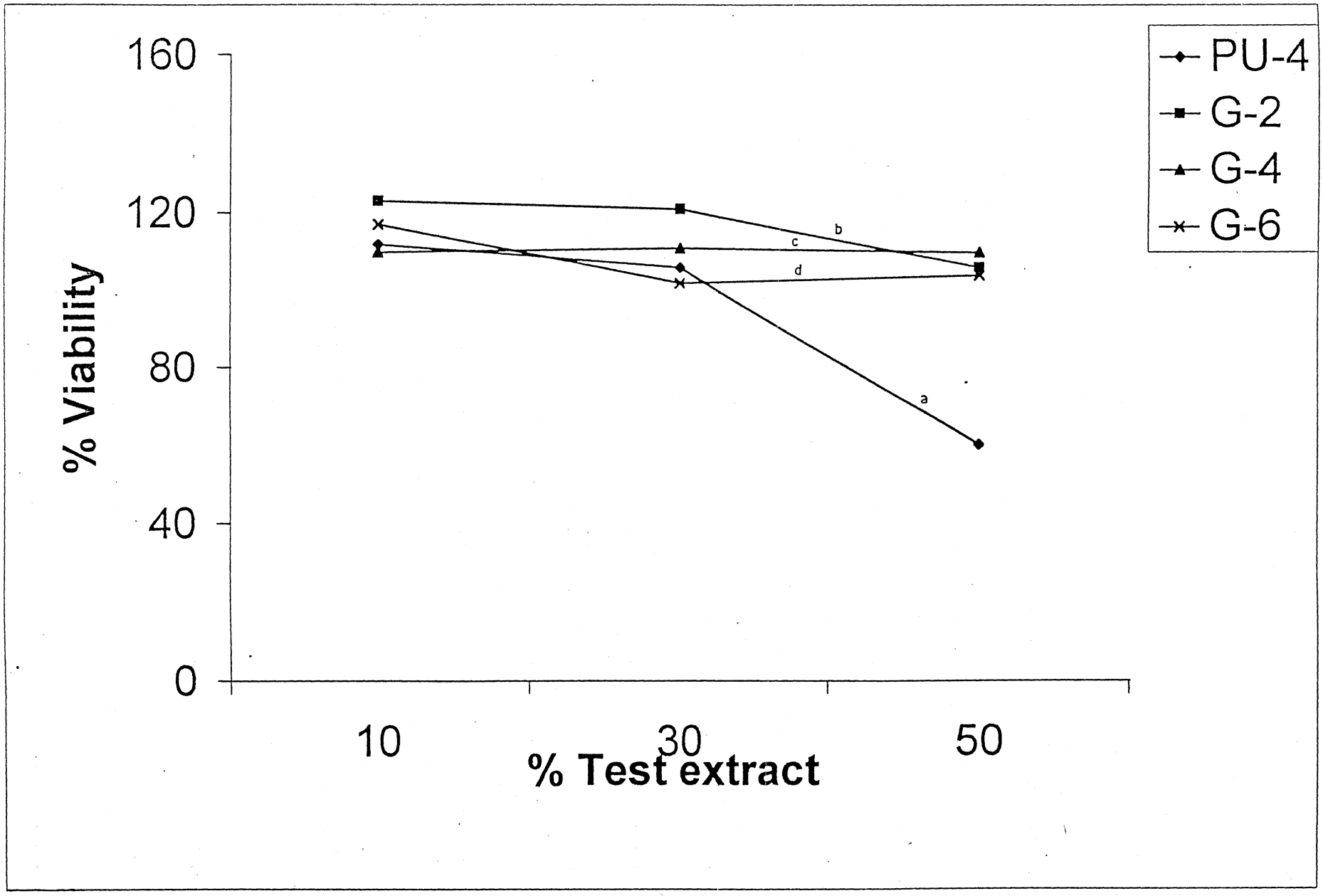

Fig. 3.29 Cytotoxicity evaluation of ungrafted and grafted polyurethane membranes by MTT assay using VERO cells **(a)** ungrafted PU-25% (PU-4) **(b)** PU-g-

in presence of test extract of grafted membranes is almost similar to ungrafted membrane.

2. By NR assay:

Neutral red (3-amino-7-dimethyl-2-methylphenazine hydrochloride) is a water soluble, weakly basic, supravital dye that accumulates in lysosomes of viable cells. The neutral red (NR) assay is an in vitro cell viability test that was developed and extensively studied for in vitro cytotoxicity determinations. Earlier in vitro tissue culture studies using the NR dye were developed for assessments of viral cytopathogenicity and for immunotoxicity assays . The NR assay is based on the incorporation of NR into the lysosomes of viable cells after their incubation with test agents. Cellular uptake of dye is accomplished by passive transport across the plasma membrane. Accumulation of NR within lysosomes occurs either from the binding of NR to fixed acidic charges, such as those of polysaccharides, within the lysosomal matrix, or from the trapping of the protonated form of NR within the acid milieu of the lysosomes. In damaged or dead cells NR is no longer retained in the cytoplasmic vacuoles and the plasma membrane does not act as barrier to retain the NR within the cells (Clifford & Downes, 1996).

NR assay also revealed no cytotoxic effects of leach out products from grafted membranes. Fig 3.30 shows the viability of VERO cells as tested by NR assay. All grafted membranes show higher viability than that of the ungrafted membrane. They also show almost similar viability independent of the type of the monomer used for surface grafting.

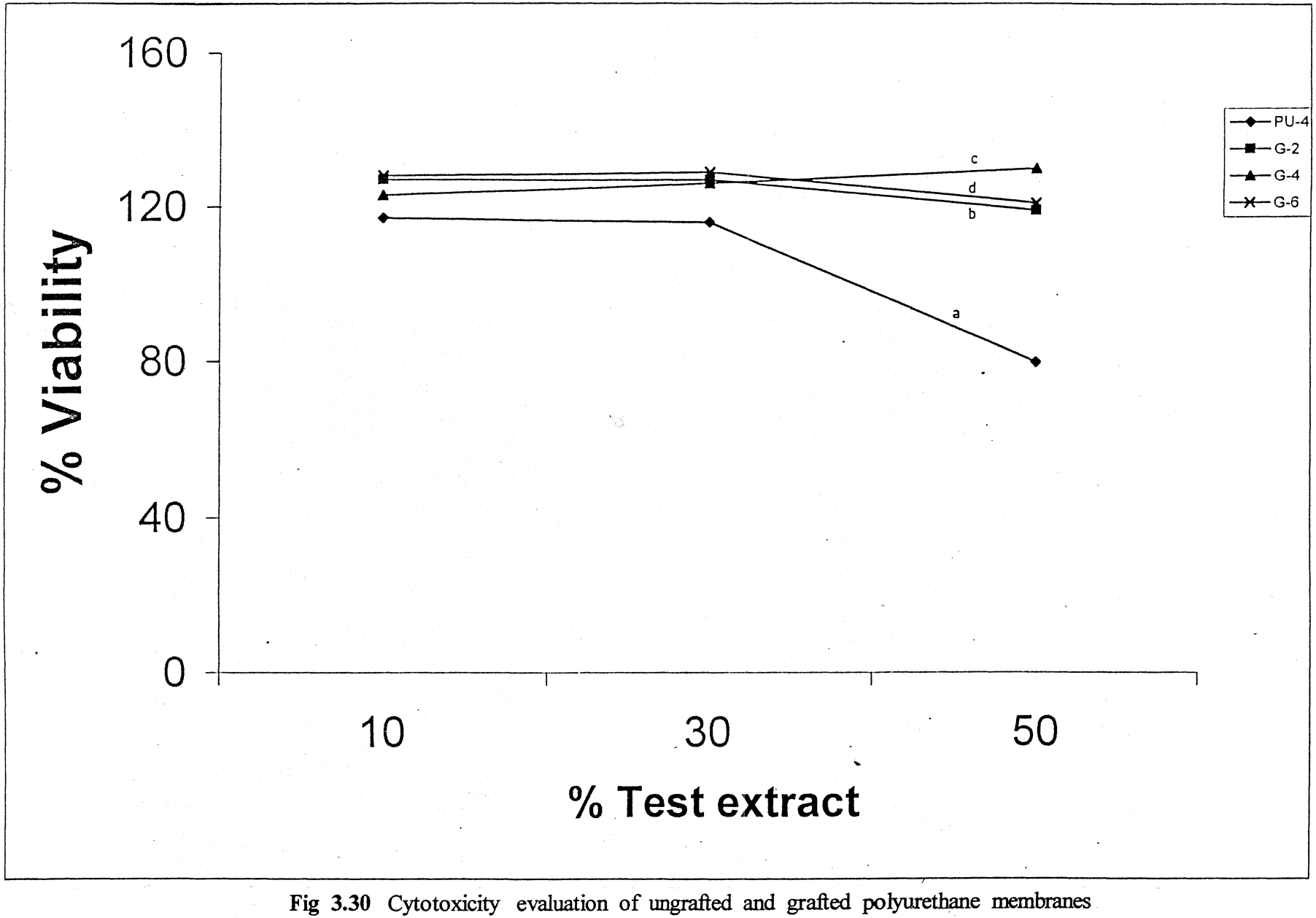

Fig 3.30 Cytotoxicity evaluation of ungrafted and grafted polyurethane membranes by NR assay using VERO cells **(a)** ungrafted PU-25% (PU-4) **(b)** PU-g-HEMA (G-2)

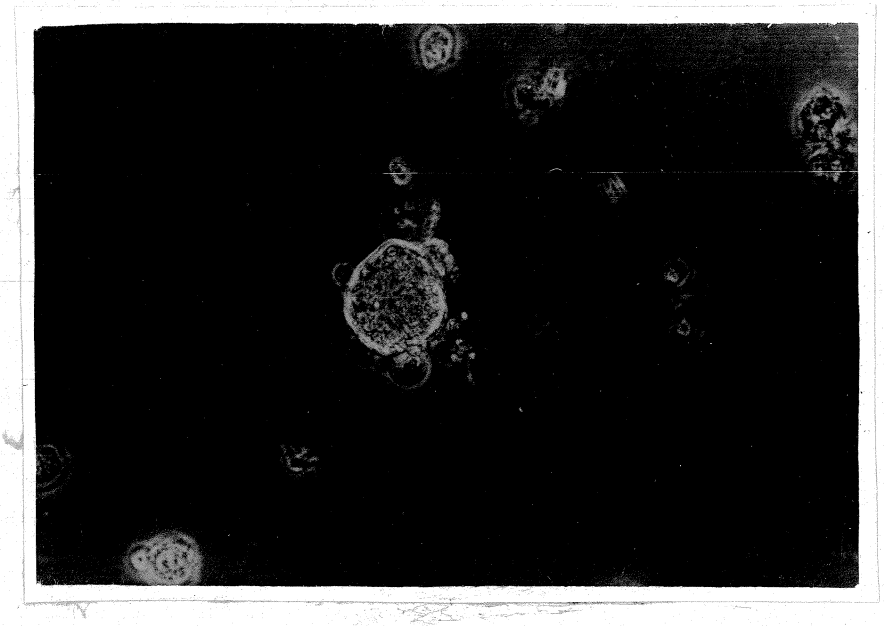

Fig 3.31 Representative phase contrast photomicrograph showing the morphology of islets in contact with **(a)** TCPS control

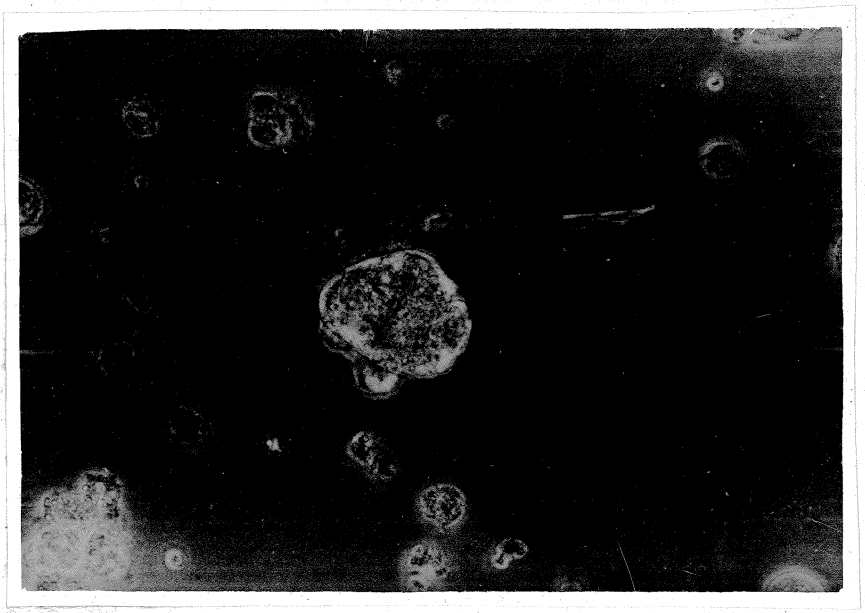

Fig 3.31 Representative phase contrast photomicrograph showing the morphology of islets in contact with **(b)** Grafted membrane

3.3.3.2 Compatibility with Islet of Langerhans:

Islets cultured on all grafted membranes showed normal morphology and architecture and after 24 hrs. of culture on these grafted membranes, they showed viability, which was high and comparable to control. Figure 3.31a shows the phase contrast photomicrograph of the islets cultured on TCPS control. The grafted membranes showed exactly the same morphology as that of the islets cultured on the control. Fig. 3.31b shows the representative phase contrast photomicrograph of islets in contact with one of the grafted membranes (G-6). Fig. 3.32 shows the graphical representation of the viability of the islets cultured on the ungrafted and grafted membranes. It is observed from Fig. 3.31 that the grafted membranes, PU-g-HEMA (G-2) and PU-g-EHA (G-6) shows higher viability of islets than the ungrafted membrane, however, PU-g-NVP (G-4) showed lesser compatibility (74%) though it scored well in the cytotoxic determination using MTT assay and NR assay.

3.3.3.3 Islet Morphometry:

Morphometry of islets cultured on the grafted membranes is shown in Table 3.XII. The islets show comparable area and diameter to islets cultured on TCPS control ($p>0.05$).

The islets in contact with the grafted polyurethane membranes showed exactly same morphology of the islets as in contact with TCPS control and better morphology than the ungrafted membranes indicating no lysis or degeneration of the cells.

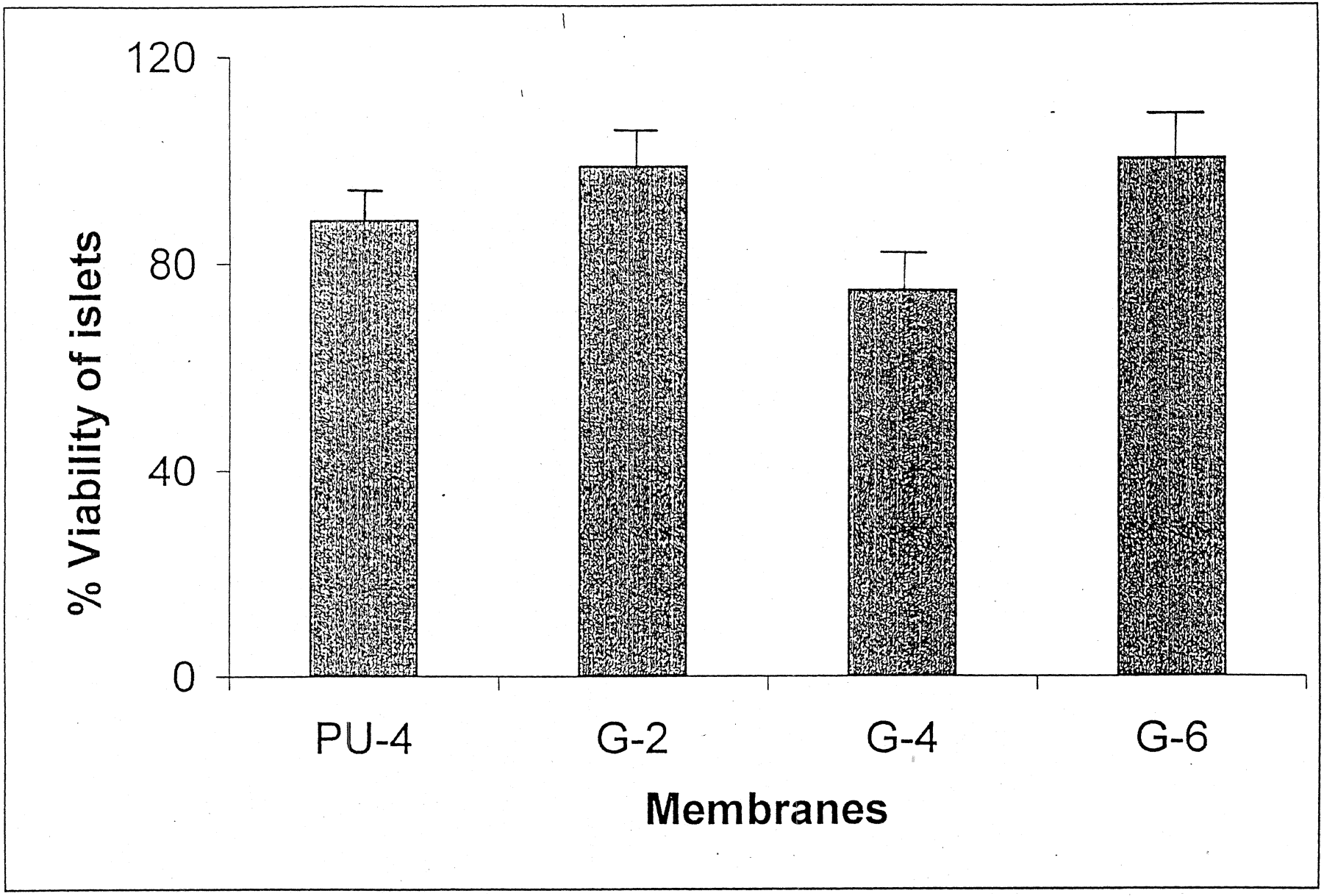

Fig. 3.32 Viability of islets cultured on ungrafted and grafted membranes

Table 3.XII

Islet morphometry studies of islets cultured on PU-4, the grafted materials and TCPS control (n =100).

	TCPS (control)	PU-4	PU-g-HEMA (G-2)	PU-g-NVP (G-4)	PU-g-EHA (G-6)
Area(μm^2)	11779 ± 984.2	12468± 1135	13465± 1234	11789 ± 1123	11213± 1034
Diameter (μm)	118.42± 3.57	115± 2.3	117.2± 2.6	109 ± 1.3	107 ± 2.4

Polyurethanes were synthesised and characterised using physicochemical and biocompatibility studies as discussed in the previous chapters 2 & 3. One of the best compositions (PU-4) was grafted with various monomers. These grafted membranes were further characterised using various physicochemical techniques. Desirable physicochemical properties, selective permeability of the grafted membranes as well as enhanced permeation rates for less than 10,000 mol. wt. metabolites have been discussed in section 3.2. The membranes were seen to have a balance of hydrophilic-hydrophobic character with membrane PU-g-NVP (G-4) showing higher overall hydrophilicity and the membranes PU-g-EHA (G-6) showing overall hydrophobocity. The grafted membranes did not induce deleterious effects on VERO cells as seen through both MTT and NR assay (Figures 3.29 & 3.30), indicating better tolerance of the cells and also absence of cytotoxicity for material leach out, if any. These grafted membranes on further testing with islet cells were found to be compatible and similar to the control islets cultured on TCPS control. Islet morphology studies, Table 3.XII, revealed comparable area and diameter grafted membranes and on control TCPS plates. The viability of islet cells on the grafted membranes were greater than that of the bare PU membrane except for membrane G-4 (Fig. 3.32). The viability of islet

cells on almost all materials was high 88-100% and even material G-4 showed 74% viability, which is considered compatible.

Grafted membranes G-2 and G-6 also have the crosslinker (EGDMA) incorporated; their higher viability could be a result of higher molecular weight of the crosslinked materials. While the lesser compatibility of G-4 could be due to the extensive phase separation showing distinct domains, as can be seen in SEM studies shown in Fig 3.14 (b). Since the membrane G-4 shows noncytotoxic response in MTT and NR assay using VERO cells, it can be tested for other biomedical applications employing different primary cell culture systems. Though earlier it was proposed for nonporous polyurethane membranes as discussed in section 3.3.2, that minimum surface free energy and surface hydrophilicity are vital factors influencing biocompatibility, especially with reference to islet cells. But from the present studies on grafted membranes, we observe that from the surface parameters (Table 3. VII) that PU-g-NVP is relatively more hydrophilic among the grafted composition. Also the surface free energy of PU-g-NVP is the least. Accordingly PU-g-NVP should have better biocompatibility but from the biological evaluation especially with reference to islet cells, PU-g-EHA shows better compatibility than PU-g-NVP. Thus, it is not only the surface hydrophilicity but also an optimum balance of hydrophilic and hydrophobic nature, which plays a major role in biocompatibility evaluation. It is further evident from the present studies that biocompatibility is a very complex process, which involves a number of parameters, and there is no direct influence of any single parameter.

Section 3.4

Blood compatibility studies of nonporous ungrafted polyurethanes and grafted polyurethane membranes

3.4.1 Background:

During recent years a large number of investigations have been undertaken to develop blood compatible biomaterials which offer a variety of applications in medicine, surgery and artificial organs. The physiology of the human body is such that it can recognize any foriegn surface and promote clot formation on it. In many extracorporeal devices, blood contact invariably occurs thus requiring polymer to be biocompatible with blood. Blood clotting can be prevented by anticoagulants (e.g. heparin). In other cases, the blood contacting material must be blood compatible. The mechanism of blood clotting due to polymeric material is not well understood at present. Very few polymeric materials have good blood compatibility. However none of them completely prevents blood clotting, even if it mimics a limited feature of the living blood vessel. Some of the current blood compatible materials are segmented Pus (Lelah & Cooper, 1986; Ito & Imanishi, 1989; Fiala et al, 1987; Grasel et al, 1987), polytetrafluoroethylene (Kottke et al, 1986), Low temperature isotropic (LTI) carbon (Haubold et al, 1982), natural polymers such as cellulose and its derivatives (Ito et al, 1986; Woffindin & Hoenich, 1988) and some kinds of polymeric hydrogels are in practical used in medical products with comparatively good blood compatibility (Tsuruta et al, 1993).

The reason for this difficulty is due to the complicated behaviour of blood components at interfaces. The first event is the adhesion of proteins on the surface, followed by adhesion and activation of platelets, activation of leucocytes, complement

systems etc., activation of plasmatic proteins triggering the coagulation cascade and leading to the formation of blood clot. Blood coagulation and thrombosis are induced through both cascade reactions of intrinsic clotting factors in blood and through platelet adhesion followed by its activation on the foreign surface. These reactions are affected not only by the surface properties of the foreign surface, but also by the serum proteins adsorbed on that surface. It may be due to the extremely diverse nature of the components of the whole blood in size and amount allowing manifold interactions. Table 3.XIII shows a simplified view of the size and amount of blood components. As it can be seen from this table that the components have diverse affinities and interactions and hence it leads to complex and manifold interactions.

Table 3.XIII

Size and Amount of Blood Components - A Simplified view

Component ratio	Size	No. of cells/ Molecules per mm^3
Blood cells 40-50%(Vol)		
WBC	7-20(μm)	10^3
RBC	8-9 (μm)	10^6
Platelets	2-4 (μm)	10^5
Plasma 50-60% (Vol)		
Proteins 7%(wt.)	100(nm)	10^{14}
Organics 2%(wt.)	1 (nm)	10^{16}
Electrolytes 1% (wt.)	0.2(nm)	10^{17}
Water 90%(wt.)	0.3(nm)	10^{19}

The suggested sequence of events (Lyman & Knutson, 1980) at the polymer interface leading to the formation of thrombus is depicted in Fig. 3.33. In addition, it is also reported that the decrease of intracellular cyclic AMP, the rapid adsorption or desorption of denatured thrombin, haemolysis of red cells, adhered white blood cells,

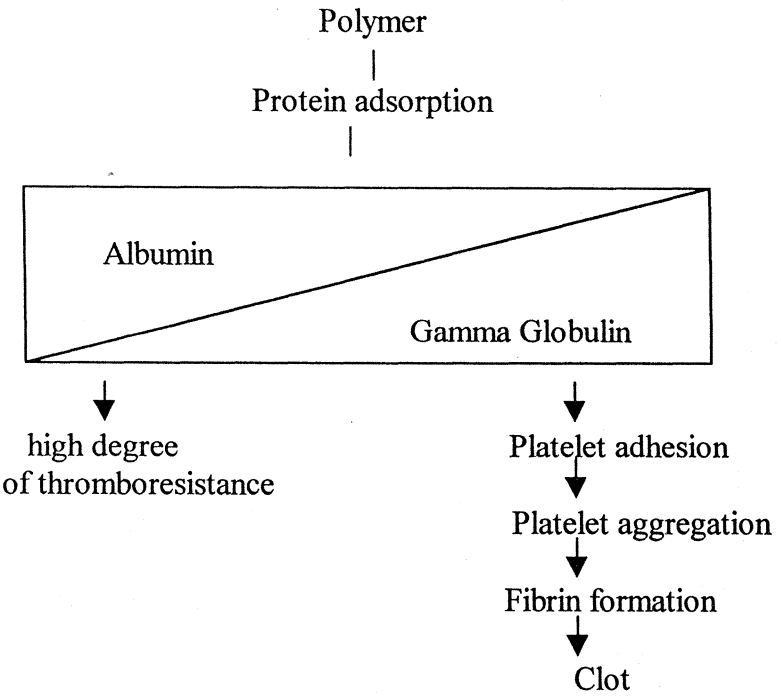

Fig. 3.33: Sequence of events at the polymer interface

surface activation of intrinsic coagulation factors and/or the release of adsorbed fatty acids may cause the formation of thrombus (Kim & Lee, 1979). A thrombus is then the end result of a complex interaction of various processes. At the site of vascular damage the coagulation cascade is rapidly activated to produce thrombin and finally to generate solid fibrin from insoluble fibrinogen, reinforcing the primary platelet plug. The coagulation cascade is shown in Fig.3.34.

The compatibility of a material with blood appears to be influenced by a number of factors, mainly related to the surface of the material which contacts blood (Lyman, 1966, Ratner, 1982; Scarborough, 1971). Surface free energy, surface molecular motions, surface topography, critical surface tension, electrical conductivity

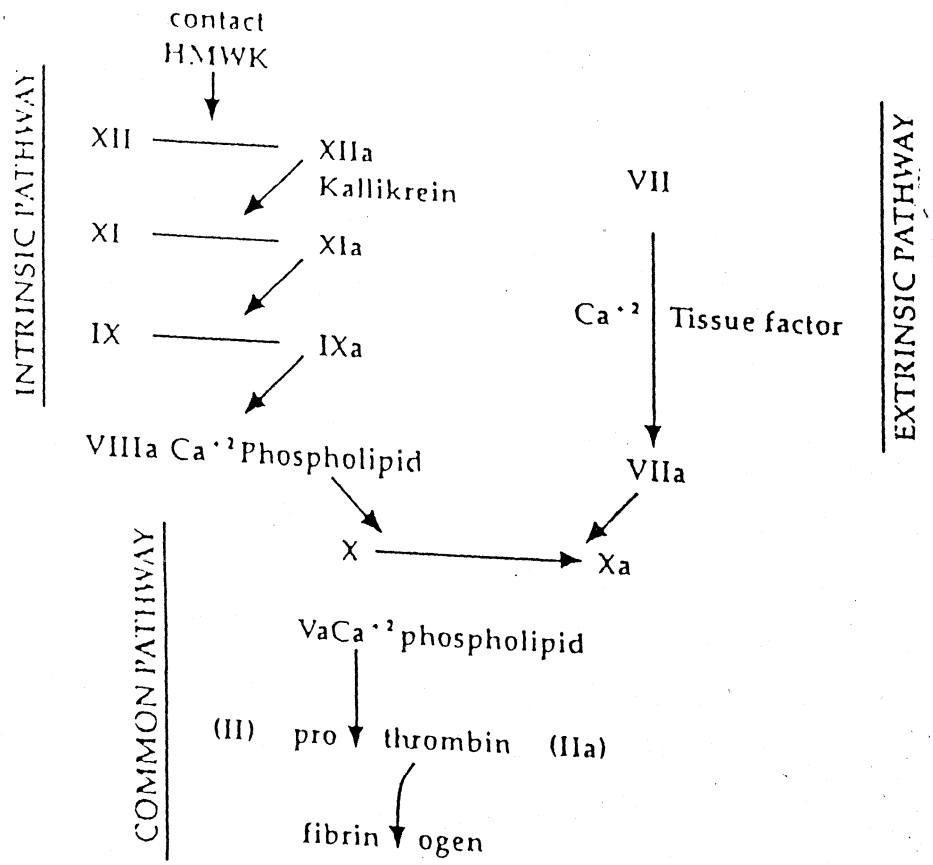

Fig.3.34 Intrinsic, extrinsic and common pathways of coagulation

(Adapted from Davis& Lewis, 1991)

and water content are the determining factors of blood compatibility (Barrenberg & Mauritz, 1982; Lyman et al, 1974). Andrade (1973) put forward the hypothesis that the material surface with an interfacial energy of zero would be expected to be highly nonthrombogenic. Hydrogels possessing minimum interfacial energy (Holly & Refoji,

1975) attract lower thrombus adherence and validate the hypothesis. Kaelble and Moacanin (1977) have indicated that dispersive and polar components of polymer surface play an important role in interfacial interactions. Lyman et al (1974) put forward the hypothesis of optimum hydrophilic-hydrophobic balance. It is based on the fact that segmented polyetherurethanes which have been used as artificial hearts are multiphase with a microphase-separated structure of polar and nonpolar segments, and that the excellent antithrombogenicity of segmented polyetherurethanes originates from the selective adsorption of albumin and is inactive to platelets. Several of these theories are however in conflict with one another.

Other properties of the surface which seem to affect blood interactions, especially in the case of polymeric materials is the chemical nature of the surface. Distribution of chemical groups on the surface could affect interctions such as thrombus formation, emboliformation, hemolysis of red cells and distribution of proteins. The surface concentration and the type of hard segment (Lelah et al, 1986) and relative concentration of hard segment (Lelah et al, 1986 & Okano et al, 1981) are seen to be of importance in the determination of the blood responses of PUs.

3.4.2 Blood compatibility studies of polyurethane membranes (ungrafted and grafted):

For the possibility of the use of these polyurethane membranes as a blood contacting device for future applications, these synthesised PU membranes as well as the surface grafted membranes was subjected to preliminary blood compatibility assessment. Materials used for blood contacting applications require characterization for blood compatibility to confirm their safety.

The protocol for blood compatibility testing was selected from ISO 10993 as discussed in section 2.6. The composition which were found noncytotoxic towards the fibroblast cells (PU-1, PU-4 and PU-5) as well as the grafted membranes were subjected to preliminary blood compatibility studies. Blood -cell adhesion on the ungrafted and grafted polyurethane nonporous membranes after contact with whole blood was evaluated by SEM analysis. Fig.3.35(a) shows SEM photomicrograph of nonporous PU membranes after 1 hour contact with the whole blood. Many adherent blood cells were observed on PU membranes after contact with whole blood and some aggregates were also observed. We evaluated the following parameters: i)RBC consumption ii) Plasma heamoglobulin (Hb) iii) WBC consumption iv) Platelet consumption as discussed in Table 3.XIV.

3.4.3 Evaluation of erythrocytes (RBC) on ungrafted & grafted PUs:

Erythrocytes or RBC, because of their very high number density, are major determinants of the flow properties of the blood, and affect the transport and distribution of all of the formed elements and macromolecules. When a red blood cell makes contact with a surface, it may attach at one or more points to the surface.red blood cells, while highly deformable, are actually fragile. As seen in Table XIII, the RBC consumption was not significant in any of the samples. From SEM analysis (Fig. 3.35 a,b and c) it is seen that the morphology of RBC was unchanged on all the ungrafted as well as the grafted samples.

In all the samples, plasma haemoglobin content showed the same range as that of the blank, indicating no haemolysis and RBS consumption was also not significant.

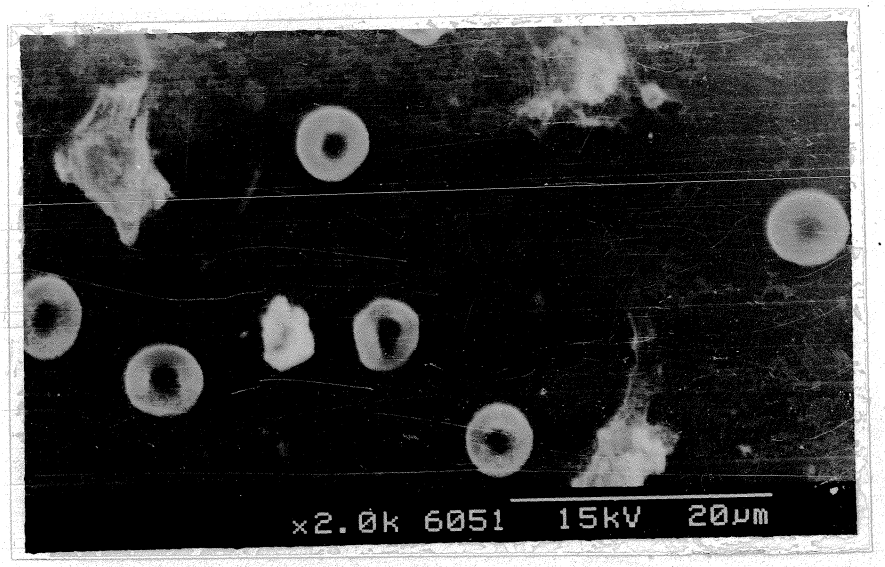

Fig. 3.35 SEM analysis of the preliminary blood compatibility studies **(a)** Membrane PU-4 which shows some microaggregates of platelets

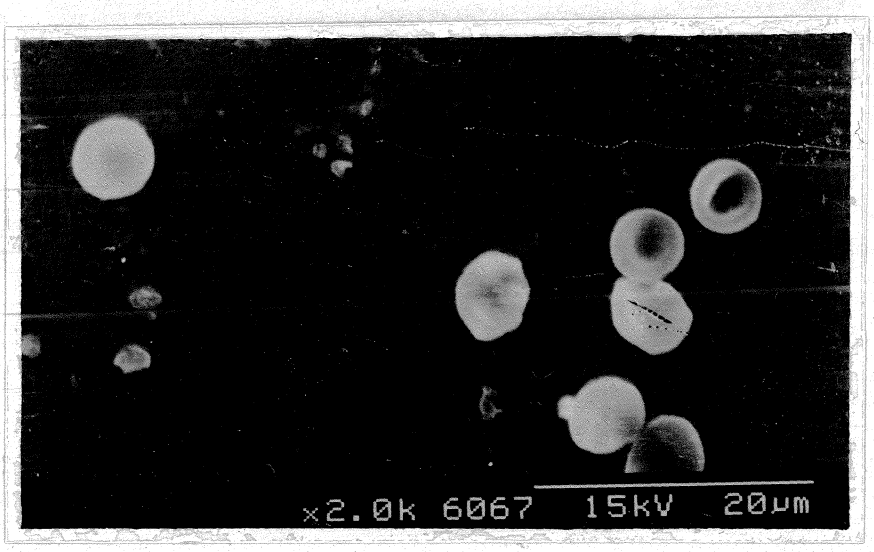

Fig. 3.35 SEM analysis of the preliminary blood compatibility studies **(b)** Grafted membrane (PU-g-EHA)

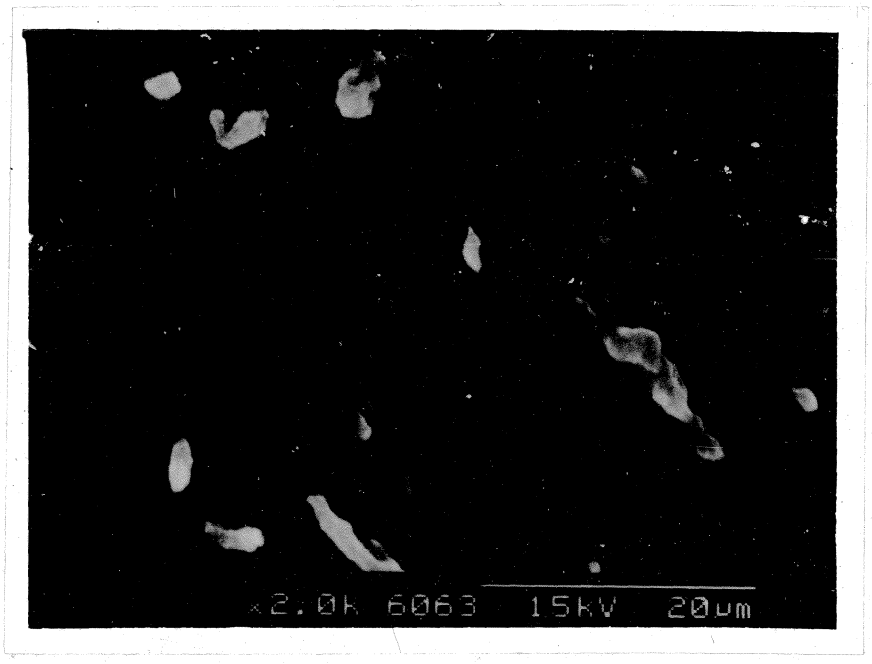

Fig. 3.35 SEM analysis of the preliminary blood compatibility studies **(c)** PU-g-NVP membrane

3.4.4 Evaluation of leucocytes (WBC) adhesion on ungrafted & grafted PUs:

Leucocytes are a central cell type in directing host inflammatory and immune processes: thus, its response to biomaterials is extremely important in understanding material-host interaction. Blood contacting biomaterials may activate the complement cascade, thus promote leucocyte adhesion and activation to the biomaterial surface. First, leucocytes recognize adhered proteins on the biomaterial surface and may adhere onto the surface via several adhesion ligand -receptors (Ziats et al, 1988).

Table 3.XIV

Data on Preliminary studies on Blood Compatibility

Sample Code	Composition	RBC consump-tion (%)	Plasma Hb	WBC consump-Tion (%)	Platelet consump-Tion(%)	LDH value as against Blank value of 174 IU/L
PU-1	PPG-25%	Not significant	Same range as blank	5	17	266 IU/L
PU-4	PTMG- 25%	Not significant	Same range as blank	4	13	407 IU/L
PU-5	PTMG -32%	Not significant	Same range as blank	Not significant	Not significant	336 IU/L
G-2	PU-g-HEMA	Not significant	Same range as blank	Not significant	Not significant	169 IU/L
G-4	PU-g-NVP	Not significant	Same range as blank	Not significant	Not significant	202 IU/L
G-6	PU-g-EHA	Not significant	Same range as blank	Not significant	Not significant	318 IU/L

This function could be modulated by the presence of active cytokines and growth factors (Anderson, 1988). However, the exact interrelationship among material physicochemical properties, adsorbed proteins, adherent cells, and inflammatory cytokines and growth factors is not yet fully characterised.

As can be seen from the Table 3.XIV that WBC consumption is almost nil in all the ungrafted and grafted samples except PU-1 and PU-4 which are 5% and 4% repectively. The SEM analysis also shows that in ungrafted samples (Fig.3.35a), the cells were flattened with cytoplasmic spreading and slight pseudopodial extensions. However, the SEM analysis of grafted samples (Fig. 3.35 b and c) showed only few leucocytes adhered on the surface. This shows that the grafted membranes had better blood compatibility from that of the ungrafted material by evaluation of leucocytes adhesion.

3.4.5 Evaluation of platelet adhesion on ungrafted & grafted PUs:

Platelets are seen as dots (Fig 3.35 a,b,c). In ungrafted samples (e.g. PU-4), platelets seen are like microaggregates but the morphology has not changed (Fig.3.35a).

Platelet contact activation is followed by adhesion, spreading, aggregation and sometimes granule secretion. If fully activated on the surface, platelet will exhibit a progression of morphologies from dendritic to fully spread forms (Allen et al,1979; Goodman et al, 1989; Barnhardt et al, 1972). The SEM photomicrographs of the membranes of the ungrafted samples shows that though the platelets are adhered on the surface as rounded body but no platelet activation by change in the morphology by pseudopod formation had taken place. Sample PU-4 shows some microaggregates of

platelets(Fig 3.35a). This shows that all the membranes can be considered as blood compatible in their preliminary studies.

When PU-4 was grafted with various vinyl monomers such as HEMA, NVP and EHA, it is observed that grafting reduced the number of adhered platelets and prevented platelet spreading on the surface. Hence, surface composition of the material in contact with blood greatly influences the extent of platelet adhesion and activation (Pelzer et al, 1986). Among the grafted samples, on analysis of SEM photomicrographs, the surface of PU-g-NVP (Fig.3.35 c) depicts less number of cells. In PU-g-EHA (Fig.3.35 b), platelets are present but no microaggregates or spread platelet are seen. According to Table 3.XIV, the platelet consumption is not significant in grafted samples. However, the ungrafted sample, PU-1 and PU-4 shows negligible amount of platelet consumption.

Thus the blood compatibility of the grafted materials are enhanced. Platelet adhesion on the ungrafted material was relatively high, and in some cases even microaggregate like structures were seen. By surface grafting, the number of adherent platelets were reduced and platelets were not that spread, thus enhancing the blood compatibility.

3.4.6 Lactic dehydrogenase(LDH) release analysis:

Enzyme assay was done using 0.1 ml of plasma obtained by spinning PC for 10 minutes at 5000 rpm. Assay was done as described in Worthington's manual. A Shimadzu UV-Vis 240 spectrophotometer with thermostated cuvette chamber was used to measure the reaction velocity as a decrease in absorbance at 340 nm resulting from oxidation of NADH made as described by Wacker et al (1956). Enzymes present

in the plasma, immediately after the concentrate preparation, was compared to the enzymes released to the plasma of WBC or platelets.

Lysis of platelets/ WBC will result in the discharge of platelet/ WBC lactate dehydrogenase (LDH) and this is the marker enzyme to predict cell lysis (Synder et al, 1981). As observed in the Table 3.XIV, LDH was not released into the plasma excessively and hence significant platelet/WBC lysis was absent and it was comparable to that of the blank, except ungrafted samples PU-4 and PU-5 which showed slightly higher value from that of the blank. It may due to the lysis of WBC or platelets in contact with these materials which is not significant in PU-5 and grafted materials.

From the preliminary blood compatibility analysis it is evident that the materials can also be used as a blood contacting device. Though their seems to be no direct coorelation of the physicochemical properties with the blood compatibility but it can be concluded that the surface has a very important role to play in devices used for blood contacting applications. As from the above results it is confirmed that by surface grafting, the blood compatibility had improved. However, elaborate studies needs to be done in future for a definite conclusion to be derived.

Section 3.5

Enzymatic degradation of polyurethane (*in vitro* analysis)

3.5.1 Background:

The subject of polymer degradation is of considerable importance in medical engineering and pharmaceutical technology. But for devices used for log-term application, the study of degradation is very significant. The most widely used method for studies of degradation of medical devices is enzymatic degradation.

Traditional application of synthetic polymers are mostly based on the relative inertness to biodegradation compared with natural macromolecules such as cellulose and proteins. The physicochemical environment - blood or extracellular fluid is a complex environment. Hydrolysis is an important degradation mechanism in the biological environment. The easiest method for studying biodegradation is to use model environments *in vitro*. The bioabsorption rate of biodegradable polymers depends on the factors shown in Table 3.XV.

Table 3.XV

Factors affecting the biodegradation of polymers

(Tsuruta et al, 1993)

Factor	Method of Control
Chemical structure of the main chain and functional groups	Selection of chemical bonds and side groups
Aggregation state	Processing, copolymerization
Crystalline state	Polymer blend
Hydrophilic / hydrophobic balance	Copolymerization , introduction of functional groups
Surface Area	Micropores
Shape and Morphology	Fiber, film, composite

The rate is strongly affected by the primary and higher order structures of the polymers, although the rate-structure profile is different for the enzymatically and nonenzymatically degradable polymers. A relatively fast degradation rate is shown in the polymers consisting of easily hydrolysable bonds. Their degradability depends on how easily the interaction between the enzymes and the relevant chemical bonds takes place, rather than the nature of the chemical bonds to be involved in the degradation reaction.

The solid state structure of polymers is another important factor affecting the bioabsorption rate. Generally, polymers are classified into 3 groups with respect to the aggregation structures of the polymer chains in solid state i.e. crystalline, glassy, and

rubbery polymers (Kimura, 1993). This classification is valid for bioabsorbable polymers. The rubbery polymers whose segment motion is active in solid state can adsorb water inside as well as ions, catalyst and enzymes to allow degradation from the internal region of the materials(Fig 3.36a). In the glassy polymers, however, such adsorption is not allowed, or is very slow because of their frozen segment motion, therefore degradation starts only from the surface and undergoes in such a way as to induce lateral surface etching (Fig.3.36b). In case of the crystalline polymers, which generally form polycrystalline states, the degradation takes place preferentially in the amorphous phase present in between the crystalline phase, and the reaction steps in the crystalline phase gradually after the amorphous region of the polymer has been degraded. Sometimes the remaining crystallites are peeled off as small pieces (Fig.3.36c). It is therefore admitted that if the chemical structure are similar to each other, the absorption rate should increase in the order of crystalline polymers < glassy polymers < rubbery polymers.

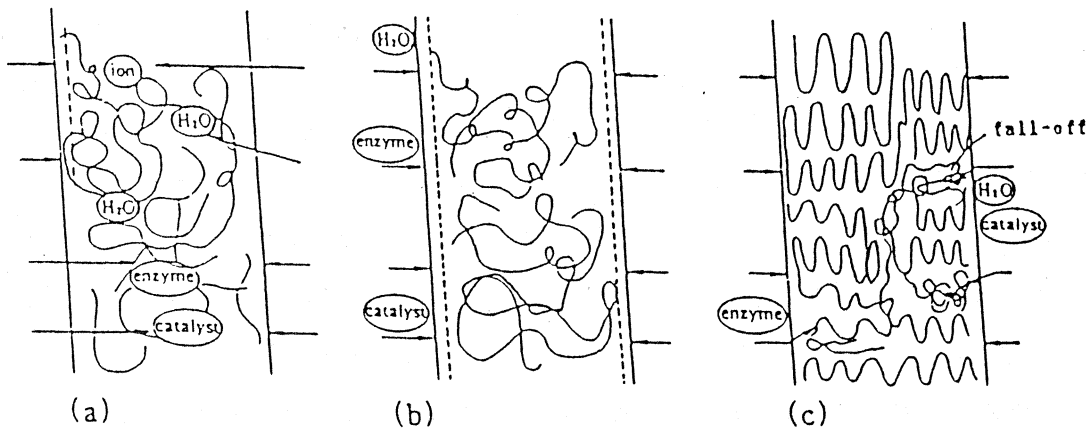

Fig: 3.36 The phenomenon of degradation : Decomposition mechanisms of the (a) rubbery, (b) glassy, and (c) polycrystalline polymers.

Since the surface should be the first site of attack in the heterogeneous hydrolysis reaction of polymeric materials, the surface structure and area are also important factors affecting the degradability. The hydrophilic and hydrophobic balance of polymer is also an important factor. An increased degradation is usually observed in the more hydrophilic polymers by virtue of easier absorption of water, catalyst and enzymes. Especially, the polymers containing hydroxyl and carboxyl side groups show an enhanced degradability not only due to the self - catalysis by the side groups. Contrarily, the hydrophobic polymers containing long chain alkyl and aromatic groups in both the main and side chains have a porous degradability.

With these factors considered, structural design by chemical modification and morphological control by polymer processing should be done carefully in order to control the degradability and absorbability of the polymers.

There are certain exceptions in the literature with polyethylene (Oppenheimer et al, 1955 & Smith & Williams), polypropylene (Liebert et al, 1976), poly(methyl methacrylate) (Oppenheimer et al, 1955), and polystyrene (Oppenheimer et al, 1955), all apparently suffering some degree of degradation.

The possible involvement of leucocyte-derived lysosomal enzymes (i.e. those enzymes with esterase or amidase activity) mediating biodegradation of biomedical polymers including polyesters, polyamides, polyolefins and segmented polyurethanes was studied by several researchers (Williams, 1984; Smith et al, 1987 and Phua et al, 1987). However, while the effects of the enzymes have been clearly shown, no direct evidence for a particular mechanism between an enzyme and a solid synthetic polymer substrate has been demonstrated.

Considering the wide applications and extensive use of polyurethane in biomedical and nonbiomedical systems, our present understanding of the degradation and stability of polyurethanes is incomplete. The field of polymer degradation has

extensive and sometimes different definitions for biodegradation. Potts et al, 1973 defined biodegradable materials as those which because of their chemical structure are susceptible to be assimilated by microorganism such as fungi and bacteria. Lemm et al, 1981 considered changes in surface properties or loss in mechanical strength to be biodegradation. Marchant et al, 1984 presented biodegradation as occurring on many different structural levels i.e. molecular, macromolecular, microscopic depending on the mechanism. Huang et al, 1979 defined biodegradation as enzyme induced degradation.

Due to advances in the design and materials choice of biomedical prostheses, implants, and devices, such biostability information becomes vital for the extension of the in vivo life of polyurethanes in biomedical applications.

In this section, the definition of biodegradation is restricted to degradation caused by enzymes in vitro.

3.5.2 Enzymatic degradation:

The procedure for *in vitro* treatments is discussed in section 2.7. Briefly, the stability of polyurethanes in enzymes was studied using enzymes (Trypsin, Bromalein and Papain). PU films of size 8 x 1 cm^2 were immersed in appropriate buffers - 100 BAEE units/ ml Trypsin enzyme in tris buffer (pH 8.1) solution, 100 units/ ml Bromalein enzyme in citrate buffer (pH 6.0) solution and Papain enzyme was dissolved in HEPES buffer. The composition of the buffers and enzymes are given in Appendix A. The experiment was carried out for a period of one month. After a month the polymer pieces were removed, cleaned with distilled water and dried. The change in the physical properties was determined.

Wt. Change Measurements:

The percentage wt. changes of polyurethane are summarised in Table 3.XVI

Table 3.XVI

Percentage weight change measurements of polyurethanes, PU-4 (PTMG- 25%) and PU-5 (PTMG- 32%)

Medium	% wt. Change after 1 month treatment (PU-4)	% wt. change after 1 month treatment (PU-5)
Tris Buffer	0.37	0.55
Trypsin in Tris buffer	0.16	0.52
Citrate Buffer	0.14	0.58
Bromalein in citrate buffer	0	0.51
HEPES Buffer	0.23	0.33
Papain in HEPES Buffer	0.40	0.56

As observed from the Table 3.XVIthe percentage weight change is negligible for the enzyme treated samples ranging between 0-0.6 %.

3.5.3 Infra-red Spectra:

Figures 3.37a shows the representative IR spectra of the enzyme treated ungrafted polyurethane samples. The spectra of enzyme treated samples of polyurethanes revealed no significant differences when compared with the spectra of

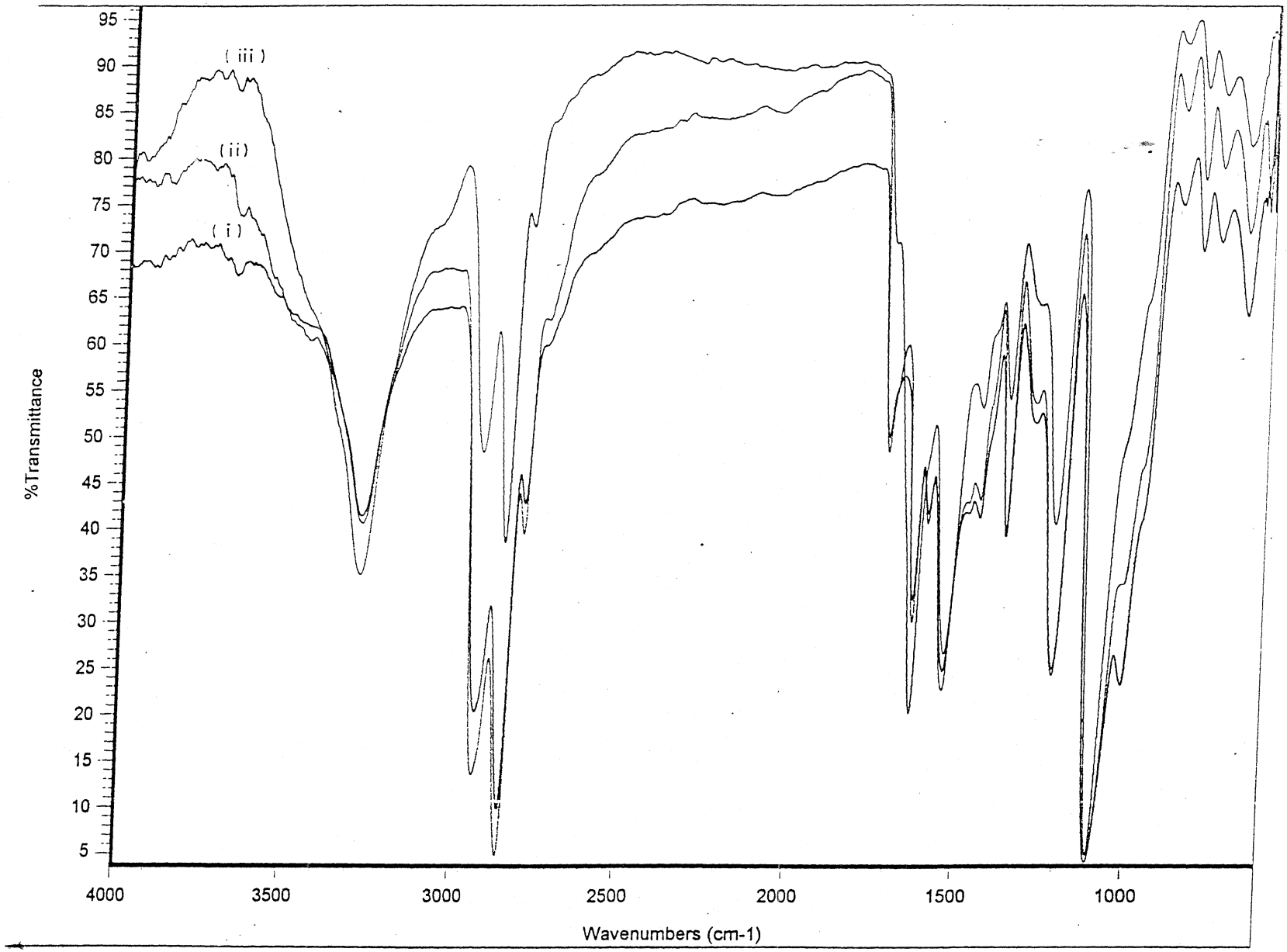

Fig. 3.37 Representative Infrared spectra of enzyme treated and untreated (a) ungrafted polyurethane (i) PU-4 (PTMG-25%) – untreated (ii) PU-4)PTMG – 25%)

untreated and buffer treated polyurethanes. PU shows characteristic peaks at 3300, 2900, 1400, 1200, 950-1000 cm^{-1} in case of trypsin, bromalein and papain enzymes. The absence of the protein characteristic bands in the spectra of enzyme treated PU denoted that enzymes were not adsorbed by these samples. However, these indications are not necessarily a measure of the actual changes experienced in the samples.

3.5.4 Mechanical Properties:

Table 3.XVII shows the percentage change in mechanical properties of the enzyme treated samples with the nontreated samples. It is observed that the materials show only negligible decrease in mechanical properties from the untreated material.

Table 3.XVII

Percentage change in mechanical properties of the enzyme treated polyurethane membranes with that of the untreated samples

Medium	% change in stress of the treated sample with the untreated sample(PU-4)	% change in strain(%) of the treated sample with the untreated sample (PU-4)	% change in stress of the treated sample with the untreated sample (PU-5)	% change in strain (%) of the treated sample with the untreated sample(PU-5)
Tris Buffer	0.43	0.61	0.75	0.91
Trypsin in Tris buffer	0.40	0.55	0.70	0.92
Citrate buffer	0.58	0.86	0.80	1
Bromalein in citrate buffer	0.72	0.58	1	1
HEPES Buffer	0.67	0.67	1	1
Papain in HEPES Buffer	0.67	0.52	1	1

Thus, it can be concluded from the biodegradation studies that the materials (PU-4 & PU-5) which showed appropriate physicochemical properties and also showed compatibility with the islet cells showed nondegradable characteristics for a period of 1 month. They can be proposed as suitable candidates for immunoisolation purposes.

The biodegradation studies of the surface modified materials are dealt in the next section of this chapter.

3.5.5 Biodegradation studies of surface modified grafted polyurethane membranes:

The degradation studies of the grafted polyurethane membranes was done by the same procedure as that of the ungrafted polyurethane membranes. The in vitro biodegradation studies indicated that, even after grafting the stability of the polyurethane is not deteriorated substantially. Table 3.XVIII shows the percentage weight change measurements of the grafted polyurethane membranes. As observed in this table, negligible change in weight was observed in the enzyme treated grafted polyurethane material from that of the untreated materials. Table 3.XIX (a) and (b) shows the percentage change in mechanical properties of the grafted materials after treatment with enzymes. As is evident from these Tables that the loss in mechanical properties is negligible. Fig. 3.37 b shows the representative IR spectra of the grafted material after treating it with enzyme. The spectrum of the enzyme treated sample is almost the same as that of the untreated material.

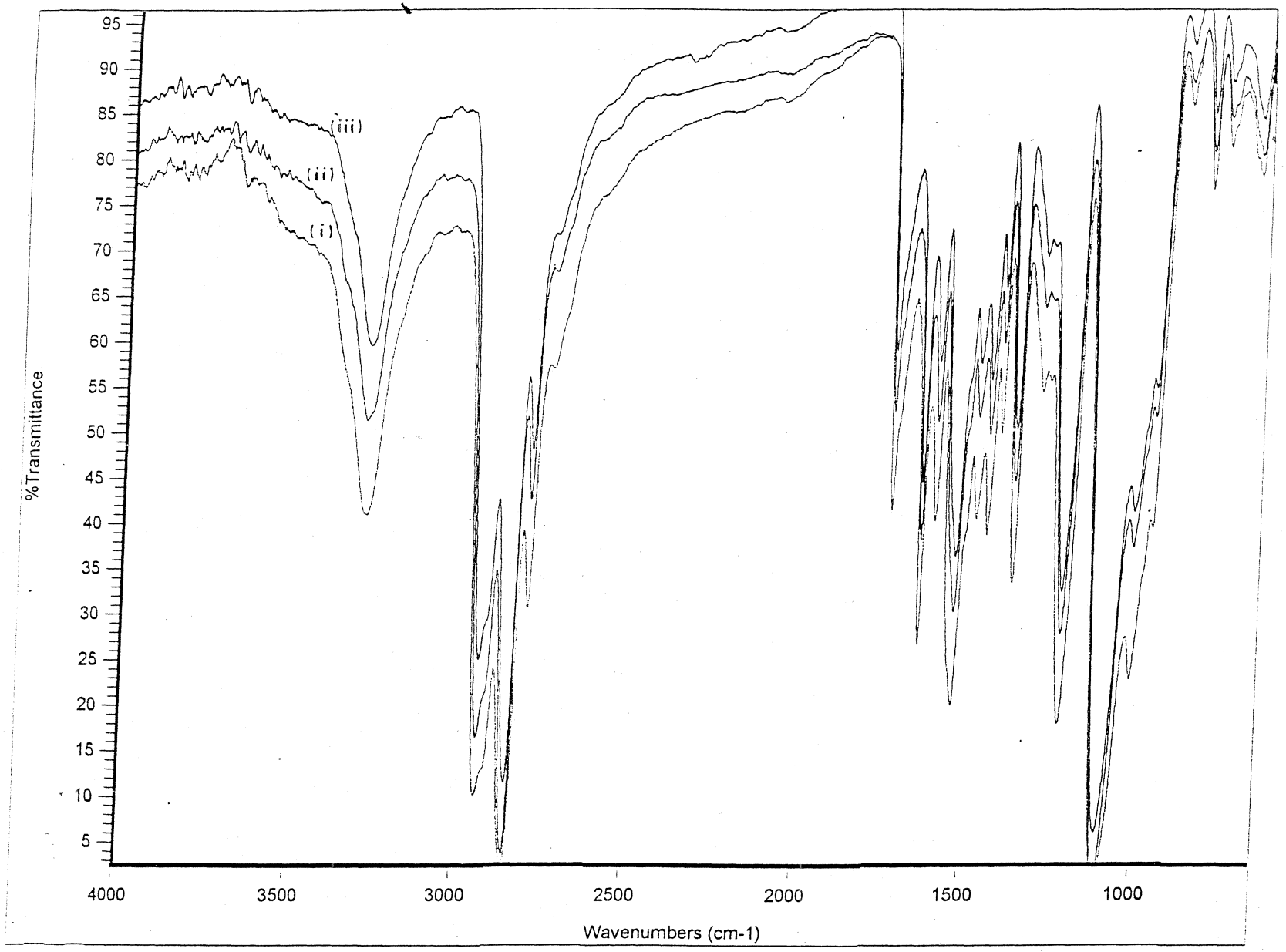

Fig 3.37 Representative Infrared spectra of enzyme treated and untreated (b) grafted polyurethane (i) PU-g-EHA (untreated) (ii) PU-g-EHA (citrate buffer treated

Table 3.XVIII

Percentage weight change measurements of grafted polyurethane membranes

	% wt. change of the enzyme treated grafted materials with that of the untreated materials→		
Medium↓	G-2	G-4	G-6
Tris Buffer	0.15	0.2	0.11
Trypsin in Tris buffer	0.11	0.21	0.12
Citrate Buffer	0.22	0.3	0
Bromalein in citrate buffer	0.5	0.5	0.01
HEPES Buffer	0.23	0.33	0.02
Papain in HEPES Buffer	0.36	0.46	0.1

Table 3.XIX

a) % Change in mechanical properties(stress) of the enzyme treated grafted material with that of the untreated material

	% change in mechanical properties(stress) of the enzyme treated grafted material with that of the untreated material→		
Medium↓	G-2	G-4	G-6
Tris Buffer	0.40	0.6	0.36
Trypsin in tris buffer	0.40	0.61	0.37
Citrate buffer	0.4	0.59	0.35
Bromalein in citrate buffer	0.5	0.71	0.35
HEPES buffer	0.62	0.69	0.5
Papain in HEPES buffer	0.6	0.71	0.51

Table 3.XVIII

b) % Change in mechanical properties (%strain) of the enzyme treated grafted material with that of the untreated material

	% change in mechanical properties(%strain) of the enzyme treated grafted materials to that of the untreated material→		
Medium↓	G-2	G-4	G-6
Tris Buffer	0.52	0.63	0.52
Trypsin in tris buffer	0.50	0.63	0.50
Citrate buffer	0.7	0.55	0.7
Bromalein in citrate buffer	0.5	0.70	0.5
HEPES buffer	0.4	0.70	0.4
Papain in HEPES buffer	0.6	0.71	0.6

Hence, the preliminary biodegradation studies of the ungrafted and the grafted materials shows that the materials are stable upto a period of about one month. However, elaborate studies on biodegradation for longer periods needs to be conducted for the realistic picture of biodegradation of materials in the long term.

CHAPTER 4

SUMMARY, CONCLUSIONS AND FUTURE PROSPECTS

SUMMARY, CONCLUSIONS AND FUTURE PROSPECTS

4.1 Summary and Conclusions:

Encapsulation is based on the premise that cells, once sequestered within a semipermeable membrane, are isolated from the immune system and therefore cannot be recognised and/or destroyed by normal host defenses. The membrane also presents a barrier to any potential outgrowth of cells into the host parenchyma, thus permitting the use of mitotically active cell lines. Bidirectional diffusion through the membrane of small molecular weight molecules such as oxygen, glucose, cellular secretogues and bioactive cellular products allows for continued maintenanance of encapsulated cell viability and sustained release of the molecules of interest. The need for immunosuppressive drugs, however, may lead to a variety of serious side effects. One approach to minimizing or eliminating systemic immunosuppression is immunoisolation of the transplanted tissue by a semipermeable membrane in order to protect it from immune rejection, thereby creating what has been termed an implantable biohybrid artificial organ. Devices of this type are under study for treatment of variety of diseases, including secretion of insulin in diabetes, treatment of kidney failure, liver failure etc., neurogenerative disorders such as Parkinson's disease etc. Since polymers can be tailor made to match the mechanical and physical characteristics of many parts of the body, they find maximum applications as biomaterials.

Transplantation of islets or insulin secreting cells has been attempted to restore a normal pattern of insulin secretion in diabetic individuals and thus prevent the complications of diabetes that occur inspite of the long-term insulin injection therapy. The relatively low availability of human islets and the problem of immunorejection have

made the pancreatic islet transplantation in humans very limited. A possible solution might be to transplant xenogenic islets surrounded by a permselective synthetic membrane, which isolates the transplant from its environment. The protection of cell transplants from being destroyed by the host's immune system is the main purpose of immunoisolation, thereby eliminating costly and dangerous immunosuppressive drug therapy. The membrane surrounding the islets should be permeable to nutrients, glucose, and insulin and should prevent the entry of antibodies and lytic factors of the complement, as well as the inward migration of white blood cells. Several permselective membranes such as alginate-polylysine alginate, polyvinyl chloride acrylic copolymer etc. have been used without satisfactory results. The dominant issue that remains is the inadequate membrane material biocompatibility. An alternative might be represented by polyurethane materials, which have shown good blood and tissue compatibility in a variety of applications such as vascular prosthesis, blood filters etc. but it has not been extensively explored for immunoisolation purposes.

The thesis comprises the authors efforts to synthesise and evaluate linear segmented polyurethane membranes based on toluene diisocyanate, polyols such as polypropylene glycol and polytetramethylene glycol and butanediol with varying percentage of hard segment, for biomedical applications, particularly for islet cell immunoisolation purposes. These synthesised polyurethane membranes were characterised for islet cell immunoisolation purposes using various physicochemical and biological assays. The chapter 2 comprises the details of the synthetic procedures and the experimental details of the synthetic characterisation. The procedures used to study the in vitro biocompatibility, blood compatibility and stability are also discussed.

Chapter 3 summarises the results and the discussions of the characterisation of the synthesised polyurethane membranes. The infrared spectra confirmed that the synthesised membrane is of polyurethane type. The scanning electron microscopy SEM)

helps to study morphological structure of polymers and to assess the gross uniformity of the samples. SEM of the synthesised PUs shows no imperfections and a smooth, homogeneous system at all compositions. The mechanical properties were seen to be influenced by the type of polyol used and the percentage of hard segment. The thermoanalytical data obtained from Differential scanning calorimetry gave an idea about soft segment and hard segment glass transitions (Tg) of polyurethanes (PUs). The PUs synthesised showed 2 Tgs, in which the lower value is attributed to soft segment Tg. This data confirms the synthesis of segmented PUs. The thermogravimetric curves show a gradual weight loss as temperature increases. These results are summarised in section 3.1.5. A decrease of surface air-water contact angle is suggestive of the hydrophilicity of the system. The measurement of air-water contact angle shows that the PUs with PTMG as glycol is more hydrophilic than PUs with PPG as glycol. The results of relative hydrophilicity and hydrophobicity are summarised in section 3.1.4. WAXD and polarised microscopic studies reveals the semicrystalline nature of PTMG based PUs. This semicrystalline nature was coorelated with the sharp intense peak in the DSC curves of the PTMG based polyurethanes which was absent in the PPG based PUs.

Permeation studies shows that the synthesised PU membranes are readily permeable to glucose and insulin while impermeable to albumin and immunoglobulins.

An attempt was made to surface modify one of the polyurethane membrane, which was found compatible with the islet cells. Surface modification of polymers have developed into an important way to develop materials having appropriate surface properties and bulk properties. Several techniques have been used for surface modification of polymers. Physicochemical modifications like coating the surface with other polymers, surface chemical reactions, grafting other polymers on the surface and biological methods like incorporation of biologically active molecules have all been attempted by various researchers. Radiation grafting which is one of the easiest and

simplest technique have been attempted to surface modify the PU. Grafting was carried out using vinyl monomers such as hydroxyethylmethacrylate, N- Vinyl pyrrollidone and ethyl hexyl acrylate. These membranes was also characterised using various physicochemical and biological assays. Section 3.2 discusses the results of the physicochemical analysis. The grafted membranes showed better permselective characteristics as discussed in section 3.2.8, while retaining the desirable physicochemical properties.

As for use as a biomaterial, biological evaluation is necessary.In the biological assays as discussed in sections 3.3.1 and 3.3.2, the grafted polyurethane membranes showed better viability and morphological characteristics of the islets. The results of the in vitro biocompatibility analysis such as viability studies using fibroblast cells and islet cells, islet morphometry studies etc. of the synthesised polyurethane membranes and the grafted membranes are discussed in sections 3.3.1 and 3.3.2 respectively.

For the future use of the synthesised and surface grafted Pus for blood contacting applications, blood compatibility evaluations were carried out. Preliminary blood compatibility studies shows that the grafted polyurethane membranes were found to be better for blood contacting applications. The results are discussed in section 3.4.

As the material is intended for long term applications, degradation studies were also done using enzymatic degradation analysis as discussed in section 3.5. Preliminary biodegradation studies in various enzymes showed that the ungrafted and grafted membranes are relatively stable for a period of 1 month.

In conclusion, the grafted polyurethane membranes are good candidate islet immunoisolation matrices which may be considered for further in vivo applications.

4.2 Future Prospects:

The study indicates that polyurethane can be tailor made with specific properties for specific biocompatibility requirements. The future work on these polyurethane is mainly concerned with the applications to which it would be utilized. These membranes will be further tested in invivo animal models. Since the membranes showed better blood compatibility in preliminary studies, more experiments for invivo blood contacting applications are to be carried out, so that these membranes can be further tested as a blood contacting device or as coatings. The synthesised polyurethanes and the grafted polyurethane membranes can be used for the encapsulation of other cells such as hepatocytes etc. to develop a range of tissue engineered devices.

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APPENDIX-A

A.1 ACD

Trisodium citrate	= 2.2 g
Citric Acid	= 0.8 g
Dextrose	= 2.5 g
Dissolved in 100 ml of distilled water, pH	= 5 to 5.1

A.2 Tris Buffer

Dextrose	= 1.000 g
Magnesium chloride	= 0.199 g
Potassium chloride	= 0.402 g
Sodium chloride	= 8.120 g
Calcium chloride	= 5.5 g
Tris(hydroxymethyl amine tris buffer)	= 1.756 g

A.3 Phosphate Buffer saline (0.15 M)

Disodium hydrogen phosphate. 2H2O	= 21.42 g
Sodium hydrogen phosphate.2H2O	= 4.689 g
Sodium chloride	= 8.599 g

Total volume made upto 1 litre by dissolving in distilled water, pH = 7.4

A.4 Citrate Buffer stock, pH 6.0

Sodium Citrate = 147 g

Distilled water = 600 g

Adjust the pH to 6 with 50% citric acid and then make up the volume to 1 litre with distilled water

Working buffer : Dilute the stock buffer 1 in 10.

A.5 The compositon of buffers and enzymes

Enzymes	Source	Buffer
Trypsin	Bovine pancreas	0.46M Tris-HCl pH 8.1 with 0.0115 M $CaCl_2$ and 0.2% NaN_3
Bromalein	Pineapple	0.1M citrate pH 6.0 with 0.2% NaN_3
Papain	Papaya latex	0.1M HEPES 0.2% NaN_3 0.005M Cystene 0.01 M EDTA 0.06 M Mercaptoethanol

List of Publications

Publications **:**

The following publications have resulted from the work reported in this thesis:

1. Sheela George and Prabha D. Nair, 'Permselectivity of nonporous polyurethane membranes for immunoisolation I. The influence of Hydrogen bonding', *J. of Appl. Poly. Sci.* Vol 73, 1949-1954,1999.

2. Sheela George and Prabha D. Nair, 'Novel Polyurethane Materials for Islet cell immunoisolation', Proceedings of *XII Kerala Science Congress(Part I)*, 244-249, 2000.

3. Sheela George & Prabha D. Nair 'Synthesis and Characterisation of Grafted Polyurethane Membranes for Immunoisolation', Proceedings of *Recent Advances in Polymers and Composites* (Macro 2000)(Allied Publishers Limited), 2000, 327-330.

4. Sheela George & Prabha D. Nair 'Grafted Polyurethane Membranes For Islet Cell Immunoisolation- Biocompatibility Studies', Proceedings of the *Annual general Meeting of SBAIO and BEDPSI and National Conference on Tissue Replacement Materials & Devices & Biodegradable Polymers & Composites for the Mellinium Ahead*, 2000, 74-76.

5. Sheela George, M.Risbud, Prabha D.Nair & R.R. Bhonde. 'Nonporous polyurethane membranes as immunoisolation matrices - Biocompatibility Studies', *J. Of Biomedical Application (Polymer Edition)* (Under revision).

6. Sheela George & Prabha D. Nair Surface modified Polyurethane membranes for Immunoisolation (To be communicated).

7. Sheela George & Prabha D. Nair'Biocompatibility studies of surface modified Polyurethane for Islet Cell Immunoisolation' (To be communicated).

8. Sheela George & Prabha D. Nair 'In vitro blood compatibility studies of synthesised and grafted Polyurethane Membranes'(To be communicated).

APPENDIX-C

LIST OF ABBREVIATIONS:

ACD	- Acid citrate dextrose
BD	- Butanediol
DBTL	- Dibutyl tin dilaurate
DMF	- Dimethyl formamide
DMEM	- Dulbecco's Minimum Essential Medium
DSC	- Differential Scanning Calorimetry
DTG	- Differential thermogravimetry
DTG	- Differential thermogravimetry
EHA	- Ethyl hexyl acrylate
EGDMA	- Ethylene glycol dimethacrylate
HEMA	- Hydroxyethylmethacrylate
IR	- Infrared Spectroscopy
MTT	- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
μm	- micrometer
NR	- Neutral Red(3-amino-7-dimethyl-2 methylphenazine hydrochloride)
NVP	- N-vinyl pyrollidone
PBS	- Phosphate buffer saline
PPG	- Polypropylene glycol
PTMG	- Polytetramethylene glycol
PU	- Polyurethane
PU-1	- Polyurethane based on TDI-PPG-BD with 25% hard segment
PU-2	- Polyurethane based on TDI-PPG-BD with 32% hard segment
PU-3	- Polyurethane based on TDI-PPG-BD with 40% hard segment
PU-4	- Polyurethane based on TDI-PTMG-BD with 25% hard segment
PU-5	- Polyurethane based on TDI-PTMG-BD with 32% hard segment

PU-6	- Polyurethane based on TDI-PTMG-BD with 40% hard segment
PRP	- Platelet rich plasma
PU-g-HEMA	- Polyurethane grafted with hydroxyethylene methacrylate
PU-g-NVP	- Polyurethane grafted with N-vinyl pyrollidone
PU-g-EHA	- Polyurethane grafted with ethyl hexyl acrylate
SEM	- Scanning Electron Microscopy
TCPS	- Tissue Culture Polystyrene
TDI	- Toluene diisocyanate
TGA	- Thermogravimetric Analysis
UV	- Ultra voilet
WAXD	- Wide Angle X-ray Diffraction