**ELASTOMERIC NETWORKS BASED ON TRIMETHYLENE
CARBONATE POLYMERS FOR BIOMEDICAL APPLICATIONS**

PHYSICAL PROPERTIES AND DEGRADATION BEHAVIOUR

Erhan Bat

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Elastomeric networks based on trimethylene carbonate polymers for biomedical applications
– Physical properties and degradation behaviour

Erhan Bat

PhD Thesis , with references; with summary in English and in Dutch

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DISSERTATION

to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus,
prof.dr. H. Brinksma,
on account of the decision of the graduation committee,
to be publicly defended
on Friday the 28th of May at 16:45

by

Erhan Bat

born on the 16th of January 1981
in Nusaybin, Turkey

This dissertation has been approved by:

Promotores: Prof. Dr. J. Feijen
Prof. Dr. D. W. Grijpma

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In 2005, I started a “new” life in a foreign country, with a new job, a new home, new friends and colleagues. Doing my Ph. D. at the Polymer Chemistry and Biomaterials (PBM) group has been an unforgettable and very pleasant experience for me. It has been a long time with lots of ideas, hard work, disproved-confirmed hypotheses, discussions, nice friendships and fun. Finally, I am almost there! Despite all the intrinsic difficulties of doing a Ph. D., (and all the nasty jokes the Simpsons family makes about graduate students such as having made a terrible life choice!), I am glad to say that I don’t recall any moment that I regretted pursuing a PhD. In this long journey, I met many people from whom I received valuable support. Now, I would like to take the opportunity to thank to those people who have made this work and my life better with their support.

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PART I

**GENERAL INTRODUCTION
&
BACKGROUND**

Chapter 1

General Introduction

The development of synthetic polymeric biomaterials has been one of the major driving forces in advancing biomedical technologies, particularly in the fields of tissue engineering and controlled/targeted drug or gene delivery ¹⁻³. In many cases, implants are subjected to dynamic mechanical environments *in vivo*, under which they must sustain and recover from repeated deformation without causing mechanical irritation to the surrounding tissue ⁴. Therefore, biodegradable elastomers receive increasing attention especially in soft tissue engineering and in drug delivery ^{5, 6}.

In the most commonly followed tissue engineering approach, porous biodegradable scaffolds play a pivotal role and act as a temporary replacement of the extracellular matrix onto which (stem-) cells can adhere and grow ^{3, 7-9}. The physical-, chemical-, and biological properties and the degradation behaviour of these scaffolds are of vital importance in the success of the tissue engineered constructs. The materials used to prepare the scaffolds and their degradation products must be biocompatible. They should allow adhesion, proliferation, and proper functioning of cells. Ideally, the mechanical properties of the scaffold should resemble that of the tissue to be regenerated and the erosion rate of the scaffold should closely match new tissue formation without causing an adverse tissue response.

The major advantages of using elastomeric polymers as scaffolding materials in soft tissue engineering are their ability to sustain dynamic mechanical environments, to allow mechanical stimulation during culture, and to transmit mechanical signals to the developing tissue. In addition, their low elastic modulus values which approximate those of soft and elastic tissues are also desired properties.

Poly(trimethylene carbonate) (PTMC) is an amorphous polymer with a low glass transition temperature (T_g , approximately $-17\text{ }^{\circ}\text{C}$) ^{10, 11}. Owing to its low T_g and amorphous nature PTMC is very flexible with an elastic modulus of approximately 6 MPa, but it can also creep in an undesired manner under stresses, making materials prepared from this polymer not

form-stable ¹²⁻¹⁴. By gamma irradiation under vacuum, form-stable and elastomeric PTMC networks having low elastic modulus values can be formed ¹². Another remarkable property of PTMC is its degradation by surface erosion, in which non-acidic products are formed. These properties make this biocompatible polymer an attractive material for soft tissue engineering and drug delivery applications. However, this biocompatible polymer erodes relatively rapidly *in vivo*, which limits its use to short-term applications ^{14, 15}. Therefore, there is a need to develop trimethylene carbonate-based materials having lower erosion rates.

Aim and Structure of the Thesis

The work described in this thesis aims at developing slowly eroding elastomeric polymer networks based on trimethylene carbonate polymers in an efficient and practical manner to be utilised in biomedical applications.

This thesis is divided into four parts. In **Part I**, a general introduction and a brief literature overview is presented providing the background to the study. The aim of the work is stated and the structure of the thesis is outlined in **Chapter 1**. An introduction to biodegradable polymers, tissue engineering, and a short review on biodegradable elastomers is given in **Chapter 2**. The implications of the different degradation profiles and mechanical properties of biodegradable polymers on the performance of tissue engineering scaffolds and drug delivery systems are discussed. In **Chapter 3**, gamma irradiated PTMC is evaluated as a scaffolding material for cardiac tissue engineering. It is demonstrated that these materials are suitable substrates for cardiomyocyte adhesion and proliferation, and scaffolds prepared from these materials have low elastic modulus values that approximate those of myocardium. Implantations on the heart of rats showed that these angiogenic scaffolds erode rapidly.

In **Part II**, an attempt is made to obtain slowly eroding elastomeric networks by copolymerisation of trimethylene carbonate (TMC) with ϵ -caprolactone (CL) and by variation of the gamma irradiation dose. **Chapter 4** is an *in vitro* study focusing on the crosslinking of (co)polymers based on TMC and CL (0 to 30 mol %) by gamma irradiation, their mechanical properties and their enzymatic (surface) erosion behaviour. The effects of copolymer composition and gamma irradiation dose on mechanical properties, enzymatic surface erosion behaviour, and cell compatibility are discussed. In **Chapter 5**, the *in vivo* tissue response to networks based on TMC and CL (co)polymers and their erosion behaviour are investigated qualitatively and quantitatively. Adjusting the network density by variation of the gamma irradiation dose and by copolymerisation with up to 30 mol % CL were not much effective in

obtaining slowly eroding networks. Therefore, new approaches were taken as described in the following parts of the thesis.

In **Part III**, a macrophage culture model for the investigation of the erosion behaviour of networks based on TMC polymers is introduced. To obtain slowly eroding elastomeric networks, a new route is followed to crosslink these polymers. In **Chapter 6**, we report an *in vitro* macrophage culture as a versatile platform to investigate the erosion behaviour of PTMC networks. The possible involvement of macrophage-derived enzymes and reactive oxygen species in the erosion of gamma irradiated PTMC films is also assessed and discussed.

In **Chapter 7**, we present the crosslinking of PTMC polymers by gamma irradiation in the presence of pentaerythritol triacrylate (PETA) as a crosslinking aid. First, the effect of molecular weight and irradiation dose on gel content and network density of PTMC networks without PETA is investigated. It is demonstrated that very high gel contents and network densities are practically not attainable even for polymers having a very high initial molecular weight. Therefore, the use of PETA was introduced and densely crosslinked networks with high gel contents (up to 96 %) could be obtained. The effects of incorporation of PETA on compatibility with cells, and on improvement of mechanical properties, and reduction of enzymatic erosion rates of PTMC networks are assessed.

Chapter 8 focuses on crosslinking TMC and DLLA (co)polymers that either degrade or crosslink very inefficiently during sterilization by gamma irradiation. By incorporating PETA in these films, it is shown that even PDLLA can be efficiently crosslinked. The effects of copolymer composition and crosslinking in the presence of PETA on the thermal- and mechanical properties, cell compatibility, and macrophage-mediated erosion of the networks are presented and discussed. Materials with a wide range of mechanical properties and degradation behaviours were obtained by copolymerisation and gamma irradiation in the presence of PETA.

In **Chapter 9**, we describe a practical in-house method of crosslinking TMC polymers by ultraviolet light. PTMC polymers having a range of molecular weights have been used to prepare networks by photocrosslinking in the presence of PETA and a photoinitiator. Also the modification of properties of TMC-based networks formed by irradiating blends of PTMC with block copolymers having PTMC and PCL or poly(ethylene glycol) blocks is demonstrated. The networks are characterised with respect to their gel contents, network densities, wettabilities, and enzymatic erosion behaviour. Their potential as scaffolding materials in tissue engineering is assessed by preparing porous scaffolds by fused deposition modelling and by human mesenchymal stem cell culturing.

We bring a novel approach to photocrosslinking high molecular weight PTMC by incorporating only a small amount of a relatively biocompatible photoinitiator or a photoinitiator and PTMC macromers into PTMC films. This work is presented in **Chapter 10**. The effects of photoinitiator concentration and PTMC macromer concentration are discussed with regards to gel formation and network density. The improvement in mechanical properties of the networks are demonstrated. The effect of macromer content on reducing macrophage-mediated erosion rates of PTMC networks is discussed. Also, the erosion rates of these materials in aqueous- cholesterol esterase or potassium superoxide solutions are investigated. The compatibility of the PTMC networks with cells is assessed.

Part IV includes an appendix to **Chapters 4** and **5** and a preliminary study on physically crosslinked hydrogels. In **Appendix A**, (co)polymers of TMC and CL having higher CL contents than those investigated in **Chapters 4** and **5** were prepared. The effects of composition and gamma irradiation on the mechanical properties are investigated. The erosion behaviours of the (co)polymer networks are assessed *in vivo* by subcutaneous implantations in rats, as well as *in vitro* using macrophage cultures. In **Appendix B**, we report on the thermoresponsive behaviour of hydrogels prepared from triblock copolymers of PTMC and polyethylene glycol (PEG) (PTMC-PEG-PTMC). The effects of polymer composition and polymer concentration in the aqueous gel on gelation behaviour and on rheological properties are investigated.

Most of work described in this thesis has been published ¹⁶⁻¹⁸ or is expected to be published in the near future ¹⁹⁻²⁵. A patent application based on the work described in **Part III** has been filed.

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Chapter 2

Biodegradable Elastomers for Biomedical Applications

Synthetic Biodegradable Polymers

Synthetic biodegradable polymers are of great value for the preparation of implants that are required to reside only temporarily in the body. Use of biodegradable polymers obviates the need for a second surgery to remove the implant, which is often the case when a non-degradable implant is used. Moreover, as a biodegradable implant will be eventually resorbed, concerns regarding long-term safety of implants are eliminated when using biodegradable implants. Synthetic biodegradable polymers have been used for the preparation of resorbable sutures, drug/gene delivery systems, fracture fixation devices, anti-adhesion membranes, temporary vascular grafts, polymeric stents and tissue engineering scaffolds. The design and synthesis of new biodegradable polymers is currently an important research topic. The impetus behind this is the emergence of new application areas for these materials such as tissue engineering, regenerative medicine and gene therapy that require materials with a wide range of different material properties ^{1, 2}.

Degradation and Erosion of Synthetic Biodegradable Polymers

Understanding the degradation and erosion behaviour of synthetic biodegradable polymers is essential for successful design of biomaterials. The process of polymer chain cleavage is referred to as degradation and the loss of material mass is referred to as erosion ³.

Degradation of polymers can occur by hydrolysis ⁴⁻⁸, thermolysis ^{9, 10}, radiolysis ^{6, 11, 12}, or as a result of mechanical- ¹³ and oxidative stress ¹⁴⁻¹⁶. Most synthetic biodegradable polymers contain hydrolysable bonds such as ester-, anhydride-, amide- and carbonate bonds in their main chains that can be cleaved either enzymatically or non-enzymatically upon reacting with water. The rate of hydrolysis is influenced by the nature of the labile bond, the accessibility of bonds to water or enzymes and by several other factors such as glass transition temperature, hydrophilicity, morphology, crosslinking, pH and the presence of proteins.

Erosion of polymers implies the loss of material as a result of diffusion and dissolution of monomers and other low molecular weight compounds that are formed upon degradation³. Polymer erosion is a complex process that depends on many factors including polymer degradation, swelling, changes in morphology, polymer molecular weight, and diffusion of water, monomers, and oligomers¹⁷.

Surface- and Bulk Erosion

Biodegradable polymers can be classified as surface- or bulk eroding materials¹⁸. Both processes are schematically illustrated in Figure 1.

Figure 1. Schematic illustration of surface- and bulk erosion processes.

Surface eroding polymers, among which are poly(anhydride)s¹⁹, poly(orthoester)s²⁰, poly(trimethylene carbonate)²¹⁻²³, poly(ethylene carbonate)^{24, 25}, poly(hydroxy alkanate)s^{26, 27}, and poly(glycerol sebacate)²⁸, lose material from the surface only. The mass and dimensions of a device made of a surface eroding polymers decrease proportionally to its surface area whereas the molecular weight of the polymer in the bulk of the material remains essentially unchanged. For bulk eroding polymers such as lactone (co)polymers⁴⁻⁷ however, degradation and erosion occurs throughout the bulk of the material. The mass and dimensions of a device remain constant for a considerable portion of its application time, although the molecular weight of the polymer starts to decrease essentially from the beginning of the bulk

hydrolysis process. Mass loss usually sets in upon reaching a critical low molecular weight, leading to a rapid release of degradation products within short periods of time²⁹. It should be noted that loss of mechanical properties of the material precedes the loss in mass, leading to a catastrophic mechanical failure of the device^{6, 30}. The change of properties in time for bulk- and surface eroding polymers is schematically shown in Figure 2.

Figure 2. Representative plots showing the relative change of mass, strength, and molecular weight in time for the bulk- (A) and surface erosion (B) processes of degradable polymers³⁰.

The predictability of the erosion process in the case of surface eroding polymers is a great advantage in controlled release applications. This allows sequential- and zero-order release of bioactive molecules. In addition, rapid release of degradation products in the case of bulk eroding polymers can be harmful to cells cultured in tissue engineering scaffolds prepared from these materials or can evoke a severe tissue response to implants prepared from these materials. Moreover, the structural integrity and the mechanical properties of implants are better preserved in the case of surface erosion, this is especially desirable in tissue engineering applications.

In vivo Tissue Response and Biodegradation

In vivo, interactions of cells with implanted polymers are complex due to the presence of blood, other body fluids, and multiple cell types³¹. These interactions are very important not only with regard to the biocompatibility of these materials but also with regard to their functionality and biodegradation. Unlike biostable implants, degradable implants have only a temporary function, and are designed to be eliminated from the body after degradation. This implies that the degradation products should also be biocompatible and easily resorbable.

Once a biomaterial is introduced into the body of a patient, a sequence of events takes place in the tissue surrounding the biomaterial that leads to a foreign body reaction³²⁻³⁵. Upon

implantation, proteins immediately adsorb to the surface of polymeric implants. These adsorbed proteins dictate the adhesion of inflammatory cells to these surfaces and their survival. In general, neutrophils are the predominant type of cells at the tissue-biomaterial interface in the first few days following implantation. Monocytes/macrophages extravasate and migrate to the implant site following the neutrophils and then become the predominant cell type. At later stages, lymphocytes and fibroblasts are recruited and macrophages adhering to the surface of the biomaterial fuse to form foreign body giant cells. Macrophages are able to produce a large number of biologically active compounds such as chemotactic factors, reactive oxygen species, hydrolytic enzymes, cytokines, growth factors, etc. Therefore, they are regarded as highly influential cells in orchestrating the host response to biomaterials and their biodegradation. Some biodegradable polymers have been shown to degrade faster *in vivo* than *in vitro* ^{21-23, 28, 36, 37}. This has been attributed to hydrolytic compounds secreted by macrophages.

Polyurethanes intended for use in long-term applications for instance, have been shown to be degraded by the action of hydrolytic enzymes and reactive oxygen species that are secreted by macrophages ^{16, 38-40}. Several studies have demonstrated macrophage-mediated degradation and erosion of biodegradable polymers as well ⁴¹⁻⁴⁵.

Engineering of (Soft-) Tissues

Every year millions of people face health problems related to tissue loss and end-stage organ failure ⁴⁶. Physicians have used organ transplantation, surgical reconstruction and artificial prostheses for the treatment of damaged tissues and organs. Though being a good alternative to organ restoration, transplantation has constraints regarding donor shortage and immune response ⁴⁷. Reconstructive surgery on the other hand, can cause serious problems (such as tumors) in the long term because of the abnormal interaction of the tissue at its new location ^{46, 47}. Artificial prostheses also lack some requirements in treating organ failure such as durability and deficiency in fully performing all organ functions ⁴⁶. Therefore, tissue engineering has emerged as an alternative approach. Tissue engineering is defined as an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function ⁴⁶. Different strategies have been proposed for the engineering of tissues ⁴⁸⁻⁵⁰. The most common approach uses cells associated within a matrix or scaffolding to guide tissue development ^{51, 52}. Tissue engineering research includes four main areas: Cells, scaffolds, signalling systems, and bioreactors ^{53, 54}.

Cells

Identification of a cell source from which clinically viable and suitable cells can be isolated and produced in sufficient numbers for tissue engineering is a major challenge ⁵⁵. Various sources of cells have been utilised for tissue engineering. Cells can be obtained directly from the patient (autologous cells) and expanded without any immune response. However, limited numbers of autologous cells are available and there is a risk of morbidity at the donor tissue. Besides, autologous cells may not be the most suited in diseased or elderly patients ^{56, 57}. Allogeneic (from another individual of the same species) or xenogeneic (from an individual of different species) cells are alternatives for autologous cells. However, they have the risk of transmitting infections. A promising option is the use stem cells or progenitor cells. Stem cells are found in many tissues of the body and are capable of not only renewing themselves, but also of differentiating into many phenotypes ^{58, 59}. For instance, mesenchymal stem cells present in adult bone marrow can differentiate into osteogenic, chondrogenic, adipogenic, and myogenic cell lineages. While stem cells are promising for tissue engineering applications, it is still challenging to isolate and differentiate these cells in an efficient and reproducible manner ^{55, 60}. A very promising recent development in cell biology with regards to tissue engineering is the programming of human somatic cells to a pluripotent state ⁶¹⁻⁶⁴.

Scaffolds

The cells are in contact with a complex network of extracellular macromolecules that are physically and chemically crosslinked. The presence of this so called extracellular matrix (ECM) can be considered the actual difference between isolated cells and a tissue ⁶⁵. The ECM serves to organize cells in space, to provide them with environmental signals to direct site-specific cellular regulation, and to separate one tissue space from another. The cells are constantly accepting information regarding their environment from cues in the extracellular matrix, and are frequently remodeling their extracellular matrix ^{66, 67}. The ECM has roles in maintaining homeostasis, guiding development and directing regeneration. It provides adhesion signals, growth factor binding sites, and degradation sites to give way to enzymatic activity of cells as they migrate ^{68, 69}.

As most primary organ cells require anchorage sites and specific environments that provide support and act as a template for growth, a tissue engineering scaffold that mimicks the ECM is essential for cells to proliferate and form tissues ^{55, 70-72}. Several fabrication techniques such as particulate leaching, gas foaming, phase separation, stereolithography, and

fused deposition modelling have been developed and applied to shape polymers into complex architectures for the preparation of tissue engineering scaffolds ⁷². The requirements often depend on the intended application. However to be considered for use in tissue engineering, some general properties must be fulfilled by a material ^{55, 70-73}:

- The material and the degradation products of the scaffold must be biocompatible and completely resorbable.
- The material should allow cell adhesion, -proliferation, and -migration, and the incorporation of bioactive molecules.
- To facilitate transfer of nutrients and biological waste products, and to guide new tissue formation, the material should be processable into a porous scaffold with interconnected pores.
- The scaffold should have mechanical properties that suit the intended application.

Several materials have been used in preparing scaffolds for tissue engineering. These were of biological origin, of synthetic origin or hybrid materials. Natural polymers, such as collagens, starch, glucosaminoglycans, chitin and chitosan have been used ⁷⁰. Natural polymers may closely mimic the ECM quite well, but they show large batch-to-batch variations upon isolation from biological tissues and often poor mechanical performance. Besides, it is challenging to control the degradation times over wide time ranges. Polymers of biological origin may also provoke an immunological response ^{60, 74}.

Synthetic biodegradable polymers such as poly (α -hydroxy ester)s, polyorthoesters, polyanhydrides, and polyphosphazenes have been used as alternatives to natural polymers ^{60, 65, 66, 68-70}. Polylactide (PLA), polyglycolide (PGA), poly(ϵ -caprolactone) (PCL) and their copolymers are the most widely used synthetic polymers for the preparation of biodegradable medical devices. The physical properties and degradation rates of PGA, PLA and PCL polymers can be tuned by copolymerization ^{60, 70, 75, 76}. The synthetic polymers that were initially used for tissue engineering applications were not designed for a specific application, but the available aliphatic polyesters that were used in the preparation of degradable medical devices were used instead ⁷⁷⁻⁸⁰. These polymers served well in establishing the feasibility of the tissue engineering concept. However, bulk hydrolysis of these polymers can lead to rapid formation of acidic products that can be pro-inflammatory ⁸¹. Another drawback of their erosion by bulk hydrolysis is the dramatic deterioration in material strength. Moreover, these

materials are very stiff (elastic moduli ranging from several hundred MPa to several GPa) and deform plastically upon application of stress ⁷⁰.

The mechanical properties of a scaffold are a function of the properties of the polymer itself, the geometry of the scaffold and the fabrication technique. Ideally, the mechanical properties of the scaffolds closely resemble those of the tissues. Therefore, especially for soft tissue engineering applications, novel materials need to be developed. Soft tissues such as skin, muscle and blood vessels are highly flexible and elastic in nature. The mechanical properties of several human tissues are presented in Table 1. Tissue cells feel the stiffness of their substrate and behave differently on substrates of different stiffness ⁸². Many aspects of cell function have been shown to be mediated by the stiffness of the cell culture substratum or matrix, examples are: cell spreading ⁸³, -proliferation ^{84, 85}, -migration ⁸⁶, and the formation of focal adhesions ⁸⁷ and protein expression ^{88, 89}. It was reported recently, that the matrix stiffness also regulates the differentiation of stem cells ⁹⁰, the preservation of the phenotype of smooth muscle cells ⁹¹, and the delivery of genes to the cells ⁹².

Table 1. Mechanical properties of several human soft tissues.

Natural Tissue	E-modulus (MPa)	Stress at break (MPa)	ϵ_{break} (%)
Smooth muscle ⁹³	0.006-0.010	-	300
Articular cartilage ⁹⁴	2.1-11.8	-	-
Carotid artery ⁹⁵	0.084	-	-
Inferior cava vein ⁹⁶	-	1.17	84
Ascending aorta ^{96, 97}	0.1±0.06-	0.069	81
Aortic heart valve ⁹⁸	15±6	-	21±12
Myocardium ⁹⁹	0.02-0.5	-	-
Pericardium ¹⁰⁰	20.4±1.9	-	34.9±1.1
Cerebral artery ¹⁰¹	15.7	3.97	50
Cerebral vein ¹⁰¹	6.9	2.40	83
Ulnar peripheral nerve ¹⁰²	-	0.5-0.6	8-21
Spinal cord ¹⁰³	0.089	-	-
Cortical bone ^{a,104}	6000-30 000	-	-

^aIncluded for comparison purposes

In engineering of soft tissues that are subject to cyclic mechanical stresses *in vivo*, such as cardiovascular tissues and cartilage, the mechanical properties of the scaffold should also provide sufficient mechanical support to protect the cells from detrimental tensile- or compressive forces. They should also allow transmission of mechanical stimuli to and from

the cells upon culturing the constructs in a bioreactor or upon implantation. In this respect, flexible polymers with low modulus values and an elastic nature, such as hydrogels or elastomers, are the most suited materials for the engineering of soft tissues.

Another important aspect of scaffolds is their degradation and erosion behaviour. Preferably, the degradation and erosion should take place on an appropriate time scale: the new tissue formed should replace the eroding scaffold. Degradation and erosion rates can also influence cell spreading¹⁰⁵ and -proliferation⁸⁵, as well as protein expression⁸⁸. When cells are encapsulated in hydrogels, degradation leads to an increasing porosity which facilitates the diffusion and deposition of molecules secreted by the cells¹⁰⁶. While rapid erosion can promote tissue ingrowth¹⁰⁷, rapid release of degradation products can inversely effect viability of cells and their function^{106, 108}. Moreover, the mechanical integrity of the construct might be lost too quickly, leading to failure of the tissue-engineered construct.

Signalling Systems

Biological recognition between cells and their surrounding extracellular matrix is bi-directional and dynamic: the extracellular matrix presents adhesion signals to the cells, it provides bound growth factors, which the cells liberate using locally activated enzymes at their cell surface, the cells thereby induce further remodelling at the interface between the cells and the extracellular matrix⁶⁸. The adhesion proteins of the extracellular matrix that bind to adhesion receptors at the surfaces of cells have been mimicked by synthesizing oligopeptide ligands such as arginine-glycine-aspartic acid (RGD) and incorporating them either in bulk or using them as a substrate surface modification^{69, 109}. Large surface concentrations of a strong ligand will lead to very strong adhesion of the cells and correspondingly to very low migration rates¹¹⁰. Thus moderate concentrations or moderate binding strengths balance good adhesion with normal cell migration rates. To mimic the presentation of growth factors by the extracellular matrix, bioactive molecules have been immobilized within biodegradable matrices or on their surfaces. This may result in controlled release of these bioactive molecules¹¹¹⁻¹¹⁴ upon degradation.

Bioreactors

In tissue engineering, bioreactors are used to create controllable, mechanically-active environments for the incubation of cell-seeded scaffolds¹¹⁵. Early work has shown that by using bioreactors, the cell density and -homogeneity during seeding can be improved and also larger constructs with enhanced cellularity upon cultivation can be obtained¹¹⁶⁻¹¹⁹.

Bioreactors are important tools in investigating the effects of mass transport, hydrodynamic conditions, and mechanical stimulation on tissue formation. Especially for the engineering of tissues where mechanical function is important (load bearing tissues, dynamic mechanical environments), mechanical stimulation of tissues during culture is essential ^{115, 120}. Application of dynamic compressive stresses to cartilage constructs leads to increased extracellular matrix formation and improved biomechanical properties ^{121, 122}. Cyclic stresses have been applied during the culturing of cardiac- and smooth muscle constructs ¹²³⁻¹²⁸ (Figure 3). This resulted in increased contractility of the cardiac constructs formed. In addition, when embryonic stem cells were cultured under cyclic stretching conditions, higher cardiac gene expression was observed when compared to non-stretched cells ^{129, 130}. Recently, it was reported that the stiffness of stem cells is also an important parameter that dictates stress-induced spreading and differentiation ¹³¹. The apparent need for mechanical stimulation implies that the scaffolding materials intended for use in such applications should possess the required extensibility and elasticity to allow (long term) mechanical stimulation of cells ^{132, 133}.

Figure 3. Application of mechanical stimulation during the culturing of heart muscle constructs ^{126, 127}. To improve their contractile properties, unidirectional phasic stretching (left) and auxotonic loading (right) was applied to engineered heart tissues prepared by mixing and culturing isolated neonatal rat heart cells with Collagen type I from rat tails.

Biodegradable Elastomers for Biomedical Applications

Elastomers

Materials that show high extensibility and essentially complete shape recoverability are termed elastomers^{134, 135}. The terms rubber and rubberlike material are also used. As the elastic properties of these materials are very important, elastomers are currently much in use in many applications. For a material to exhibit such elastic properties, three criteria must be fulfilled: the material must be constituted of long polymer chains (for extensibility), these chains must have a high degree of flexibility and mobility (low glass transition temperature for deformability), and they must be joined into a network structure (for recovery).

Figure 4. Schematic illustration of an elastomeric network and imperfections. (A) dangling chain end, (B) loop, (C) trapped entanglement, (D) chain contamination, (E) entrapped chain (sol).

A hypothetical perfect network may be defined as one having no free chain ends, in which all the chains contribute to the elasticity of the network¹³⁵. However, upon crosslinking a polymer, several imperfections can be incorporated into the network structure and elastically ineffective structures might be formed^{135, 136} as shown in Figure 4. If a chain is connected to a junction point at only one end, this will result in a dangling chain. If both ends of a chain are attached to the same junction point, a loop is formed. Chain contaminations result for example from the reaction of initiator molecules with the chains. There might also be some chains that are not incorporated into the network (sol), but remain trapped inside the network structure. Only trapped entanglements are elastically effective.

Biodegradable Elastomers

Biodegradable elastomers are gaining increasing interest in the biomedical field, especially in preparing flexible and elastomeric tissue engineering scaffolds and depots for controlled drug delivery systems ¹³⁷⁻¹³⁹. These elastomers can be either physically- or chemically crosslinked systems.

Physically Crosslinked Biodegradable Elastomers (Thermoplastic Elastomers)

Phase separation of a block copolymer into hard and soft domains on a micron scale results in polymers having an elastomeric nature. While the soft domains provide flexibility to the material, the hard domains constitute the physical crosslinks. The advantage of thermoplastic elastomers is that they can be processed thermally. On the other hand, phase separation leads to heterogeneous degradation of implants prepared from these materials. This can potentially lead to failure of implants due to loss of mechanical integrity. Biodegradable thermoplastic elastomers based on several polymers have been investigated.

Segmented polyurethanes are thermoplastic elastomers that have been used in the preparation of implants for several decades ¹³⁹⁻¹⁴². The biodegradation behaviour and the physical properties of polyurethanes can be tailored by the choice of hard segments (diisocyanate and chain extender) and soft segments (polyester, polyether, polycarbonate) and their weight ratio. Several chemical processes such as passive hydrolysis ¹⁶, enzymatic hydrolysis ¹⁴³, and oxidative degradation ¹⁴⁴ have been shown to be involved in the biodegradation of polyurethanes. Biodegradable polyurethane elastomers have been used for the preparation of scaffolds for soft tissue engineering ¹⁴⁵⁻¹⁵⁰.

Poly(ether ester) block copolymers consist of a polyether soft segment and a polyester hard segment. The most investigated poly(ether ester)s are block copolymers of poly(ethylene oxide) and poly(butylene terephthalate). These poly(ether ester) elastomers have been used as anti-adhesion barriers ¹⁵¹, in controlled release systems ^{152, 153} and in preparing scaffolds for tissue engineering ¹⁵⁴⁻¹⁵⁶.

Copolymers having lactide or glycolide hard segments and ϵ -caprolactone or trimethylene carbonate soft segments also show elastomeric behaviour ¹⁵⁷⁻¹⁵⁹. Recently, thermoplastic elastomers were obtained by stereocomplexation of D-lactide or L-lactide containing block copolymers having poly(trimethylene carbonate) as a soft segment ¹⁶⁰. Trimethylene carbonate or ϵ -caprolactone is used to obtain polymers with a low glass transition temperature, which are thus flexible at physiological conditions. To make use of their elastic properties, cells were cultured in scaffolds prepared from lactide/caprolactone

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Chemically Crosslinked Biodegradable Elastomers

Several synthetic routes have been followed to prepare chemically crosslinked biodegradable elastomers¹³⁷. One of the first approaches utilized ring opening polymerisation of lactone monomers in the presence of bislactones^{165, 166}. With this method, surface eroding and flexible (elastic modulus < 5 MPa) polyester elastomers were prepared by copolymerising ϵ -caprolactone and δ -valerolactone in the presence of 2,2-bis(ϵ -caprolactone-4-yl)propane (BCP)¹⁶⁵. To vary the mechanical properties of such networks in a predictable manner, an alternative crosslinking approach was taken. Hydroxyl functionalised prepolymers of varying compositions and molecular weights were crosslinked with BCP^{167, 168}, but unfortunately this approach yielded networks with high sol contents and poor mechanical properties.

Polyester elastomers prepared by polycondensation of polyols and multifunctional carboxylic acids are of interest for controlled release and tissue engineering applications. Poly(polyol sebacate)s^{169, 170} (PGS) and poly(polyol citrate)s¹⁷¹ (POC) have been mostly investigated, as sebacic acid and citric acid are metabolic intermediates. Drawbacks of preparing elastomers by a polycondensation approach are the high temperatures and long reaction times required for curing. *In vivo*, PGS networks show surface erosion and a favourable tissue response, although its relatively high erosion rate limits its use to short-term applications^{28, 172, 173}. To slow down the erosion rate of PGS, amino alcohol based poly(ester amide) networks have recently been developed^{174, 175}. The suitability of PGS elastomers has been assessed for several applications such as drug delivery¹⁷⁶, the engineering of cardiac muscle^{99, 177}, of blood vessels¹⁷⁸, and as nerve guides¹⁷². Poly(polyol citrate)s have also demonstrated cell and tissue compatibility¹⁷⁹ and have been used as a scaffolding material in the tissue engineering of blood vessels^{171, 180}.

Network formation by free radical polymerisation is a very popular method of obtaining biodegradable elastomers. Crosslinking of prepolymers that contain unsaturated groups can be achieved by either thermal- or photo-initiated polymerization reactions. Methacrylate-functionalised prepolymers based on D,L-lactide, ϵ -caprolactone, trimethylene carbonate were thermally cured to obtain elastomeric networks^{181, 182}. Thermal initiation usually requires relatively high temperatures and long reaction times. In addition, the biocompatibility of the initiators used for this purpose such as benzoyl peroxide and 2,2'-azobis (2-methylpropionitrile) is a concern.

Photoinitiated crosslinking is more advantageous compared to thermal initiation. Relatively rapid and efficient crosslinking reactions at low temperatures reduce the risk of denaturation of biological compounds and allow the development of minimally invasive injectable systems. This method has been widely used in preparing hydrogels^{183, 184} and recently also in preparing elastomers based on lactide, ϵ -caprolactone, and trimethylene carbonate monomers¹⁸⁵⁻¹⁸⁹. To improve their processability, photocurable poly(glycerol sebacate) was developed^{190, 191}. These materials have found use as tissue adhesives¹⁹² and in the engineering of cardiac muscle¹⁹³. Also in the case of photo-induced crosslinking, the biocompatibility of the initiators is a concern, but some photoinitiators have been shown to be well-tolerated by a range of mammalian cells^{194, 195}.

Some biodegradable polymers can be chemically crosslinked by gamma- or e-beam irradiation¹⁹⁶⁻¹⁹⁹. Gamma irradiation of poly(trimethylene carbonate) results in flexible, elastomeric networks. The next section of this review deals with trimethylene carbonate-based (co)polymers and networks.

Trimethylene Carbonate Based Elastomers

The first synthesis of poly(trimethylene carbonate) (PTMC) by Carothers and van Natta dates back to 1930²⁰⁰. PTMC is synthesized by ring opening polymerisation of the cyclic trimethylene carbonate monomer^{23, 200-203}. This aliphatic polycarbonate with a glass transition temperature of approximately -17 °C, is amorphous and flexible at physiological temperatures. Currently, trimethylene carbonate-containing copolymers are used in preparing sutures (Maxon™, Biosyn™, Caprosyn™), tissue fixation devices (Suretac™, Endofix™), and biodegradable meshes for hernia repair (Gore BIO-A®).

In vitro, the non-enzymatic hydrolysis of PTMC is very slow^{21, 23, 204}. Even after 8 weeks of incubation in highly acidic (pH=1) or in highly basic (pH=13) buffers, the molecular weight of PTMC remained unchanged and less than 2 % mass loss was reported²¹. Previous studies have shown that *in vivo* PTMC degrades by erosion at a rate much faster than that observed *in vitro*. The erosion rates of films and rods implanted subcutaneously in rats or in femur and tibia bones of rabbits were 21 and 7.7 $\mu\text{m/day}$, respectively. The more rapid erosion *in vivo*, suggests that enzymes or other active species released by cells can be involved in degradation of this polymer *in vivo*²¹⁻²³. We have previously shown that lipase from *thermomyces lanuginosus* effectively catalyses the hydrolysis of PTMC²¹. Upon degradation of PTMC, water soluble and non-acidic products such as 1,3-propanediol, TMC monomer, and oligomers with an average degree of polymerisation of 2.9 were formed. (It

should be noted that PTMC oligomers with an average degree of polymerisation lower than 3.7 are water soluble ²¹).

Initially, the mechanical strength of PTMC was considered to be too low for biomedical applications ⁷⁵, and the TMC monomer was only used for copolymerisation with lactides ^{158, 205-208}, glycolide ²⁰⁹ and ϵ -caprolactone ²¹⁰⁻²¹². PTMC was also used for blending with rigid polylactides or polyglycolide to tune their properties. However, our group has shown that high molecular weight PTMC ($\overline{M}_n > 200$ kg/mol) is a tough, flexible and elastomeric polymer with an elastic modulus of approximately 6 MPa ¹⁹⁶.

The degradability of PTMC into non-acidic products by surface erosion and its flexible and elastomeric nature make trimethylene carbonate based (co)polymers interesting for drug delivery and soft tissue engineering applications ²¹²⁻²²⁰. Moreover, *in vivo* studies showed that, PTMC and copolymers of TMC with D,L-lactide or ϵ -caprolactone are biocompatible materials, inducing a mild tissue response upon subcutaneous implantation in rats ^{22, 211}. These trimethylene carbonate-based polymers also allow adhesion and proliferation of several mammalian cells including human Schwann cells ²¹⁴, human umbilical vein endothelial cells, and rat cardiomyocytes ²¹³.

Poly(trimethylene carbonate) and its amorphous copolymers with ϵ -caprolactone or D,L-lactide with low glass transition temperatures are flexible materials. But due to the lack of chemical crosslinks, even high molecular weight TMC (co)polymers have low form-stability and flow irreversibly as can be seen in Figure 6. It was observed that porous scaffolds prepared from these polymers shrink upon incubation in physiological buffers at 37 °C ²¹³. This is undesired, as it would decrease the size of the pores and reduce the porosity of the scaffolds. In addition, in drug delivery, changes in the surface area might lead to changes in drug release rates, as these polymers show surface erosion behaviour.

Figure 6. Effect of crosslinking on the form-stability of high molecular weight PTMC. Specimens prepared from the linear polymer (left) and PTMC specimens crosslinked by gamma irradiation (right), which initially were disk-shaped with a diameter of 10 mm, were incubated in phosphate buffered saline (pH=7.4) for one week.

It was found that gamma irradiation under vacuum leads to crosslinking of high molecular weight PTMC and improves its creep resistance ¹⁹⁶. Creep-resistant trimethylene carbonate-based networks have also been prepared by photocrosslinking of methacrylate- or fumarate encapped oligomers ¹⁸⁶⁻¹⁸⁹. Stereocomplexation of trimethylene carbonate-based triblock copolymers containing either D- or L-lactide blocks ¹⁶⁰ has also been employed to prepare form-stable materials. However, the preferential rapid enzymatic degradation of TMC blocks may lead to premature disintegration of scaffolds prepared from these stereocomplexed block copolymers ²²¹.

The work described in this thesis is directed to the preparation of trimethylene carbonate-based networks with excellent elastomeric properties and (tuneable) slower surface erosion behaviour.

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Chapter 3

Flexible Scaffolds Based on Poly(trimethylene carbonate) Networks for Cardiac Tissue Engineering^{*}

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Abstract

Poly(trimethylene carbonate) (PTMC) crosslinked by gamma irradiation was evaluated as a scaffolding material for cardiac tissue engineering. The PTMC networks allowed adhesion and proliferation of neonatal mouse cardiomyocytes and preservation of their phenotype. Highly porous structures having tuneable mechanical properties were prepared. The stiffness of these structures was close to that of myocardium. Upon implantation on rat hearts, gamma irradiated porous scaffolds were found to be eroded by macrophages and foreign body giant cells.

Introduction

In engineering of soft tissues such as cardiovascular tissues, the stiffness of the scaffolds is of great importance in maintaining proper function of cells and controlling the differentiation of stem cells ^{1, 2}. Cyclic stresses are often applied to cell-seeded constructs to control cell phenotype and to enhance production of extracellular matrix ³, which also requires flexible and elastic materials. PTMC is a biocompatible and biodegradable polymer with a low elastic modulus of approximately 6 MPa that can be crosslinked into an elastomeric network by gamma irradiation under vacuum ^{4, 5}. In this study, we assessed the suitability of PTMC networks in preparing flexible porous scaffolds for cardiac tissue engineering.

Materials and Methods

PTMC was synthesized by ring opening polymerization of TMC monomer ^{5, 6}. To characterise the networks formed upon gamma irradiation, equilibrium swelling experiments were performed on compression moulded films using chloroform ⁶.

For cell culturing studies, PTMC spin-coated glass discs (15 mm in diameter) and non-coated glass discs (controls) were gamma irradiated at 50 kGy under vacuum. Also controls in which fibronectin (1 % in gelatine solution in phosphate buffer) was used to coat the films were employed. Neonatal cardiomyocytes (CMs) isolated from one-day old mice were seeded on the materials at a density of 5×10^4 cells/cm² (n=3 per time point). The number of adhering CMs was determined by counting the nuclei stained with 4',6-diamidino-2-phenylindole on eight pictures per disc at 200X magnification. To determine the percentage of proliferating cells, 5-bromo-2-deoxyuridine was added to the cell cultures one day prior to fixation.

Porous PTMC scaffolds were prepared by a process involving polymer coagulation, compression moulding and particulate leaching. Salt particles previously sieved to a size range of 106-250 μ m were dispersed in polymer solutions in chloroform and then precipitated into a non-solvent. Porogen-to-polymer ratios were 85:15, 90:10, and 93:7 w/w. After drying, the salt-polymer composites were compression moulded and gamma irradiated (0, 50, 100 kGy). The salt particles were leached out using Milli-Q water at 4 °C for 3-4 days. The porosities of the scaffolds were determined by measuring their size and mass. Their pore size and pore interconnectivity were assessed by scanning electron microscopy (SEM). Tensile measurements were performed on rectangular slabs of the porous specimens (n=5, measuring approximately 100×5×1 mm) according to ASTM-D 882-91.

For supra-epicardial implantations on the hearts of Albino Oxford rats, salt leaching was done with cold sterile water under sterile conditions. All scaffolds were endotoxin-free. The disk-shaped scaffolds ($n=4$, 10 mm in diameter and approximately 500 μm in thickness) were implanted supra-epicardially through a small incision made on the pericardium and then fixated with a Safil® suture to the left ventricle. After 5 days of implantation, the hearts with the scaffolds were explanted. Histological and immunohistochemical analyses were performed to evaluate the response to the porous PTMC scaffolds. Histological sections were stained with toluidine blue. Macrophages and T-lymphocytes were stained using ED1 and R73 antibodies, respectively and the extent of vascularisation was assessed using anti-collagen IV.

Results and Discussion

The purified PTMC polymer had a number average molecular weight of 306 kg/mol with a polydispersity index of 1.33. Gamma irradiation led to simultaneous sterilisation and crosslinking of the PTMC films. Upon irradiation at 50 kGy, networks with gel contents of $48\pm1\%$ and swelling ratios of 84 ± 1 vol/vol were formed. At 100 kGy, the respective values were $61\pm1\%$ and 43 ± 1 vol/vol.

Figure 1. Light micrographs (200X) showing cardiomyocytes on non-coated glass discs controls (A,C) and on PTMC spin-coated glass disc (C,D) after 1- and 14 days of culturing. Both surfaces were gamma irradiated at 50 kGy. Cells stained in red are proliferating cells.

Initially, the adhesion and proliferation of neonatal cardiomyocytes (CMs) on gamma irradiated PTMC surfaces was evaluated. The CMs adhered well to the PTMC networks, in a time dependent fashion. After 2, 4, and 8 h of culturing, the total numbers of CMs adhering to the PTMC networks were 528 ± 57 , 603 ± 360 , and 1314 ± 169 , respectively. The values for the non-coated glass disc controls were lower at 2h and 4h (299 ± 42 and 451 ± 335 , respectively), but comparable to those of the PTMC networks at 8h (1626 ± 137). It should be noted that, the adhesion of CMs onto PTMC networks was comparable to the adhesion onto the fibronectin-coated PTMC networks. The values were 607 ± 380 , 887 ± 67 , and 1559 ± 667 after 2, 4 and 8h of culturing, respectively.

The CMs had a more spread morphology on the PTMC networks than when compared to non-coated glass discs, especially on the early days of culturing (Figure 1A and 1B). The CMs also proliferated very well on the networks, almost reaching confluency after 14 days of culture (Figure 1D). Importantly, the cells beated spontaneously, suggesting that they had preserved their phenotype. The percentage of proliferating cells increased during culturing, reaching 20.9 ± 3.3 % for PTMC networks and 25.1 ± 4.2 % for the non-coated controls.

Figure 2. Effect of the porogen-to-polymer ratio and the gamma irradiation dose on the elastic modulus (A) and stress at break (B) values of porous PTMC structures.

Porous PTMC structures were prepared by a salt-leaching technique. Porous structures with high porosities ranging from 70.8 ± 1.7 to 86.1 ± 0.1 % depending on the porogen-to-polymer ratio were obtained. SEM analysis of the porous structures revealed that the pores were interconnected and their sizes ranged from 100 to 200 μm (data not shown). Crosslinking by gamma irradiation resulted in form-stable porous structures: upon incubation in PBS at 37 °C for two days, non-irradiated porous structures shrunk by 35 to 55 vol %

depending on the porogen-to-polymer ratio, whereas no significant shrinkage was observed for the crosslinked structures. Shrinkage of scaffolds is not desired, as it would lead to lower porosities and pore sizes.

The porous PTMC structures were highly flexible, with elastic moduli that could be varied from 373 ± 60 to 84 ± 12 kPa by adjusting the porogen-to-polymer ratio (porosity) and (to a lesser extent) by adjusting the irradiation dose (Figure 2A). This allowed the preparation of structures with stiffness values close to those of human myocardium (20-500 kPa) ⁷. The stress at break values of the porous structures also depended on porosity and the applied irradiation dose, the values ranged from 469 ± 30 to 72 ± 8 kPa. All materials exhibited high elongations at break ranging from 145 ± 20 to 209 ± 24 %. A significant dependence on porosity or irradiation dose was not observed.

The host response to tissue engineered constructs is a key factor in determining their success ^{8, 9}. These responses can be towards the biomaterial component and/or the implanted cells, which can also influence each other. Moreover, the biodegradation process and its rate can modulate these responses. We investigated the response to bare PTMC scaffolds (porosity of approximately 80 % and irradiated at 50 kGy) which were supra-epicardially implanted on the hearts of rats. After five days of implantation, macroscopically no abnormalities were observed on the explanted hearts. At the implant site large numbers of newly formed blood vessels were present, which is highly desired in tissue engineering (Figure 3A). The density of lymphocytes at the implant site was not high (115 ± 27 cells/mm²). However, already at this early time point, high numbers of macrophages and foreign body giant cells had infiltrated the scaffolds and their surroundings (Figure 3B and 3C). These cells were found to have phagocytosed fragments of the scaffolds. These histological and immunohistochemical evaluations show that the PTMC scaffolds were angiogenic and had been rapidly degraded upon implantation on rat hearts.

Figure 3. Light micrographs showing the extent of vascularisation (stained brownish-red) at the implant site (A), the phagocytosis of gamma irradiated PTMC scaffold material by macrophages and foreign body giant cells (B), and the macrophages (stained brownish-red) at the interface between the scaffold and the heart. P indicates polymer, and arrow heads in (B) indicate phagocytosed PTMC fragments. (Magnifications: (A) 100X, (B) and (C) 200X)

Conclusions

Networks based on PTMC are useful materials for cardiac tissue engineering, as they allow the adhesion and proliferation of neonatal cardiomyocytes and can be easily processed into flexible porous scaffolds with tuneable mechanical properties. However, the erosion of these materials needs to be tuned to achieve an appropriate balance between scaffold degradation and tissue regeneration.

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PART II

**GAMMA IRRADIATION CROSSLINKED
TMC AND CL (CO)POLYMERS**

Chapter 4

Trimethylene Carbonate and ϵ -caprolactone Based (co)Polymer Networks: Mechanical Properties and Enzymatic Degradation^{*}

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Abstract

High molecular weight trimethylene carbonate (TMC) and ϵ -caprolactone (CL) (co)polymers were synthesized. Melt pressed (co)polymer films were cross-linked by gamma irradiation (25 kGy or 50 kGy) in vacuum, yielding gel fractions of up to 70 %. The effects of copolymer composition and irradiation dose on the cytotoxicity, surface properties, degradation behaviour, and mechanical and thermal properties of these (co)polymers and networks were investigated. Upon incubation with cell culture medium containing extracts of (co)polymers and networks, human foreskin fibroblasts remained viable. For all (co)polymers and networks cell viabilities were determined to be higher than 94 %. The formed networks were flexible with elastic moduli ranging from 2.7 to 5.8 MPa. Moreover, these form-stable networks were creep resistant under dynamic conditions. The permanent deformation after 2

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hours relaxation was as low as 1 % after elongating to 50 % strain for 20 times. The *in vitro* enzymatic erosion behaviour of these hydrophobic (co)polymers and networks was investigated using aqueous lipase solutions. The erosion rates in lipase solution could be tuned linearly from 0.8 to 45 mg/(cm² x day) by varying the TMC to CL ratio and the irradiation dose. The copolymers and networks degraded essentially by a surface erosion mechanism.

Introduction

An important aspect of tissue engineering is the design of scaffolding materials which mimic the properties of extracellular matrix. In this respect, the physical-, chemical- and biological properties and the biodegradation behaviour of the materials are decisive for their suitability. In soft tissue engineering such as cardiovascular applications, scaffolds are usually subjected to mechanical stimuli, either during culturing in a bioreactor or upon implantation in the body. As the scaffold and the engineered tissue should be able to deform without dilation under cyclic pulsatile conditions, resorbable, flexible and elastic materials that allow cell adhesion and proliferation, are likely the most suited to form functional soft-tissue constructs ¹⁻⁴. In addition, surface eroding polymers might be better suited as a scaffolding material, than polymers which degrade by bulk erosion, as mass loss is not preceded by significant deterioration of mechanical properties and structural integrity ^{5, 6}.

Due to their biocompatibility and their tuneable degradation rates, polymers of lactide, glycolide, and ϵ -caprolactone and their copolymers have initially been used as scaffolding materials ⁷⁻¹¹. But these polymers are relatively rigid, with elastic moduli ranging from approximately 400 MPa for poly(ϵ -caprolactone) to 3000 to 3500 MPa for poly(glycolide) and poly(lactide), while soft tissues, such as myocardium, have elastic moduli below 1 MPa ¹²⁻¹⁴. At higher elongations, these polymers either fracture in a brittle manner or by plastic deformation. Furthermore, their hydrolytic degradation takes place by bulk erosion, which could lead to a rapid loss of mechanical integrity and a burst release of acidic degradation products.

High molecular weight poly(1,3-trimethylene carbonate) (PTMC) is a flexible, rubbery polymer which can be crosslinked into an elastic network upon gamma irradiation in an inert atmosphere ^{15, 16}. This is an important feature, as networks are highly resistant to creep under long term cyclic (pulsatile) deformation. The polymer is quite stable towards hydrolysis in buffers ranging from pH 1 to 13, whereas it degrades relatively rapidly *in vivo* by enzymatic surface erosion ¹⁷⁻²¹. Several enzymes have been shown to degrade PTMC in *in*

vitro experiments as well ²¹⁻²³. We compared the degradation behaviour of PTMC of different molecular weights *in vivo*, in tibia of rabbits and *in vitro*, in aqueous lipase solutions (from *Thermomyces Lanuginosus*) ²¹. In both cases PTMC degraded by a surface erosion process, but the rate of mass loss depended on polymer molecular weight. In the lipase solutions, PTMC with a molecular weight of 29.1×10^4 g/mol eroded at rate of $6.7 \mu\text{m/day}$, while PTMC of molecular weight of 6.9×10^4 g/mol eroded at a rate of $1.4 \mu\text{m/day}$. Besides this effect of polymer molecular weight on erosion rate, *in vivo* experiments showed that crosslinking and network formation upon gamma irradiation also decreases the rate of mass loss ¹⁵.

Poly(ϵ -caprolactone) (PCL), is a semi-crystalline polymer with a melting point of approximately 65°C and a low glass transition temperature of approximately -65°C . In a manner similar to PTMC, PCL can also be crosslinked to form networks by gamma- and electron beam irradiation ²⁴⁻²⁶. *In vitro*, several enzyme solutions have been shown to effectively degrade PCL and PCL networks ²⁶⁻²⁸. Here also, the rate of mass loss was lower for networks than for linear PCL polymers. While PCL erodes within four days in *Pseudomonas lipase* (Lipase PS) solutions ^{27, 28}, the *in vivo* degradation process is markedly different. Animal implantation studies have shown that it takes years for a high molecular weight PCL specimen to resorb ^{29, 30}.

In this study, we aimed at developing flexible and elastic form-stable networks that mimic the properties of soft tissue more closely. In order to tune the degradation properties of TMC polymers, copolymers were prepared with CL. Furthermore, as these copolymers are expected to also crosslink upon gamma irradiation, flexible creep-resistant networks could readily be prepared in this manner. The gamma irradiation dosage and the TMC to CL ratio were varied to investigate their effects on the chemical-, physical-, and biological properties of TMC-CL (co)polymer networks, and their *in vitro* enzymatic degradation behaviour.

Materials and Methods

Materials

Polymer grade 1,3-trimethylene carbonate was obtained from Boehringer Ingelheim (Germany). ϵ -Caprolactone (Aldrich, UK) was dried over ground CaH_2 and purified by distillation under reduced argon atmosphere. Stannous octoate (Sigma, U.S.A.) was used as received. Lipase from *Thermomyces Lanuginosus* (EC 3.1.1.3, min. 100.000 Units/g) was purchased from Sigma (Denmark) and used as received. This aqueous enzyme solution further contains 25 wt % propylene glycol, 0.5 wt % CaCl_2 and 2 wt % enzyme concentrate.

Phosphate buffered saline (PBS, pH=7.4) was obtained from B. Braun Melsungen AG, Germany. Solvents (Merck, Germany) were of analytical grade.

Polymer Synthesis

Poly(1,3-trimethylene carbonate), poly(ϵ -caprolactone), and poly(1,3 trimethylene carbonate-co- ϵ -caprolactone) were synthesized by ring opening polymerization of the corresponding monomers under vacuum at 130 ± 2 °C for three days using stannous octoate (2×10^{-4} mol per mol of monomer) as catalyst. The polymers were purified by dissolution in chloroform and precipitation into a ten fold volume of ethanol, washing with ethanol and drying at room temperature under vacuum.

Polymer Characterisation

Monomer conversion and copolymer composition were determined by proton nuclear magnetic resonance (^1H -NMR) spectroscopy (300 MHz, Varian Innova, USA) using CDCl_3 (Merck, Germany).

Glass transition temperatures (T_g) and melting temperatures of purified polymers and irradiated polymer films were determined by differential scanning calorimetry (DSC). Samples (5-10 mg) were analyzed at a heating rate of 10 °C/min in a temperature range of -100 to 100 °C using a Perkin Elmer Pyris 1 DSC. After the first scan, samples were quenched to -100 °C at 300 °C/min and a second scan was recorded after 5 minutes. Reported values were determined from the second scan. Indium, lead, and cyclohexane were used as standards for temperature calibration.

Number average- and weight average molecular weights ($\overline{M}_n$ and $\overline{M}_w$, respectively), molecular weight distributions (MWD) and intrinsic viscosities ($[\eta]$) of the purified polymers and extracts of irradiated polymers were determined by gel permeation chromatography (GPC, Viscotek USA). The setup was equipped with ViscoGEL I-guard-0478, ViscoGEL I-MBHMW-3078, and ViscoGEL I-MBLMW-3078 columns placed in series and a TDA 302 Triple Detector Array with refractometer-, viscometer-, and light scattering detectors, allowing the determination of absolute molecular weights. All determinations were performed at 30 °C, using chloroform as the eluent at a flow rate of 1.0 ml/min.

Polymer Processing

Purified polymers were compression moulded at 140 °C in 500 micrometer thick stainless steel moulds using a laboratory press (Fonteijne THB008, The Netherlands). The films were moulded at approximately 25 kg/cm² and quenched to room temperature using cold water.

Gamma Irradiation, Network Formation and Network Characterization

The compression moulded films were sealed under vacuum in laminated polyethylene/polyamide bags (Hevel Vacuum B.V., The Netherlands) and exposed to 25 or 50 kGy gamma irradiation from a ⁶⁰Co source (Isotron B.V., Ede, The Netherlands). This leads to crosslinking and network formation^{15, 16}.

To determine equilibrium swelling and gel contents, disk-shaped specimens (500 μ m thick, 10 mm in diameter) were punched out from the irradiated films and placed in 30 mL CHCl₃ for 1 week, the solvent was refreshed once after 3 days. This procedure ensured complete removal of the sol fraction. Then the swollen gels were weighed, dried to constant weight at room temperature in vacuo and weighed again. The gel and the sol fractions were calculated according to equations (1) and (2) respectively:

$$\text{Gel fraction (\%)} = \frac{m_d}{m_0} \times 100 \quad (1)$$

$$\text{Sol fraction (\%)} = \left(1 - \frac{m_d}{m_0} \right) \times 100 \quad (2)$$

where m_d is the mass of dried (extracted) samples and m_0 is the mass of the specimens before swelling. The volume degree of swelling (q) was calculated according to equation (3).

$$q = 1 + \rho_p \times \left(\frac{m_s}{m_d \times \rho_s} - \frac{1}{\rho_s} \right) \quad (3)$$

where m_s is the mass of the extracted and swollen samples and ρ_s and ρ_p are the densities of chloroform³¹ (1.48 g/cm³) and the (co)polymers, respectively. The densities of the copolymers were determined by measuring the mass and dimensions of compression moulded films. The densities of TMC and CL copolymers containing 100 %, 89 %, 79 %, 70 %, and 0 % TMC were 1.31, 1.29, 1.27, 1.25, and 1.09 g/cm³, respectively.

Cell Viability Assay

Possible cytotoxicity of the polymer films irradiated at 0, 25 or 50 kGy was evaluated using a [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay³². MTS is a tetrazolium compound that is reduced by living cells into a formazan product, the absorbance of which can be measured at 490 nm. The quantity of formazan produced is directly proportional to the number of living cells in culture.

Human skin fibroblasts (PK 84 cell line) were cultured in a 96 wells plate (5000 cells/well) in RPMI medium (Roswell Park Memorial Institute medium). Of each sample, two disks (diameter 6 mm, thickness 500 μ m) were extracted at 37 °C and 5 % CO₂ with 400 μ l RPMI for 24 hours. The cells were incubated at 37 °C and 5 % CO₂ for three days, the medium was then replaced by RPMI medium containing the polymer extract. After two days, the absorbance of formazan, which is soluble in the culture medium, was measured. The mean value obtained for cell cultures incubated with RPMI medium only was standardized as 100 % cell viability.

A medical grade polyurethane (2363-55D-Pellethane[®] resin from Dow Chemical, Midland, USA) and latex rubber (Hilversum Rubber Factory, Hilversum, The Netherlands) were used as negative and positive controls, respectively. Prior to the MTS assays, it was confirmed that the controls and the (co)polymer samples were essentially endotoxin-free.

Mechanical Properties

The mechanical properties of melt pressed and irradiated (0 kGy, 25 kGy, and 50 kGy) (co)polymers were determined in triplicate according to ASTM-D 882-91. The specimens were not extracted after irradiation and measured 0.5×10×0.05 cm³. A Zwick Z020 tensile tester (Ulm, Germany) equipped with a 500 N load cell was operated at a crosshead speed of 500 mm/min. The initial grip to grip separation was 50 mm and a preload of 0.01 N was applied. The strain was measured using extensometers.

To assess their behaviour under dynamic loading conditions, the specimens were repetitively (20x) elongated to 50% strain at 50 mm/min in cyclic tests. After a 2 h recovery period, the permanent deformation was estimated from the stress-strain diagram of the 21st cycle. In these experiments a preload of 0.01 N was applied, the deformation was derived from the grip to grip separation. The error in the values is approximately 0.5 % strain.

Wettability and Water Uptake

Captive bubble contact angles were determined using ultra-pure water (MilliQ Plus-Millipore, France) at room temperature and a video-based system (OCA 15 DataPhysics Instruments GmbH, Germany). Glass discs ($n=3$), were spin-coated with (co)polymer solutions in chloroform (2-3 wt/vol. %), dried in vacuum and crosslinked by gamma irradiation as described before. Measurements (three per disk) were performed immediately after immersion in the water and after conditioning for a week.

The equilibrium water uptake of disks (500 μm thick, 10 mm in diameter) was determined after conditioning at 37 °C in PBS (pH=7.4) for one week. Water uptake was defined as the mass increase of the disks.

In Vitro Enzymatic Degradation

The *in vitro* enzymatic degradation of TMC-CL (co)polymers and networks was investigated using aqueous lipase solutions (Lipase from *Thermomyces Lanuginosus*, min. 100.000 Units/g). Non-extracted, disk-shaped specimens (500 μm thick, 10 mm in diameter) ($n=3$ /time point) were placed in vials containing 0.5 ml of the enzyme solution and conditioned at 37 °C. The medium was refreshed twice a week, except for PCL polymers and networks, where the medium was refreshed every 8 hrs. Control experiments without enzyme were performed using PBS (pH 7.4) ($n=1$ per time point). At predetermined times, the mass and thickness of wet specimens was determined after blotting the surface. The same measurements were then performed after rinsing, and drying the specimens to constant weight *in vacuo* at room temperature.

Results and Discussion

To prepare flexible and form-stable (co)polymer networks by gamma irradiation, a range of (co)polymers based on TMC and CL were synthesized. The TMC to CL ratio and the gamma-irradiation dose were varied to obtain networks with tuneable properties.

Synthesis of (co)Polymers

High molecular weight (co)polymers were synthesized by ring opening polymerisation of the corresponding monomers. In all cases, the monomer conversion was quite high (>98 %). Table 1 gives the characteristics of the purified and of the compression moulded (co)polymers.

Table 1. Characteristics of synthesized TMC-CL (co)polymers before and after compression moulding. The values for compression moulded specimens are given between brackets.

TMC/CL charged (mol ratio)	TMC/CL ^a actual (mol ratio)	T _g ^b (°C)	$\overline{M}_n$ ^c (kg/mol)	MWD ^c	[η] ^c (dl/g)
100/0	100/0	-14.8	590 (586)	1.31 (1.32)	7.5 (7.2)
90/10	88.9/11.1	-23.4	149 (136)	1.60 (1.62)	2.8 (2.6)
80/20	79.2/20.8	-29.2	264 (218)	1.39 (1.56)	4.3 (4.0)
70/30	69.9/30.1	-35.8	261 (230)	1.35 (1.43)	4.2 (4.1)
0/100	0/100	-64.3	234 (228)	1.35 (1.37)	4.7 (4.6)

^a Determined by ¹H-NMR on purified specimens.

^b Determined by DSC from the second scan.

^c Determined by GPC at 30 °C using chloroform as the eluent.

The molecular weights of the (co)polymers were relatively high (149 to 590x10³ g/mol), although the copolymer containing 89 mol % TMC had a somewhat lower molecular weight than the other polymers. The comonomer ratios determined for the purified copolymers were in accordance with the amounts charged. The glass transition temperatures (T_g) of all (co)polymers were below 37 °C. PTMC and TMC-CL copolymers were amorphous and therefore in the rubbery state at physiological temperatures. Only PCL was semi-crystalline, with a maximum melting temperature of 55.6 °C and a heat of fusion of 53.4 J/g.

Gamma Irradiation and Network Formation

Upon gamma irradiation, all polymers crosslinked. The networks were characterized by performing equilibrium swelling tests using chloroform as the solvent. Gel fractions and degrees of swelling of the networks are given in Table 2. Especially at the higher irradiation doses, networks with gel fractions up to 70 % could be prepared. These values are comparable to those of networks prepared from high molecular weight PTMC^{15, 16}.

The table shows that for all (co)polymers the gel fraction of the networks increases, and the degree of swelling decreases, as the irradiation dose is increased from 25 to 50 kGy. In assessing the effect of the copolymer composition on the network characteristics, no major differences in swelling behaviour can be observed at irradiation doses of 50 kGy. However, at 25 kGy networks with significantly lower gel contents are obtained from the copolymers. This may be due to the relatively low initial molecular weights of the copolymers as compared to PTMC^{15, 16} (See Table 1). Also in the case of PCL networks, the gel fractions are much higher than those previously reported²⁵. Although the molecular weight of this polymer is also relatively low ($\overline{M}_n$ =248 kg/mol), it is significantly higher than that used in the earlier

experiments ($\overline{M}_n=128$ kg/mol). Apparently, in the case of crosslinking of semi-crystalline PCL by gamma irradiation, higher gel fractions can be obtained at lower molecular weights when compared to amorphous PTMC.

Table 2. Effect of (co)polymer composition and irradiation dose on network characteristics of formed TMC-CL (co)polymer networks.

		TMC content (mol %)				
	Irradiation dose	100	89	79	70	0
Gel fraction (%)	25	57 $\pm$ 9	32 $\pm$ 3	32 $\pm$ 2	27 $\pm$ 9	57 $\pm$ 2
	50	70 $\pm$ 3	62 $\pm$ 1	63 $\pm$ 1	70 $\pm$ 11	69 $\pm$ 1
Degree of swelling ^a (vol/vol)	25	58 $\pm$ 24	163 $\pm$ 17	163 $\pm$ 15	177 $\pm$ 17	55 $\pm$ 5
	50	26 $\pm$ 4	36 $\pm$ 2	35 $\pm$ 1	33 $\pm$ 6	28 $\pm$ 9
$\overline{M}_n$ (sol fraction) ^b (kg/mol)	25	45.9	71.7	79.3	113.5	33.7
	50	21.8	18.7	15.5	16.5	13.9
MWD (sol fraction) ^b	25	2.64	3.23	2.99	3.15	3.35
	50	3.27	4.50	5.23	6.85	3.75

Values are expressed as mean $\pm$ standard deviation, (n=3).

^a Determined by using chloroform at room temperature.

^b Determined by GPC at 30 °C using chloroform as the eluent.

Gamma irradiated (co)polymer films were extracted using chloroform, the molecular weights of the sol fractions are also given in Table 2. The sol fractions had lower $\overline{M}_n$ values and a broader MWD compared to the corresponding non-irradiated (co)polymers. As the irradiation dose was increased, the $\overline{M}_n$ values of the sol fractions decreased, while the molecular weight distribution became broader. This indicates that upon gamma irradiation, degradation, branching and crosslinking occur simultaneously. The exact reaction mechanisms of the chain scission and crosslinking processes involved are not known.

High energy irradiation can lead to radical formation due to excitation of the polymer molecules. In the case of polyethylene ³³ radicals can be formed by breakage of (1) a C-C bond in the main chain or (2) by breakage of a C-H bond, the latter reaction will lead to simultaneous formation of a radical on the polymer chain and a hydrogen radical. In the case of TMC-CL (co)polymers, a C-O bond in the main chain can be broken as well (3). These different radicals can react with each other and with the polymer chains:

Recombination of type (1) or type (3) radicals will result in the formation of either C-C bonds or C-O-C bonds, both leading to linear chains. Combination of type (2) radicals will result in crosslinking, while combination of a type (2) radical with either a type (1) or type (3) radical will yield branched structures. Branched structures also form by reaction of type (1)

and (3) radicals with the linear chain. Chain scission can happen as a result of the combination of a hydrogen radical with a type (1) or type (3) polymer chain radical. Furthermore, a hydrogen radical can abstract a hydrogen atom from the main chain to form hydrogen gas and a type (2) or type (3) polymer chain radical, or a double bond in the main chain. We have observed the formation of gas bubbles within PTMC films, especially at relatively low polymer molecular weights and at higher irradiation doses. This can indicate the formation of hydrogen gas, although it can not be excluded that CO or CO₂ are formed. The elucidation of the mechanism of the scissioning and crosslinking reactions will require more detailed studies.

Cell viability

An MTS assay was used to assess the effect of extracts of (co)polymer and network films on the viability of human foreskin fibroblasts. The (co)polymers and networks were extracted using RPMI medium. The metabolic activity of the fibroblasts was determined after incubating with the medium containing the polymer extracts. A medical grade polyurethane and latex rubber were used as negative- and positive control, respectively. The mean value obtained for cell cultures incubated in medium that did not contain polymer extract was standardized as 100 % cell viability (blank). Figure 1 shows the results of the MTS assays.

Figure 1. Cytotoxicity scores of TMC-CL (co)polymers and networks: (■) 0 kGy, (■) 25 kGy (dark gray), and (■) 50 kGy (light gray). The first three bars indicate the results of the blank (culture medium only), negative- and positive controls, respectively.

The results of the MTS assays showed that the viability of human skin fibroblasts after incubation with extracts of the TMC and CL (co)polymer networks was quite high (>94 %).

All (co)polymers can be considered non-cytotoxic, regardless of their monomer composition and the irradiation dose used in network formation.

Thermal- and Mechanical Properties

The effects of comonomer ratio and gamma irradiation on the thermal and mechanical properties of the (co)polymers and networks are shown in Table 3. The (co)polymers and networks containing 100, 89, 79, and 70 % TMC were rubbery at physiological temperatures, with T_g 's ranging from -14.8 to -36.0 °C. With decreasing TMC content, T_g 's decreased. The T_g 's decreased very slightly with increasing irradiation dose. PCL and PCL networks were semi-crystalline at physiological temperatures. The maximum melting temperatures were 55.6, 55.7, and 55.9 °C with heats of fusion of 53.4, 56.9, 56.5 J/g for specimens treated with 0 kGy, 25 kGy or 50 kGy gamma-irradiation, respectively. The glass transition temperatures were approximately -64 °C.

Table 3. Effect of copolymer composition and irradiation dose on glass transition temperature and mechanical properties of TMC-CL (co)polymers and networks.

TMC content (mol %)	Irradiation Dose (kGy)	T_g (°C)	E (MPa)	σ_{yield} (MPa)	ϵ_{yield} (%)	σ_{max} (MPa)	ϵ_{break} (%)	Permanent Set (%) ^a
100	0	-14.8	7.2±0.4	3.2±0.1	183±15	15.9±4.2	469±26	1.3
100	25	-15.6	5.8±0.1	2.4±0.1	150±9	9.8±1.0	628±2	1.0
100	50	-15.5	4.8±0.3	1.9±0.1 ^b	136±3 ^b	5.9±0.6	708±6	1.2
89	0	-23.4	4.1±0.1	1.5±0.1	107±5	1.5±0.1	284±109	11.9
89	25	-23.4	4.2±0.2	1.6±0.1	141±8	≥1.9±0.3	≥1200	1.8
89	50	-23.5	3.3±0.1	1.4±0.1 ^b	125±5 ^b	≥6.6±0.4	≥985	1.3
79	0	-29.2	4.6±0.1	1.7±0.1	95±3	1.7±0.1	103±6	11.0
79	25	-30.1	3.8±0.1	1.4±0.1	133±4	≥1.6±0.1	≥1230	1.2
79	50	-29.8	3.1±0.1	1.3±0.1 ^b	126±5 ^b	≥7.1±0.4	≥1010	1.4
70	0	-35.8	4.2±0.2	1.5±0.1	84±2	1.5±0.1	91±5	- ^c
70	25	-35.7	3.3±0.1	1.2±0.1	137±3	≥1.3±0.1	≥1235	1.4
70	50	-36.0	2.7±0.1	1.1±0.1 ^b	125±5 ^b	≥4.9±0.5	≥973	1.4
0	0	-64.3	458±18	17.1±0.2	11±2	38.3±2.1	442±2	34.3
0	25	-64.2	493±21	18.3±0.1	12±1	35.7±2.0	467±12	35.0
0	50	-64.3	514±5	19.0±0.3	13±1	30.9±1.7	414±6	36.4

Values are expressed as mean ± standard deviation, (n=3).

^a Result of a single measurement. Permanent set is estimated from the 21st cycle performed after two hours of recovery period. The error is approximately 0.5 % strain.

^b Estimated from the intersection of tangents to stress-strain diagrams as the curves did not show a distinct yield point.

^c: Sample broke during the cyclic loading experiment.

For PTMC, the elastic modulus, yield strength, yield strain, and tensile strength decreased with increasing irradiation dose, while the elongation at break increased. Relatively low molecular weight chains formed by chain scissioning upon gamma irradiation may have a plasticizing effect which will decrease the elastic modulus, yield strength and -strain. The relatively higher tensile strength of the non-crosslinked samples can be related to strain induced crystallization ¹⁶. The presence of crosslinks lowers the crystallinity that can be attained upon elongation of PTMC.

The amorphous copolymers and networks containing 89, 79, and 70 % TMC were also very flexible with elastic moduli ranging between 2.7-4.6 MPa. The elastic moduli and the yield strengths of the copolymers and copolymer networks decreased with decreasing TMC content and with increasing irradiation dose. Upon gamma irradiation, yielding occurred at higher strains. Gamma irradiation also dramatically increased the maximum elongation of these copolymers; films of these copolymer networks did not break at strains exceeding approximately 1000 % (at this point the limits of the extensometers were reached). The maximum stress reached for non-irradiated copolymers is comparable to that of the copolymers irradiated at 25 kGy. For copolymers irradiated at 50 kGy, these values were much higher in which the stress-strain curves showed an upturn at high strains (>800 %). This could be due to denser network formation at 50 kGy compared to 25 kGy which could lead a to more limited extensibility of the networks.

The effect of gamma irradiation on the properties of PCL was different than that on the copolymers and on PTMC. The elastic modulus and yield strength of PCL increased as the irradiation dose was increased. When PCL is irradiated at room temperature, the crystallinity is retained and crosslinks are formed in the amorphous parts, leading to the higher moduli values at higher irradiation doses. However, the maximum tensile strength of PCL decreased with increasing irradiation dose. This can be related to hindered strain-induced crystallization due to the presence of crosslinks.

Creep resistance is important when materials are used under dynamic loading conditions such as in cardiovascular tissue engineering. To assess this, the (co)polymers and networks were cyclically stretched to 50 % strain (20 times). After two hours of recovery, a value for the permanent set was estimated from the 21st cycle (Table 3).

Before gamma irradiation, the permanent deformation of copolymers containing 79 and 89 % TMC were relatively high (above 11 %). The 30 % CL containing copolymer even broke during the experiment. Upon gamma irradiation and crosslinking, the creep resistance of all copolymer networks was excellent, with a permanent deformation below 2 %. The

irradiation dose itself had only a very small effect on these values. Remarkably, PTMC also had a very low permanent deformation (1.3 %) in the non-crosslinked state; this can probably be related to its very high molecular weight. Here, gamma irradiation only slightly reduced permanent deformation.

Under the same dynamic loading conditions PCL and PCL networks had poor resistance to creep, with permanent deformations at room temperature and at 37 °C, greater than 34 %. This is a result of their semi-crystalline structure. Upon heating to 70 °C, which is above the melting temperature, the films returned to their original dimensions within seconds.

Contact Angles and Water Uptake

Captive bubble contact angles of the (co)polymer and network films on glass discs were determined immediately after immersion in ultra-pure water and after conditioning for one week. Before irradiation the (co)polymers were hydrophobic, having contact angles between 58° and 65° as measured immediately after immersion in water. Upon gamma irradiation, the surfaces become more hydrophobic, as higher contact angles (63°-70°) were determined. No significant effect of the polymer composition could be observed. After one-week conditioning in water, the contact angles of all films decreased. The values ranged between 52° and 55° for non-irradiated films and between 52° and 61° for gamma-irradiated films. This is in line with the uptake of small amounts of water, allowing some reorientation or diffusion of polymer end groups at the polymer-water interface.

All the (co)polymers took up less than 2 wt % water. The water uptake of PCL was the lowest; which could be related to its chemical structure and crystallinity. TMC content and irradiation dose did not have a large influence on contact angles and water uptake.

In Vitro Enzymatic Degradation

The *in vitro* enzymatic degradation of melt pressed films irradiated at 0, 25, or 50 kGy was investigated using aqueous solutions of lipase from *Thermomyces Lanuginosus*. All (co)polymers and networks eroded in time when incubated in the lipase solutions at 37 °C. During degradation, the films preserved their integrity and the enzyme solutions remained clear. No significant mass loss was observed in control experiments where only PBS (pH=7.4) was used. This implies that the contribution of non-enzymatic hydrolysis to the degradation process was minimal.

Figure 2 shows the change in relative mass of PTMC and PCL specimens irradiated at 0, 25, or 50 kGy in time. The complete erosion of non-irradiated PTMC took approximately

eight weeks, whereas at the same time point the relative mass loss for PTMC irradiated at 50 kGy was only about 55 %. Non-irradiated PCL lost more than 90 % of its mass after 33 h, while PCL crosslinked at 50 kGy lost approximately 75 % of its mass after 50 h.

The erosion rates of (co)polymers and networks calculated from this mass loss data are given in Table 4. For PTMC, the rate of mass loss decreased from 1.3 to 0.8 mg/(cm² x day) when the irradiation dose was increased from 0 to 50 kGy. For PCL, the rates decreased from 45.0 to 24.8 mg/(cm² x day) after increasing the irradiation dose from 0 to 50 kGy. The decrease in degradation rate with increasing irradiation dose may be due to hindered chain mobility as a result of (denser) network formation. Although the use of a lipase solution was previously shown to be a good model for the *in vivo* erosion of PTMC²¹, the erosion rate of PCL is very much higher when exposed to aqueous lipase solutions than that *in vivo*^{29, 30}.

Macrophages are usually the dominant type of cells seen at the interface between the tissue and the degradable polymer. These cells can secrete many different enzymes, such as esterases and lipases, that could degrade these TMC (co) polymers and networks. However, exactly which enzymes are responsible for the *in vivo* erosion of these (co)polymers and networks is not known.

Figure 2. Effect of irradiation dose on the erosion behaviour of PTMC (A) and PCL (B) homopolymers in lipase solutions: 0 kGy (■), 25 kGy (□), 50 kGy (●). Initially, the PTMC disks measured 10 mm in diameter and approximately 530 μ m in thickness, weighing approximately 57 mg. PCL disks measured 10 mm in diameter and approximately 580 μ m in thickness, weighing approximately 51 mg.

The erosion behaviour of TMC and CL (co)polymers irradiated at 0, 25, or 50 kGy is illustrated in Figure 3. The copolymer composition had a significant effect on the rate of degradation. Upon increasing the TMC content of the polymers and networks, erosion rates decreased. At 0 kGy, the erosion rates of the (co)polymers decreased linearly ($R=0.98$) from

45.0 mg/(cm² x day) to 1.3 mg/(cm² x day) as the TMC content of the (co)polymer was increased from 0 to 100 %. See also the data presented in Table 4. When comparing the behaviour of the polymers (Figure 3), non-irradiated (0 kGy) 89 % TMC-containing copolymer eroded at a relatively low rate. This could be due to its relatively low molecular weight¹⁸.

Table 4. Effect of copolymer composition and irradiation dose on the erosion rate of TMC-CL (co)polymers and networks in lipase solutions. (Disks with initial diameters of 10 mm and thicknesses of 500-590 μ m were incubated. Initial masses ranged from 51 to 57 mg.)

Dose	0 kGy		25 kGy		50 kGy	
	Erosion rate ^a		Erosion rate ^a		Erosion rate ^a	
TMC %	mg / (cm ² x day)	μ m/day	mg / (cm ² x day)	μ m/day	mg / (cm ² x day)	μ m/day
100	1.3 $\pm$ 0.1	- ^b	0.9 $\pm$ 0.1	6.0 $\pm$ 0.2	0.8 $\pm$ 0.1	4.6 $\pm$ 0.4
89	1.3 $\pm$ 0.1	- ^b	2.0 $\pm$ 0.1	13.8 $\pm$ 0.2	2.2 $\pm$ 0.1	13.3 $\pm$ 0.3
79	5.1 $\pm$ 0.5	- ^b	4.0 $\pm$ 0.1	28.3 $\pm$ 2.3	4.1 $\pm$ 0.3	29.6 $\pm$ 2.5
70	9.7 $\pm$ 1.3	- ^b	8.0 $\pm$ 0.5	60.0 $\pm$ 6.2	9.0 $\pm$ 0.3	57.9 $\pm$ 2.7
0	45.0 $\pm$ 1.4	301 $\pm$ 13	32.5 $\pm$ 1.2	222 $\pm$ 3	24.8 $\pm$ 0.6	160 $\pm$ 4

^a Values are calculated from the initial linear region of the curves (R>0.99).

^b No reliable data could be obtained due to poor form stability of the specimens.

After irradiation and network formation, the same linear trend was observed. At an irradiation dose of 25 kGy and 50 kGy, the erosion rates of the (co)polymer networks linearly (R=0.99 for both 25 kGy and 50 kGy) decreased from 32.5 mg/(cm² x day) to 0.9 mg/(cm² x day) and from 24.8 mg/(cm² x day) to 0.8 mg/(cm² x day), respectively. The erosion rates of the copolymer networks were not much influenced by irradiation dose, which is different than in the case of PTMC and PCL networks. The effect of copolymer composition on the erosion rates was stronger than the effect of irradiation dose.

The linear increase in erosion rates with the decrease in TMC content, suggests that the catalytic activity of the enzyme in cleaving ester bonds is higher than in cleaving carbonate bonds. ¹H-NMR measurements have shown that the TMC content in the copolymers increases slightly as the films degrade. For example, the TMC content of a non-irradiated specimen, that initially contained 79% TMC, had increased to 84% after a one week degradation in lipase solution (mass loss = 56 %).

It has been shown that the activity of lipases is enhanced at the water-lipid interface and when adsorbed on hydrophobic supports³⁴. This is due to a conformational change, after which the active site of the enzyme, normally buried beneath a helical segment called “lid”,

becomes accessible. The active site of the enzyme contains an Asp-His-Ser triad, which is chemically analogous to serine proteases³⁵. Deckwer *et al.* have shown that the mobility of the polymer chain is an important factor that controls the degradability of polyesters³⁶. The decrease in Tg's (See Table 3) of the (co)polymers and networks with an increase in CL content could have an enhancing effect on the polymer chain mobility, which would make the chains more accessible to the active site of the enzyme. Despite its semi-crystalline structure, PCL, which has a glass transition temperature of -64 °C, had degraded completely and at a much faster rate than the other (co)polymers. This suggests that both amorphous and crystalline domains of PCL could be degraded in lipase solutions^{27, 28}. In the case of PCL, apparently, the effect of crystallinity was less than that of chain mobility at 37 °C.

Figure 3. Effect of copolymer composition on the erosion behaviour of TMC-CL copolymers gamma irradiated at 0 kGy (A), 25 kGy (B), or 50 kGy (C): 100 % TMC (▲), 89 % TMC (○), 79 % TMC (●), 70 % TMC (□), 0 % TMC (■). (Disks with initial diameters of 10 mm and thicknesses of 500-590 μ m were incubated. Initial masses ranged from 51 to 57 mg.)

The changes in relative thickness of the films during degradation in lipase solutions and the relationship between the change in relative mass and relative thickness are shown in Figure 4. For gamma irradiated samples the decrease in thickness is in quite good agreement with the decrease in mass, indicating that these form-stable copolymer networks are degrading by surface erosion. Upon gamma irradiation and crosslinking, the form stability of the disks in aqueous lipase solutions at 37 °C was good. The diameter of the crosslinked specimens remained essentially unchanged during degradation. For non-irradiated specimens (0 kGy), the correlation between relative thickness and mass is present, but less specific as in amorphous films, the disk-shape was not preserved. This is most likely due to deformation due to creep of these low T_g (co)polymers. This low form stability was most pronounced for the 89 % TMC containing copolymer, which has a relatively low molecular weight. A similar behaviour was reported for low molecular weight PTMC ^{20, 21}.

Figure 4. Relationship between the loss in relative thickness and relative mass of TMC-CL copolymers and networks during degradation in lipase solutions: 0 kGy (A), 25 kGy (B), 50 kGy (C); 100 % TMC (▲), 89 % TMC (○), 79 % TMC (●), 70 % TMC (□), 0 % TMC (■).

The thickness of the specimens decreased more rapidly as the CL content of the copolymers and networks was increased. By varying the TMC:CL ratio from 100:0 to 0:100, the erosion rates (decrease of thickness in time) of the networks could be tuned linearly from 6.0 $\mu\text{m}/\text{day}$ to 222 $\mu\text{m}/\text{day}$ for specimens irradiated at 25 kGy ($R=0.99$) and from 4.6 $\mu\text{m}/\text{day}$ to 160 $\mu\text{m}/\text{day}$ for specimens irradiated at 50 kGy ($R=0.99$) (See Table 4).

Increasing the irradiation dose had a larger effect on the erosion rates of PTMC and PCL homopolymer networks than of copolymer networks. As the irradiation dose was increased to 50 kGy, the erosion rate (Table 4) decreased from 6.0 $\mu\text{m}/\text{day}$ to 4.6 $\mu\text{m}/\text{day}$ for PTMC materials and from 301 $\mu\text{m}/\text{day}$ to 160 $\mu\text{m}/\text{day}$ for PCL materials.

Conclusions

High molecular weight (co)polymers of TMC and CL were synthesized by ring opening polymerization. Flexible, form-stable and elastic networks were obtained upon gamma irradiation. These hydrophobic polymers and networks were non-cytotoxic for all the compositions and irradiation doses investigated. The (co)polymers and networks degraded in aqueous lipase solutions by a surface erosion mechanism. The mechanical properties and the *in vitro* enzymatic erosion behaviour of the copolymers and networks could readily be tuned by adjusting both the gamma irradiation dose and the TMC to CL ratio. These flexible and elastic surface eroding networks are well-suited for use in soft tissue engineering scaffolds. Future studies will focus on the tissue response to these networks upon implantation and on their *in vivo* degradation behaviour.

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Chapter 5

***In vivo* Behaviour of Trimethylene Carbonate and ϵ -caprolactone Based (co)Polymer Networks: Degradation and Tissue Response^{*}**

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Abstract

The *in vivo* erosion behaviour of crosslinked (co)polymers based on trimethylene carbonate (TMC) and ϵ -caprolactone (CL) was investigated. High molecular weight poly(trimethylene carbonate) (PTMC) homopolymer- and copolymer films were crosslinked by gamma irradiation. To adjust the *in vivo* erosion rate of the (co)polymer films, both the irradiation dose (25, 50 or 100 kGy) for PTMC and composition (100 to 70 mol % TMC) at a constant irradiation dose of 25 kGy were varied. After subcutaneous implantation of irradiated films in rats, their *in vivo* behaviour was evaluated qualitatively and quantitatively. When the irradiation dose for PTMC was increased from 25 to 100 kGy, the erosion rate of non-extracted PTMC films (determined at day 5) decreased from 39.7 ± 16.0 $\mu\text{m/day}$ to 15.1 ± 2.5 $\mu\text{m/day}$, and the number of lymphocytes in the tissue surrounding the films decreased from

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235±114 cells/mm² to 64±33 cells/mm². The number of macrophages and giant cells at the tissue-polymer interface also decreased with increasing irradiation dose. All (co)polymer films eroded completely within 28 days of implantation. Variation of the TMC content of gamma irradiated (co)polymer films did not affect the tissue response to the gamma irradiated (co)polymer films and their *in vivo* erosion behaviour much.

Introduction

Resorbable elastomeric polymers have been used as scaffolding materials for the engineering of soft tissues ¹⁻⁶. Their high compliance and creep resistance are highly desired properties when culturing cardiovascular cells in a dynamic environment, such as in a pulsatile flow cell culturing bioreactor. These polymers have been applied in the controlled delivery of drugs ⁷, genes ⁸, cytokines ⁹, and growth factors ¹⁰ as well.

Poly(trimethylene carbonate) (PTMC) is a biocompatible resorbable polymer with a low E-modulus of 3 to 7 MPa ^{11, 12}. High molecular weight PTMC can be crosslinked into form-stable and creep-resistant elastomeric networks by gamma irradiation in an inert atmosphere ¹². Unlike aliphatic polyesters, the hydrolytic degradation of PTMC does not lead to acidic compounds which could be detrimental to cells, drugs or proteins ¹³. Although PTMC is rather stable towards hydrolysis in buffers of pH values ranging from 1 to 13, *in vivo* degradation of films proceeds relatively fast (8 to 21 µm per day) by surface erosion ^{11, 13}. Previous studies have shown that macrophages play an important role in erosion of PTMC and poly(ethylene carbonate) based materials by secreting hydrolytic enzymes and reactive species ¹⁴⁻¹⁸.

To be able to utilize these resorbable elastomers over longer time periods and in a broader range of applications, TMC polymers with relatively low erosion rates need to be prepared. TMC-based polymers with tuneable properties and biodegradation behaviour have been obtained by copolymerization of TMC with lactide, glycolide, and ε-caprolactone (CL) ^{11, 19-22}. While copolymers of TMC with up to 20 % glycolide and up to 30 % D,L-lactide degraded *in vivo* by surface erosion ^{21, 22}, a semi-crystalline TMC copolymer containing 89 mol % CL eroded by bulk erosion ¹¹. The erosion rate of the 89 mol % CL containing copolymer was very low: after subcutaneous implantation in rats for 52 wks a mass loss of only 7 % was observed.

Recently, we reported on the physical properties and the *in vitro* enzymatic erosion of amorphous TMC and CL based (co)polymers and networks containing 70 to 100 mol % TMC ¹⁹. For all compositions, elastomeric networks could be obtained by gamma irradiation.

Regardless of composition, denser networks with higher gel contents were obtained with increasing irradiation doses. For PTMC homopolymer films, erosion rates in aqueous lipase solutions decreased with increasing irradiation dose. However, for the copolymer networks, this effect was not significant. At a given irradiation dose, the erosion rates of the (co)polymer films decreased with increasing TMC content.

In this study, we attempted to reduce the *in vivo* erosion rate of TMC-based polymers in two ways. First, PTMC was exposed to increasing doses of gamma irradiation (25-100 kGy), which influences the network density, and second copolymers of TMC and CL were prepared and exposed to a constant dose of 25 kGy. The effect of the applied gamma irradiation dose used in the preparation of crosslinked PTMC networks and the effect of TMC content in the networks were evaluated by subcutaneous implantation of films in rats. The histological and immunohistochemical evaluation of the tissue response to these films was done qualitatively and quantitatively.

Materials and Methods

Materials

Polymer grade 1,3-trimethylene carbonate (Boehringer Ingelheim, Germany) and stannous octoate (Sigma, U.S.A.) were used as received. ϵ -Caprolactone (Aldrich, U.K.) was dried over CaH_2 and purified by distillation under reduced argon atmosphere. Solvents (Merck, Germany) were of analytical grade.

Preparation of Materials for Implantation

Poly(trimethylene carbonate) and poly(trimethylene carbonate-co- ϵ -caprolactone) copolymers were synthesized by ring opening polymerization of the corresponding monomers under vacuum at 130 °C for three days using stannous octoate as a catalyst¹⁹. The synthesized polymers were purified by dissolution in chloroform and precipitation into ethanol. The precipitated polymers were washed with fresh ethanol and dried at room temperature under vacuum.

Monomer conversion and copolymer composition were determined by proton nuclear magnetic resonance (^1H NMR) spectroscopy (300 MHz, Varian Innova, U.S.A.) using CDCl_3 . Number average- and weight average molecular weights ($\overline{M}_n$ and $\overline{M}_w$, respectively), polydispersity indices (PDI), and intrinsic viscosities ($[\eta]$) of the polymers were determined by gel permeation chromatography (GPC, Viscotek U.S.A.) at 30 °C using chloroform as the

eluent at a flow rate of 1.0 ml/min. The setup was equipped with ViscoGEL I-guard-0478, ViscoGEL I-MBHMW-3078, and ViscoGEL I-MBLMW-3078 columns placed in series and a TDA 302 Triple Detector Array with refractometer-, viscometer-, and light scattering detectors, allowing the determination of absolute molecular weights. The intrinsic viscosities of the purified polymers were also determined manually using a capillary Ubbelohde type 0C viscometer and polymer solutions in chloroform (0.05, 0.1, 0.2, and 0.3 g/dL) at 25°C.

Glass transition temperatures (T_g) of the (co)polymers were determined by differential scanning calorimetry (DSC). Samples (5-10 mg) were analyzed at a heating rate of 10 °C/min in a temperature range of -100 to 100 °C using a Perkin Elmer Pyris 1 DSC. After the first scan, the samples were quenched to -100 °C at 300 °C/min and a second scan was recorded after 5 minutes. Indium, lead, and cyclohexane were used as standards for temperature calibration. T_g values were determined from thermograms obtained in the second scan.

The purified polymers were compression moulded at 140 °C using a laboratory press (Fonteijne THB008, The Netherlands) and 500 µm thick stainless steel moulds. The polymers were moulded at approximately 25 kg/cm² and quenched to room temperature using cold water. The compression moulded films were then sealed under vacuum in laminated polyethylene/polyamide bags (Hevel Vacuum B.V., The Netherlands) and exposed to 25, 50, or 100 kGy (PTMC) or 25 kGy (copolymers) gamma irradiation from a ⁶⁰Co source (Isotron B.V., Ede, The Netherlands). This sterilization method leads to simultaneous crosslinking of the polymers ^{12, 19}.

The equilibrium swelling ratios and gel contents of the gamma irradiated films were determined using chloroform ¹⁹. Extraction of the sol fraction was done by swelling the gamma irradiated films using chloroform, and deswelling in acetone and ethanol. The extracted films were then washed in water for several days and dried under vacuum.

Cell Viability Assay

Possible cytotoxicity of the PTMC films irradiated at 25, 50 or 100 kGy was evaluated using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay ²³ as described previously ¹⁹. Briefly, human skin fibroblasts (PK 84 cell line) were cultured in a 96 wells plate (5000 cells/well) in RPMI medium (Roswell Park Memorial Institute medium). The cells were incubated at 37 °C and 5 % CO₂ for three days. Of each film, two disks (diameter 6 mm, thickness 500 µm) were incubated at 37 °C and 5 % CO₂ with 400 µl RPMI for 24 hours to extract any leachable components. The medium was

then replaced by this RPMI medium containing the leachables. After two days, the absorbance of formazan, which is soluble in the culture medium, was measured. The mean value obtained for cell cultures incubated with RPMI medium only was standardized as 100 % cell viability.

A medical grade polyurethane (2363-55D-Pellethane[®] resin from Dow Chemical, Midland, U.S.A.) and latex rubber (Hilversum Rubber Factory, Hilversum, The Netherlands) were used as negative and positive controls, respectively. Prior to the MTS assays and implantations, it was confirmed that the controls and the polymer films were essentially endotoxin-free.

Implantations

The tissue response to gamma irradiated (co)polymer films prepared from precipitated (co)polymers was investigated by subcutaneous implantation in rats. All procedures performed on the animals were done according to international guidelines on animal experiments and approved by the local committee for care and use of laboratory animals. Approximately three months old male Albino Oxford rats weighing 250-275 g were housed individually in a humidity- and temperature-controlled room with 12 hours light/dark cycles. The rats received water and pelleted diet *ad libitum*. The operations were performed under general anaesthesia using a mixture of isoflurane N₂O, and O₂. After shaving and disinfection, subcutaneous pockets were made to the right and left of two midline incisions on the back of the rats. Gamma-irradiated disk-shaped films measuring 8 mm in diameter and approximately 500 μ m in thickness were placed in each pocket.

The first set of implants consisted of PTMC homopolymer films (n=6 per time point) gamma irradiated at 25, 50, or 100 kGy. Of each material, extracted as well as non-extracted irradiated films were implanted to assess the influence of leachable components. The discs with surrounding tissue were explanted from the subcutaneous site on day 5 and 14. Immediately after explantation, half of each PTMC disk was immersion fixed in 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH=7.4) for light microscopy (n=6) while the other half was snap-frozen in liquid nitrogen for immunohistochemical analyses (n=3) and thickness determinations (n=3). The *in vivo* erosion rates of the PTMC films were determined by measuring the dry thickness of the films with a digital micrometer (Mitutoyo IP65, U.S.A.) before implantation and after explantation.

The second set of implants consisted of TMC and ϵ -caprolactone (co)polymer films (n=3 per time point) which varied in composition. The (co)polymers were gamma irradiated at a same dose of 25 kGy. The non-extracted (co)polymer disks with surrounding tissue were

explanted 5, 14, and also 28 days after implantation. Half of each disk was used for histological- (n=3), the other half for immunohistochemical analyses (n=3).

Histology

The explants were fixated in glutaraldehyde, dehydrated in graded alcohol solutions and then embedded in glycol methacrylate (Kulzer Histo-Technik Heraeus Kulzer, Germany). Histological sections (2 μm) were stained with toluidine blue. The tissue response was independently evaluated by two persons.

Immunohistochemistry

For immunohistochemical evaluation, cryo-sections (5-7 μm) were cut from snap-frozen explants. Acetone fixed sections were incubated in PBS containing 10 % serum. They were then incubated with monoclonal mouse anti-rat ED1 (Serotec, U.K.) for staining of macrophages, R73 (Serotec Ltd, U.K.) for staining of T-lymphocytes, and goat- α -human collagen IV (Southern Biotech, U.S.A.) for assessing vasculature. The endogenous peroxidase activity was blocked with 1% H_2O_2 in PBS, and colouring was performed with 3-amino-9-ethyl carbazole. Counterstaining was done with hematoxylin. Quantification was performed by counting the number of positive cells on micrographs (400X for ED-1, 200X for R73 and Coll IV). The micrographs were equally distributed over eight different areas. The extent of vascularisation was determined by computerized morphometry.

Statistical Analysis

The data are presented as mean $\pm$ standard deviation. The statistical significance of differences in the means was evaluated by one-way analysis of variance, followed by post-hoc Tukey's test.

Results and Discussion

Two different approaches were followed with the intention of reducing the *in vivo* erosion rate of TMC-based polymers. In a first series of experiments the gamma irradiation dose for treatment of PTMC (which influences the gel content and the network density) was varied from 25 to 100 kGy, in a second approach TMC was copolymerized with increasing ϵ -caprolactone contents and subsequently irradiated at 25 kGy. The effects of these approaches on the tissue response of the resulting materials were analysed qualitatively and quantitatively.

Preparation of (co)Polymer Films and Network Formation by Gamma Irradiation

High molecular weight PTMC homopolymers and copolymers of trimethylene carbonate with ϵ -caprolactone were synthesized, as follows from the GPC results in Table 1. The intrinsic viscosities determined using the viscometer detector of the GPC could be corroborated with those determined using an Ubbelohde viscometer, although the latter values are slightly higher. All polymers had low glass transition temperatures and were in the rubbery state at physiologic temperatures. The purified high molecular weight polymers were compression moulded into films and then crosslinked by gamma irradiation under vacuum. The effect of compression moulding on the molecular weight and intrinsic viscosity of the (co)polymers was only minor.

Table 1. Characteristics of the synthesized poly(trimethylene carbonate-co- ϵ -caprolactone) (co)polymers used to prepare the networks for the implantation studies. (Values between brackets were determined on compression moulded specimens.)

TMC/CL ^a (mol ratio)	Glass transition temperature, T _g (°C) ^b	Molecular weight, $\overline{M}_n$ (kg/mol) ^c	Polydispersity index ^c	Intrinsic viscosity, [η] (dl/g) ^c	Intrinsic viscosity, [η] (dl/g) ^d
100/0	-17.1	270	1.51	4.5	4.9
100/0 ^e	-14.8	590 (586)	1.31 (1.32)	7.5 (7.2)	7.8
88.9/11.1 ^e	-23.4	149 (136)	1.60 (1.62)	2.8 (2.6)	3.1
79.2/20.8 ^e	-29.2	264 (218)	1.39 (1.56)	4.3 (4.0)	4.7
69.9/30.1 ^e	-35.8	261 (230)	1.35 (1.43)	4.2 (4.1)	4.5

^a Determined by ¹H-NMR on specimens purified by precipitation.

^b Determined by DSC in the second heating scan.

^c Determined by GPC at 30 °C using chloroform as the eluent.

^d Determined by capillary viscometry at 25 °C using chloroform as the solvent.

^e See also reference ¹⁹.

To investigate the effect of the gamma irradiation dose, PTMC ($\overline{M}_n$ =270 kg/mol) homopolymer films were irradiated at 25, 50 and 100 kGy. All polymer films were crosslinked, their gel percentages were respectively 29±1, 49±1, and 63±2 % for irradiation doses of 25, 50, and 100 kGy. The swelling ratios (v/v) of the networks in chloroform were relatively high: 252±15, 68±2 and 35±1 for PTMC films irradiated at 25, 50 and 100 kGy irradiation doses, respectively. This indicates that relatively low crosslink densities were obtained. The *in vivo* behaviour of both extracted and non-extracted PTMC films was investigated by subcutaneous implantation in rats for 5 and 14 days.

For the investigation of the effect of copolymer composition, PTMC homopolymer ($\overline{M}_n=590$ kg/mol) and three copolymers having ϵ -caprolactone contents of 11, 21, or 30 mol % were used ¹⁹. Compression moulded films of these (co)polymers were all irradiated at 25 kGy, the most commonly used irradiation dose for the sterilization of medical devices ²⁴. The melt-pressed copolymer films had formed networks upon gamma irradiation, with gel contents of 32 ± 3 , 32 ± 2 , 27 ± 9 for the copolymers containing 70, 79 and 89 % TMC, respectively. The corresponding swelling ratios in chloroform were 163 ± 17 , 163 ± 15 and 177 ± 17 . After crosslinking the PTMC homopolymer ($\overline{M}_n=590$ kg/mol) had a gel content of 57 ± 9 % and a swelling ratio of 58 ± 24 in chloroform. Compared to the linear (co)polymers, crosslinking by gamma irradiation yielded flexible (co)polymer networks with significantly improved creep resistance, while thermal properties remained essentially unchanged ¹⁹. Increasing the crosslink density or varying the composition of the copolymer did not have a large influence on the elastic nature of the networks. To investigate their *in vivo* behaviour, non-extracted irradiated films were subcutaneously implanted in rats for 5, 14 and 28 days.

The Effect of Irradiation Dose on the In Vivo Behaviour of PTMC Networks

The effects of network density and gel content, which both depend on the irradiation dose, and extraction of the sol fraction, on the tissue response to PTMC networks and on their *in vivo* erosion rate were investigated using the subcutaneous rat implantation model.

Figure 1. Effect of irradiation dose and extraction of the sol fraction with organic solvents of PTMC network films on cell viability. The first three bars indicate the results of the blank (culture medium only), negative- and positive controls, respectively.

The MTS assays performed prior to the implantations demonstrated that after two days of culturing the viability of the cells was very high (87-97 %), as shown Figure 1. Neither increasing the irradiation dose nor removal of the sol fraction with organic solvents had a

significant effect on cell viability indicating that gamma-irradiation did not lead to the formation of toxic leachable compounds. All values determined for irradiated PTMC films were comparable to those of the negative control (94 %) and of non-irradiated, non-crosslinked high molecular weight PTMC ¹⁹.

Upon implantation, both extracted (extr.) and non-extracted (non-extr.) PTMC networks were found to erode. The decrease in thickness of the irradiated PTMC films after 5 or 14 days of implantation is shown in Figure 2.

Figure 2. Effect of irradiation dose and extraction of the sol fraction on the decrease in thickness of PTMC networks films after 5 days (extracted and non-extracted) and 14 days (non-extracted) of subcutaneous implantation. (* lower than 25 kGy and 50 kGy, day 5 non-extr., $p < 0.06$ and $p < 0.28$, respectively, ** lower than 25 kGy and 50 kGy, day 14 non-extr. $p < 0.06$ and $p < 0.05$, respectively, *** higher than day 5 non-extr. $p < 0.05$)

The average erosion rates of extracted network films during the first 5 days of implantation (approximately 18 μm per day) were independent of irradiation dose. Apparently, increasing the crosslink density was not very influential in slowing down the erosion of the extracted films. Although the films irradiated at the highest dose had a lower swelling ratio in chloroform, the high values of the swelling ratios indicated relatively low crosslink density for all specimens. Recently, photocrosslinked PTMC networks prepared from PTMC macromers of molecular weight 7300 g/mol have been shown to erode much slower upon implantation (33 wt % in 44 weeks) than the gamma irradiated PTMC networks we prepared ¹⁵. This suggests that PTMC networks with much higher crosslink densities than the current gamma irradiated PTMC networks are required to obtain slowly eroding PTMC networks.

The average decrease in thickness of extracted PTMC films irradiated at 25 kGy ($p < 0.09$) and 50 kGy ($p < 0.08$) was less than the non-extracted films. At 100 kGy, the degree of erosion of extracted films was comparable to that of non-extracted films, which could be

due to the higher gel content of these films (63 %). The erosion rates of all extracted PTMC network films were slightly lower than the rates previously reported for non-irradiated non-extracted PTMC films (21 $\mu\text{m/day}$)¹¹ indicating that the *in vivo* erosion of PTMC can only slightly be reduced by gamma irradiation.

During the first 5 days of implantation, the average erosion rate of non-extracted PTMC films irradiated at 100 kGy (15.1 ± 2.5 $\mu\text{m/day}$) was lower than for films irradiated at 25 kGy (39.7 ± 16.0 $\mu\text{m/day}$, $p < 0.06$) and at 50 kGy (25.9 ± 5.2 $\mu\text{m/day}$, $p < 0.28$), although the latter was not statistically significant. With increasing irradiation dose, both the gel fraction and the crosslink density increase. The faster erosion of films irradiated at the lower doses, is likely due to the more rapid degradation (and dissolution) of the sol fraction.

Erosion of the PTMC films continued after this time period. After 14 days, the PTMC films irradiated at different doses were all significantly thinner than after 5 days ($p < 0.05$). Similar to the observation at day 5, also at day 14, the erosion rate of non-extracted PTMC films irradiated at 100 kGy (22.6 ± 0.19 $\mu\text{m/day}$) was less than that of films irradiated at 25 (26.6 ± 1.5 $\mu\text{m/day}$, $p < 0.06$) or at 50 kGy (27.6 ± 1.5 $\mu\text{m/day}$, $p < 0.05$). However, these differences were less pronounced than those observed in the first 5 days.

Data on extracted irradiated PTMC films recovered after 14 days was not reliable. Due to the extraction process, these specimens were initially thinner than the non-extracted ones. Therefore, after 14 days the specimens irradiated at 25 kGy and 50 kGy could not be retrieved or were too thin to be measured accurately. The extracted films which were irradiated at 100 kGy had an average erosion rate of 20.6 ± 3.1 $\mu\text{m/day}$, close to that of non-extracted films irradiated at 100 kGy.

The tissue response to gamma irradiated PTMC films was investigated by histological- as well as by immunohistochemical analysis. This is respectively illustrated in Figure 3 and 4, while in Figure 5 the data is quantified.

Figure 3. Light micrographs (200X) of non-extracted PTMC films gamma-irradiated at 25 kGy (A,B), 50 kGy (C, D), 100 kGy (E,F) on day 5 (A,C,E) and on day 14 (B,D,F) of implantation. Toluidin blue was used for the staining of the histological sections. (Scale bars=50 μ m) (P) polymer, (fc) fibrous capsule, (*) fibrin, (g) giant cell, (→) macrophage, (L) lipid tissue, (V) blood vessel, (▲) phagocytosed intracellular fragments, (△) lymphocyte.

Figure 4. Light micrographs of non-extracted PTMC films gamma-irradiated at 25 kGy (A,B,G), 50 kGy (C,D), 100 kGy (E,F) showing the decrease in number of lymphocytes (stained red using 3-amino-9-ethyl carbazole) with increasing irradiation dose (A,C,E at 200X magnification), the decrease in number of macrophages (stained brownish-red using 3-amino-9-ethyl carbazole) at the interface with increasing irradiation dose (B,D,F at 200X magnification) and the extent of vascularisation on day 5 of implantation (G at 200X magnification) (Scale bars=50 μ m).

General overviews of the foreign body response to non-extracted PTMC network films on day 5 and day 14 of implantation are given in Figure 3. It can be seen that on day 5, fibrin that was formed as a result of wound healing and some infiltrated polymorph nuclear cells were present at the tissue-polymer interface of all implanted films. At this time point thin capsules were formed around the implants. The tissue-biomaterial interfaces mainly consisted of macrophages and foreign body giant cells, which appeared to be eroding the film surfaces by phagocytosis. The overall macrophage densities surrounding the different materials are shown in Figure 5A. Although on day 5 there was only a slight decrease in overall macrophage density with increasing irradiation dose (Figure 5A), the immunohistochemical stainings for macrophages in Figure 4 (B, D, F) showed that at the interface the number of macrophages significantly decreased with increasing irradiation dose. This is in agreement with the lower erosion rates of films crosslinked at higher irradiation doses.

High numbers of blood vessels were present at the tissues surrounding the irradiated PTMC films (Figures 3A, 3C, 3E and 4G). At the fifth day, 9 to 15 % of the tissue stained positive for collagen IV (Figure 5C). Lymphocytes and sporadic plasma cells were also present in the surrounding tissue. The lymphocyte densities are shown in Figure 5B. The number of observed lymphocytes decreased as the irradiation dose was increased (Figure 4A, 4C, 4E and Figure 5B). The erosion rate is known to influence the intensity of the tissue response to biodegradable polymers²⁵⁻²⁸, therefore the lower erosion rates of PTMC films irradiated at higher doses could be responsible for the milder tissue response to these films.

The tissue response to extracted PTMC films after 5 days of implantation was also quantified. Figure 5 shows that the overall number of macrophages and blood vessels surrounding extracted films was somewhat lower than in the case of non-extracted films, although this was only statistically significant for films irradiated at 50 kGy ($p < 0.05$). The overall number of macrophages surrounding extracted films irradiated at 50 kGy was lower than the number of macrophages surrounding extracted films irradiated at 25 or 100 kGy. At the tissue-polymer interface, however, the number of macrophages did not decrease with increasing irradiation dose. This is to be expected, as the erosion rates of extracted films do not vary with irradiation dose. The number of lymphocytes surrounding the extracted films decreased with increasing irradiation dose (Figure 5B). These numbers are comparable to those of lymphocytes surrounding non-extracted films.

Figure 5. Quantification of the tissue response to extracted (extr.) and non-extracted (non-extr.) irradiated PTMC films after 5 and 14 days of implantation. Macrophage density (A) (* lower than 25 kGy, $p < 0.09$ and 100 kGy, $p < 0.05$ day 5 extr. and 50 kGy, day 5 non-extr., $p < 0.05$, ** lower than 25 kGy and 100 kGy, day 14 non-extr., and 50 kGy, day 5 non-extr., $p < 0.05$) lymphocyte density (B) (*lower than 25 kGy, day 5 extr., $p < 0.05$ ** lower than 25 kGy, day 5 non-extr., $p < 0.09$), and the % area stained for collagen IV indicating vascularization (C) (*lower than 50 kGy, day 5 non-extr., $p < 0.05$) were evaluated.

In evaluating the tissue response after 14 days of implantation, histology could be done on non-extracted and on extracted films. Immunohistochemical analysis could only be done on non-extracted specimens: extracted films were too thin at day 14 to allow sectioning. At this time point the capsules around the implanted PTMC films were thicker than at day 5, the tissue-polymer interface mainly consisted of macrophages and foreign body giant cells. The number of cells phagocytosing fragments of the implanted polymer film is larger than at day 5. In the tissue surrounding the films some lymphocytes, sporadic plasma cells and large numbers of blood vessels were seen. In general, the tissue response to extracted and non-extracted films did not differ much, both on day 5 and day 14. This indicates that the sol

fraction of the networks formed did not contain harmful compounds that might be formed as a result of the irradiation process.

A quantified representation of the tissue response to the non-extracted films after 14 days of implantation is given in Figure 5. The tissue response to films irradiated at 100 kGy was comparable to that seen on day 5. For films irradiated at 25 or 50 kGy, the number of macrophages and lymphocytes seemed to be lower than on day 5, although this was only statistically significant for the number of macrophages surrounding the films irradiated at 50 kGy ($p < 0.05$).

Non-irradiated PTMC films induce a mild inflammatory response upon subcutaneous implantation in rats ¹¹. Histological evaluation of the tissue response to gamma irradiated PTMC films used in the current study and to the non-irradiated PTMC films previously reported ¹¹ and reevaluated, revealed that the average number of lymphocytes and plasma cells per 400X view and the overall tissue response to both materials were comparable. This indicates that gamma irradiation did not have an adverse effect on the tissue response to PTMC films. With increasing gamma irradiation dose, the erosion rates of PTMC network films were slightly reduced and the tissue response became milder (less macrophages at the tissue-polymer interface and less lymphocytes in the surrounding tissue).

The Effect of Copolymer Composition on the In Vivo Behaviour of TMC-CL (Co)polymer Networks

The effect of ϵ -caprolactone content on the tissue response to TMC-CL (co)polymer networks and on their *in vivo* erosion behaviour were investigated as well. Non-extracted, gamma irradiated (25 kGy) (co)polymer films were implanted for 5, 14, or 28 days. In a previous *in vitro* study, we showed that, regardless of their monomer composition, these TMC and CL (co)polymer networks were non-cytotoxic ¹⁹.

After 5 days of implantation, macroscopically no adverse effects were visible at the implant sites. A thin and loose capsule was formed around the implants. For this reason, some of the implants could not be retrieved with their surrounding tissue. Histological analysis could be performed for irradiated (co)polymer films containing 100 % TMC (n=3), 89 % TMC (n=2), 79 % TMC (n=2), 70 % TMC (n=1). At this time point, the tissue response to all films was comparable. Therefore, a general overview is given only for the foreign body response to the 79 % TMC containing films (Figure 6A). The interface between the films and the surrounding tissue consisted of fibrin, and mainly macrophages and foreign body giant cells (FBGCs) (Figure 6A and 6B). Small fragments of material had been phagocytosed by

these cells. Neutrophils were sporadically present at the interface. In the surrounding tissue, large numbers of blood vessels, macrophages, some lymphocytes, few FBGCs, and sporadically neutrophils were present. Quantification of the tissue response to these gamma irradiated films in Figure 7, also showed that the response to the (co)polymer films was similar. Varying the TMC to CL ratio in the (co)polymer network did not influence the overall numbers of macrophages, lymphocytes and the extent of vascularisation significantly.

Figure 6. Light micrographs showing the foreign body response to non-extracted 79 % TMC containing copolymer films gamma irradiated at 25 kGy on day 5 (A), day 14 (C) and on day 28 (D) of implantation. Toluidin blue was used for the staining of the histological sections. In (B) a light micrograph showing immuno-stained macrophages on day 5 is shown (Magnification 200X). (Scale bars=50 μ m) (P) polymer, (fc) fibrous capsule, (*) fibrin, (g) giant cell, (→) macrophage, (L) lipid tissue, (V) blood vessel, (▲) phagocytosed intracellular fragments, (△) lymphocyte.

At day 14, the inflammatory response to the (co)polymer films was also characterized by the presence of macrophages, FBGCs, lymphocytes and few plasma cells (Figure 6C). At this time point, thicker capsules than at day 5 were present around the implanted films. Also on day 14, fragments of material had been phagocytosed by macrophages and FBGCs. Phagocytosed fragments could be seen at the interface as well as in the surrounding tissue. Quantification of the tissue response to the (co)polymer films is also presented in Figure 7. The overall numbers of macrophages and lymphocytes for the different (co)polymer films did not differ significantly from each other or from the numbers after 5 days of implantation

(Figure 7A and 7B). Figure 7C shows that on day 14 the percentage of tissue that stained positive for collagen IV seemed to be somewhat lower than on day 5. Nevertheless, quite high numbers of blood vessels can be observed in the surrounding tissue.

Figure 7. Effect of composition of gamma-irradiated (25 kGy) (co)polymer films on macrophage density (A), lymphocyte density (B), and percentage vascular area (C) on day 5 and day 14 of subcutaneous implantation. (The capsule surrounding 70 % TMC containing copolymer was lost, and immunohistochemical staining at day 5 could not be done.)

After 28 days of implantation most of the implanted copolymer network films had completely eroded, and only in 1 out of 3 implanted 79 % TMC containing films, remnants could be retrieved (Figure 6D). The small fragments of this material were phagocytosed or surrounded by macrophages and giant cells. This suggests that the erosion rates of copolymer films containing more than 70 % TMC were similar to those of the PTMC homopolymer films. A statistically significant effect of the copolymerisation of TMC with up to 30 mol % of CL on the tissue response to the gamma irradiated films of these polymers and their *in vivo* erosion behaviour could not be shown.

In the tissue response to PTMC of a lower initial molecular weight ($\overline{M}_n=270$ kg/mol) and that to PTMC of a higher initial molecular weight ($\overline{M}_n=590$ kg/mol) and the copolymers (all irradiated at 25 kGy, Figures 5 and 7), the overall numbers of lymphocytes and the extent of vascularisation were comparable. The overall number of macrophages was higher for irradiated PTMC of the lower initial molecular weight; nevertheless the films are completely eroded at 28 days.

This study shows that in modulating the tissue response to PTMC (co)polymer network films and reducing their *in vivo* erosion rates, the gamma irradiation dose has a larger effect than the ϵ -caprolactone content. Although PTMC network films with much lower erosion rates than those of linear PTMC films could not be obtained, the presented data provides valuable information regarding the tissue response to these materials and their *in vivo* erosion behaviour.

Conclusions

Gamma irradiated (co)polymer networks based on TMC and CL erode upon implantation in the subcutaneous tissue of rats. Increasing the dose upon irradiation of PTMC homopolymer films leads to lower erosion rates and to a milder tissue response. Extraction of the sol fraction of the PTMC films results in slightly lower erosion rates. Variation of the network density by varying the gamma irradiation dose did not affect the erosion rate of extracted films.

In the case of irradiated (co)polymer films containing 70 to 100 % TMC, neither the tissue response to the films nor their *in vivo* erosion was significantly influenced by the (co)polymer composition. The dose at which the (co)polymer films were irradiated had a larger effect than the (co)polymer composition. These TMC and CL based (co)polymer networks still resorb quite rapidly, but can be suited for short-term controlled release applications and *in vitro* cell culturing studies. Further study is required to obtain TMC-based elastomers with considerably lower erosion rates.

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PART III

**MACROPHAGE CULTURE AS A PLATFORM TO INVESTIGATE
THE EROSION OF TMC-BASED NETWORKS**

&

**CROSSLINKING TMC-BASED (CO)POLYMERS
BY GAMMA- OR ULTRAVIOLET IRRADIATION
IN THE PRESENCE OF CROSSLINKING AGENTS/AIDS**

Chapter 6

Macrophage-mediated Erosion of Gamma Irradiated Poly(trimethylene carbonate) Films^{*}

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Abstract

A macrophage culture model was used to investigate the erosion of gamma irradiated poly(trimethylene carbonate) (PTMC) films. When the PTMC films were incubated in the culture medium, but physically separated from the cells by a membrane, no erosion occurred. In contrast, when the J774A macrophages were directly cultured on PTMC films, they adhered to the films and were found to have eroded the polymer surface. Macrophages adhered to gamma irradiated poly(ϵ -caprolactone) (PCL) controls as well, but to a lesser extent than to the PTMC films. In this case, no signs of erosion were observed. Human skin fibroblasts cultured on PTMC and PCL films as controls also adhered to the films but did not erode the surfaces. The effect of enzymes and reactive oxygen species that can be secreted by macrophages on the erosion process was assessed using aqueous solutions of cholesterol esterase, lipoprotein lipase, esterase, potassium superoxide, and hydrogen peroxide. The PTMC films eroded in aqueous enzyme solutions as well as in aqueous superoxide solutions. Cholesterol esterase and superoxide anion radicals seem to be most involved in the

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macrophage mediated erosion of PTMC. This macrophage culture model is useful in assessing the influence of macrophages on the *in vivo* biodegradability of polymers and in elucidating the biodegradation mechanisms involved.

Introduction

In the design and development of biodegradable polymeric materials, knowledge of their degradation behaviour *in vivo* is of great importance. Upon implantation, a cascade of inflammatory responses, termed a foreign body response, is initiated¹⁻³. As a result of tissue injury, chemotactic agents are released after which first neutrophils migrate to the implant site. At a later stage, macrophages migrate to the implant site as well, and become the predominant type of cells. In the case of degradable polymers, the inflammatory response is characterized by the presence of macrophages, monocytes, and lymphocytes with the proliferation of blood vessels.

Macrophages secrete over a hundred substances which modulate the inflammatory response to biomaterials and influence their degradation⁴. Among these compounds are hydrolytic enzymes, reactive oxygen species, and reactive nitrogen species that take part in the host defence mechanism⁴⁻⁶. These secretory products may also be instrumental in the degradation of implanted biodegradable polymers⁴⁻⁶.

The effect of macrophages and macrophage-derived products on the biodegradation of polyurethanes used in long-term implants has been extensively studied⁷⁻¹⁰. However, only few studies have focused on macrophage-mediated degradation of biodegradable polymers used in short term applications like drug delivery or tissue engineering¹¹⁻¹³.

Poly(trimethylene carbonate) (PTMC) is a flexible, rubbery polymer which can be crosslinked into a form-stable, creep-resistant network by gamma irradiation¹⁴. PTMC is rather stable towards hydrolysis in water or buffer solutions ranging from pH 1 to 13¹⁵, while it erodes relatively fast *in vivo*^{15, 16}. This suggests that a cell-mediated mechanism is present in the *in vivo* erosion of PTMC.

It has been suggested by us and others that macrophages and the products these cells secrete play a role in the *in vivo* erosion of PTMC and its networks¹⁵⁻¹⁹. In our previous studies, we have investigated the *in vitro* erosion of TMC-based (co)polymers and networks in buffers and enzyme solutions, as well as the *in vivo* erosion after subcutaneous implantation in rats^{15-17, 20}. We have shown that the *in vitro* erosion behaviour of TMC and ϵ -caprolactone (CL) based (co)polymer networks in aqueous lipase solutions and buffers differed from the *in vivo* degradation in the subcutaneous tissue of rats^{17, 20}. The *in vitro* erosion rate increased

linearly as the CL content of the network was increased¹⁷, whereas *in vivo* erosion rates of the networks containing 0 to 30 % CL were similar²⁰.

Amsden and co-workers recently reported on the *in vivo* and *in vitro* degradation of networks prepared by photo-polymerization of functionalized TMC oligomers¹⁹. In their experiments, it was found that the erosion rates of the networks in organic solvents containing relatively stable superoxide anion radicals were close to the *in vivo* erosion rates in the subcutaneous tissue of rats. But the erosion rates in aqueous cholesterol esterase and lipase enzyme solutions were lower than that *in vivo*. Based on these results it was concluded that oxidation plays a dominant role in the *in vivo* degradation of their PTMC networks¹⁹.

In this study, we investigated the *in vitro* erosion of PTMC networks using macrophage cultures as well as aqueous solutions of reactive oxygen species (ROS) or relevant hydrolytic enzymes. In contrast to PTMC, poly(ϵ -caprolactone) (PCL) resorbs very slowly *in vivo*²¹. Therefore, this polymer was used as a control in our experiments.

Materials and Methods

Materials

Polymer grade 1,3-trimethylene carbonate (Boehringer Ingelheim, Germany) and stannous octoate (Sigma, U.S.A.) were used as received. ϵ -Caprolactone (Aldrich, U.K.) was dried over CaH_2 and purified by distillation under reduced argon atmosphere. J774A macrophages (ATCC-TIB-67) and human skin fibroblasts (ATCC 2429; CCD 1112SK) were obtained from the American Type Culture Collection. Culture media, fetal bovine serum, GlutamaxTM and penicillin-streptomycin were obtained from Invitrogen (Gibco, U.S.A.). Culture disposables were from Nunc (U.S.A.) and Greiner (Germany). Cholesterol esterase (CE) from porcine pancreas (Sigma, U.K., 56.2 U/mg, 1 U corresponds to the amount of enzyme which liberates 1 μmol cholesterol per min at pH=7.0 and 37 °C from cholesterol acetate), lipoprotein lipase (LPL) from *Pseudomonas sp.* (Sigma, U.K., 1299 U/mg, 1 U corresponds to the amount of enzyme which liberates 1 μmol oleic acid per min at pH=8.0 and 40 °C from triolein) and esterase (E) from porcine liver (Sigma, Switzerland, 165 U/mg, 1 U corresponds to the amount of enzyme which hydrolyzes 1 μmol ethyl valerate per min at pH 8.0 and 25 °C) were dissolved in phosphate buffered saline (PBS, pH=7.4, B. Braun Melsungen A.G., Germany) for the enzymatic erosion studies. Potassium dioxide (KO_2 , Sigma, U.S.A.), cobalt chloride (CoCl_2 , Sigma, U.K.) and NaOH/NaCl buffer solution (pH=13, Scharlau Chemie, Spain) were used as received.

Preparation and Characterization of Samples for Erosion Studies

Poly(trimethylene carbonate) and poly(ϵ -caprolactone) (PCL) were synthesized by ring opening polymerization of the corresponding monomers under vacuum at 130 °C for three days using stannous octoate as a catalyst. The synthesized polymers were purified by dissolution in chloroform and precipitation into ethanol. The precipitated polymers were washed with fresh ethanol and dried at room temperature under vacuum.

Monomer conversion was determined by proton nuclear magnetic resonance (^1H -NMR) spectroscopy (300 MHz, Varian Innova, U.S.A.) using CDCl_3 (Merck, Germany). Number average- and weight average molecular weights ($\overline{M}_n$ and $\overline{M}_w$, respectively) and polydispersity indices (PDI) of the purified polymers were determined by gel permeation chromatography (GPC, Viscotek U.S.A.) using chloroform as the eluent at a flow rate of 1.0 ml/min. The setup was equipped with ViscoGEL I-guard-0478, ViscoGEL I-MBHMW-3078, and ViscoGEL I-MBLMW-3078 columns placed in series and a TDA 302 Triple Detector Array with refractometer-, viscometer-, and light scattering detectors, allowing the determination of absolute molecular weights.

The purified polymers were compression moulded at 140 °C with a laboratory press (Fonteijne THB008, The Netherlands) and 500 μm thick stainless steel moulds. The polymers were moulded at approximately 25 kg/cm^2 and quenched to room temperature using cold water. The compression moulded films were then sealed under vacuum in laminated polyethylene/polyamide bags (Hevel Vacuum B.V., The Netherlands) and exposed to 50 kGy gamma irradiation from a ^{60}Co source (Isotron B.V., Ede, The Netherlands). This sterilization method leads to simultaneous crosslinking of the polymers¹⁴.

The equilibrium swelling ratios and gel contents of the gamma irradiated films were determined as previously described using chloroform¹⁷.

Cell Mediated Erosion Studies

J774A macrophages were maintained in DMEM containing 4.5 g/L D-glucose, pyruvate, 10 % fetal bovine serum, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$ 2mM GlutamaxTM. Cells were passaged every 4-7 days by scraping. Confluency was never reached on the regular culture substratum TCPS (Greiner, Germany).

The biodegradability of gamma irradiated, non-extracted PTMC and PCL (control) films in the presence of J774A macrophages was assessed under different cell culturing configurations which are illustrated in Figure 1. In series A, macrophages were cultured on the tissue culture polystyrene (TCPS) surfaces, while disk shaped PTMC or PCL films (8 mm

in diameter) were immersed in the medium but separated from the cells using 3 μm pore-size ThinCert™ (Greiner, Germany) membranes. In series B, macrophages were cultured on the surface of disk-shaped (22 mm in diameter) gamma irradiated polymer films, while at the same time a smaller disk-shaped polymer film (8 mm in diameter) was immersed in the medium but separated from the cells using ThinCerts™. In series C and D, macrophages were directly cultured on the gamma irradiated polymer films.

Figure 1. Schematic overview of the series of cell culturing experiments conducted to investigate the effect of macrophages on the erosion of gamma irradiated PTMC and PCL films. The cells were cultured on TCPS or directly on PTMC and PCL films, as described in the text.

The seeding density was approximately 8×10^4 cells/cm² (this corresponds to 50 % of the maximum coverage reached in tissue culture flasks). The volume of medium used was 2 mL, which allowed the ThinCerts™ to be submerged in the medium. All experiments were performed in triplicate. Control experiments were performed by conditioning the gamma irradiated polymer films in the cell culture medium only, and by culturing human skin fibroblasts directly on the surface of the polymer films. Fibroblasts were cultured in RPMI-

1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 100 µg/mL 2mM Glutamax™. The cell seeding density was 10^5 fibroblasts/well or 2.6×10^4 cells/cm².

After 14 days of culturing, the films in series A, B, and C were placed in Milli-Q water to lyse the cells, then thoroughly rinsed and weighed. After drying the films to constant weight *in vacuo* at room temperature, the samples were weighed again and their thickness was measured with a digital micrometer (Mitutoyo IP65, U.S.A.). The films were sputter coated with gold and their surfaces were analysed by scanning electron microscopy (SEM, Philips XL 30 ESEM-FEG, The Netherlands) at an operating voltage of 5 kV.

The films of series D were fixed with 3.7 % para-formaldehyde in cytoskeletal stabilizing (CS) buffer (0.1 M piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer, 1 mM ethylene glycol tetraacetic acid (EGTA), pH=6.9) for 15 min, then transferred to PBS. A quarter of each film was cut and processed for SEM by further fixation with 2 % glutaraldehyde in 0.1 M cacodylate buffer (pH=7.4), dehydration in graded alcohols, and critical point drying. The rest of the sample was used for fluorescence staining of nuclei using 4',6-diamidino-2-phenylindole (DAPI) and of the actin cytoskeleton using tetramethylrhodamine iso-thiocyanide-phalloidin (TRITC-phalloidin). These specimens were then analysed by confocal laser scanning microscopy (LEICA TCS SP2, with fully water-immersed 40x objective (NA 0.80)).

Enzymatic Erosion Studies

The enzymes cholesterol esterase (CE) from porcine pancreas, lipoprotein lipase (LPL) from *pseudomonas sp.*, and esterase (E) from porcine liver were used to study the enzymatic hydrolysis of gamma irradiated films. Aqueous enzyme solutions were prepared at a concentration of 20 µg/mL using phosphate buffered saline (PBS, pH=7.4) containing 0.02 wt % NaN₃ (Sigma, U.S.A.) as a bactericide. Non-extracted, disk-shaped films (10 mm diameter, approximately 500 µm thickness, n=3 per time point) were placed in vials containing 1 ml of enzyme solution and conditioned at 37 °C. The medium was refreshed once every two days. Control experiments without enzyme were performed using PBS (pH 7.4, n=1 per time point). At predetermined time points, the mass and thickness of wet specimens were determined after rinsing and blotting their surfaces. The measurements were performed again after drying the specimens to constant weight *in vacuo* at room temperature. The surfaces of the films were analysed by SEM.

Erosion by Reactive Oxygen Species

To assess the erosion of gamma irradiated PTMC and PCL films in the presence of superoxide anion radicals, 0.25 M and 1.0 M KO₂ solutions were prepared by dissolving KO₂ in the buffer solution at pH=13. Non-extracted, disk-shaped specimens (10 mm diameter, approximately 500 μ m thickness, n=3 per time point) were placed in vials containing 1 mL of the freshly prepared superoxide solutions and conditioned at 37 °C. The solutions were refreshed daily. Control experiments were performed using the buffer solution (pH=13, n=1 per time point) only. Erosion of gamma irradiated PTMC films was also studied using a 20 wt % H₂O₂ solution containing 0.1 M CoCl₂. The solutions were refreshed once every three days. Changes in mass and thickness were determined as described before.

Results and Discussion

Preparation of Polymer Films

High molecular weight PTMC and PCL were synthesized as described previously. Monomer conversions were higher than 99 %. The $\overline{M}_n$ values of the prepared PTMC and PCL polymers were 500 kg/mol and 220 kg/mol and the PDI values were 1.51 and 1.33, respectively.

Melt pressed polymer films were gamma irradiated under vacuum at a dose of 50 kGy. Irradiation led to simultaneous sterilization and crosslinking of the films. The gel contents of the gamma irradiated PTMC and PCL films were 67 $\pm$ 3 % and 57 $\pm$ 2 % respectively, their equilibrium swelling ratios in chloroform were 35 $\pm$ 4 vol/vol and 57 $\pm$ 2 vol/vol, respectively. These results show that in both cases networks have been formed, although the high degrees of swelling indicate relatively low crosslinking densities.

The erosion of these creep-resistant and form-stable PTMC films in macrophage cultures and in different aqueous solutions containing ROS or hydrolytic enzymes was investigated. PCL films were used as controls.

Macrophage-Mediated Erosion

To assess the erosion behaviour of gamma irradiated PTMC and PCL films by macrophages, J774A macrophages were cultured on these films according to the scheme given in Figure 1.

In series A and B, the effect of products secreted by macrophages on the erosion of the polymer films was investigated by maintaining the films in the macrophage culture medium

separated from the cells by 3 μm pore-size ThinCerts™. Depending on the biodegradability of the surface to which the macrophages adhere, their activation and degradative potential can differ ²². Therefore, we cultured macrophages on biodegradable polymer films that were placed in the cell culture well (series B) as well as on the non-degradable TCPS well bottom (series A). We found that after 14 days of culturing, the mass of both the PTMC and the PCL films in the ThinCerts™ remained unchanged, regardless of the surface on which the macrophages were cultured. SEM analysis of the PTMC (Figure 2A and 2B) and PCL (not shown) films revealed that their surfaces remained smooth when macrophages were not cultured directly on the polymer films.

In series C, the effect of macrophages on the erosion of the polymer films was investigated by culturing the cells directly on the films. Contrary to our findings with the films that were separated from the cells in series A and B, erosion was evident when J774A macrophages were cultured directly on PTMC films for 14 days. Figures 2C-F, which are SEM micrographs taken after detachment of the cells from the surface, clearly show that pits with a depth of up to 50 μm had been formed on the PTMC surface. These surfaces were very similar to those that had eroded *in vivo* ²⁰. Figure 2C shows the cross section of the eroded film, and illustrates that erosion occurred only at the surface. In accordance with a surface erosion mechanism, the bulk of the film was left unchanged. The mass loss of these PTMC films after 14 days of macrophage culturing was 4.82 ± 0.19 % and the decrease in thickness was 3.67 ± 2.25 %. These values correspond to a mass loss rate of 0.20 ± 0.01 $\text{mg}/(\text{cm}^2 \times \text{day})$ and an erosion rate of 1.19 ± 0.74 $\mu\text{m}/\text{day}$. These rates are lower than our previously reported *in vivo* erosion rates of PTMC rods in tibiae of rabbits (7.7 $\mu\text{m}/\text{day}$) ¹⁵ and in subcutaneous tissue of rats (21 $\mu\text{m}/\text{day}$) ¹⁶. This can be partly explained by considering that in these *in vitro* experiments only one side of the films was in contact with macrophages, whereas *in vivo* adherent macrophages can be observed on both sides of films ^{16, 20}. In addition, we chose to study the influence of macrophages without prior activation (*e.g.* by lipopolysaccharides). Therefore, the erosion of the films is due to the intrinsic macrophage activity which results from its interaction with the PTMC polymer.

Figure 2. SEM micrographs of PTMC (A-F) and PCL (G-H) films after 14 days of macrophage culturing. Surface (A) and cross-section (B) of PTMC films placed in ThinCerts™ (series B, film placed in ThinCert™). Cross-section (C) and surface (D-F) of PTMC films after detachment of the macrophages that were directly cultured on the film (PTMC in series C). (G) surface of a PCL film after detachment of the macrophages that were directly cultured on the film (PCL in series C), (H) J774A macrophages on the surface of PCL films.

When macrophages were cultured directly on PCL films in series C, no mass loss or pitting of the surface was observed (Figure 2G). This is in accordance with the very slow resorption of PCL *in vivo*²¹. Although macrophages had adhered to the PCL films and aggregated to form clusters, they did not fuse to form giant cells (Figure 2H). After 14 days of culturing, the number of macrophages on PTMC films (Figure 3) was much higher than on PCL films (Figure 2H). In the SEM micrographs shown in Figure 3, it can be seen that the macrophages tightly adhered to the surface of the pits during erosion of the polymer. Cell-to-cell contacts were formed between the macrophages on the PTMC surface. Cell clusters, consisting of individual cells rather than of fused cells, were observed on the surface of this polymer as well. This is evident from Figure 3 and from confocal microscopy observations (data not shown). On PTMC the membrane ruffles of the macrophages were more numerous, more elongated, and much finer than on PCL. This might be an indication of a higher state of activity of macrophages on PTMC (Figures 2H and 3D).

Figure 3. SEM micrographs of J774A macrophages on PTMC films after 14 days of culturing at magnifications of 250X (A), 1000X (B), 1000X (C), 2000X (D).

The PTMC films had only eroded when the macrophages were cultured directly on the film surface and not when the films were separated from the macrophages by ThinCerts™. This implies that direct contact with macrophages is required for erosion of these polymer

films. Macrophages (and giant cells) can tightly adhere to polymer surfaces^{12, 23, 24}. It has been postulated that, in doing so, a confined environment is created where the short-lived ROS can act on the polymer surface^{23, 24}. Therefore, when the polymer film is separated from the macrophages by the ThinCert™ membranes, the ROS secreted by macrophages had likely reacted with other molecules in the medium before reaching the polymer surface. Similarly, this environment could also prevent the inhibition, inactivation, or excessive dilution of macrophage derived enzymes which might also be responsible for the erosion of PTMC.

As fibroblasts are usually abundantly present at later stages *in vivo* at the implant site as well, erosion of PTMC and PCL in fibroblast cultures was also investigated. After 14 days of culturing human skin fibroblasts on PTMC and PCL films, neither was eroded. Similarly no erosion was observed when PTMC or PCL films were placed in the culture medium without any cells. This implies a predominant role of macrophages in the biodegradation of PTMC.

The SEM observations were corroborated by confocal laser scanning microscopy (Figure 4), in which the erosion process of PTMC was clearly demonstrated to be in close association with the presence of macrophages (Figure 4A). The erosion depth after 14 days of interaction was as much as 50 µm (Figure 4E). Fibroblasts did not have an effect on erosion, as the surface reflected image was not disturbed (Figure 4F). Interestingly, fibroblast adhesion to both polymer surfaces was similar, as these cells grew to confluency (Figures 4B and 4D). Macrophages, on the other hand, did not seem to adhere to PCL (Figure 4C, and also Figure 2H) to the same extent as to PTMC (Figure 4A, and also Figure 3), despite a similar morphology of individual cells on both polymers as observed with fluorescence microscopy of the actin cytoskeleton.

Grainger and co-workers reported that macrophages of different origins show differences in responsiveness to chemical stimuli in terms of cytokine release, phenotype and intrinsic activation state^{25, 26}. Although it was suggested to use different macrophage cell lines when assessing cell-biomaterial interactions *in vitro*, the J774A cell line was used in this study, as the behaviour of these cells is similar to primary monocytic cells²⁵. The results obtained in our model with J774A cells, showed that macrophages contribute significantly to the erosion of PTMC.

Figure 4. CLSM micrographs of macrophages (A,C,E) and fibroblasts (B,D,F) adhered to PTMC films (A,B,E,F) and PCL films (C,D) after 14 days of cell culturing. Micrographs A-D represent projected images of image stacks, Micrographs E and F represent one optical section in the image stack together with cross-sectional labelling profiles along a horizontal and vertical line through the stack. In the cross-sections in E and F the PTMC surface is visualized by the white lining. Nuclei are labelled blue, the actin cytoskeleton is labelled red. Each image represents an area of 375 μm by 375 μm.

Enzymatic Erosion

The effect of a selected number of hydrolytic enzymes that can be secreted by macrophages on erosion of gamma irradiated PTMC and PCL was investigated. Solutions of cholesterol esterase (CE), lipoprotein lipase (LPL) and esterase (E) in PBS (pH=7.4) were used. In control experiments where only PBS (pH=7.4) was used, no significant erosion of PTMC or PCL was observed.

The erosion of PTMC films in these aqueous enzyme solutions is presented in Figure 5A. Cholesterol esterase was the most efficient enzyme in eroding PTMC films, while no erosion was observed using esterase solutions. During erosion studies the enzyme solutions remained clear indicating that degradation products were water soluble. After four weeks of incubation, the mass loss was approximately 90 % in CE and 8.5 % LPL solutions. In both cases, the mass loss was linear in time. Also, the decrease in thickness of the films coincided with the decrease in mass (Figure 5C). These observations are in accordance with a surface erosion process. The erosion rates were $13.68 \pm 3.26 \mu\text{m/day}$ in CE and $1.87 \pm 0.66 \mu\text{m/day}$ in LPL solutions. The mass loss rates were $1.00 \pm 0.23 \text{ mg}/(\text{cm}^2 \times \text{day})$ in CE and $0.082 \pm 0.016 \text{ mg}/(\text{cm}^2 \times \text{day})$ in LPL solutions. The surface erosion of the films conditioned in CE and LPL solutions is also evident from the SEM micrographs shown in Figure 6. Especially after erosion of the polymers in CE solutions, very large pits were visible on the film surfaces. The structure of the surface of these PTMC films was very similar to that of PTMC films eroded by macrophages *in vitro* (Figure 2C-F), and of films eroded *in vivo* ²⁰. Based on these results, it is highly likely that the macrophage-mediated erosion of PTMC can involve enzymatic hydrolysis. Especially, CE seems to be an important enzyme in this process.

Recently, Amsden and co-workers investigated the erosion of photo-crosslinked networks prepared from star-shaped TMC prepolymers ($\overline{M}_n=7800 \text{ g/mol}$) by incubating rod-shaped specimens in aqueous CE solutions having similar concentrations ¹⁹. They reported a mass loss of 19 % after 8 weeks, which is much lower than in our observations. In a way similar to that of lipases, the active site (Asp-His-Ser) of CE becomes accessible upon binding to a (hydrophobic) substrate surface ^{27,28}. Likely, the high crosslink density of their photo-crosslinked PTMC networks makes these networks less prone to enzymatic attack than the gamma irradiated PTMC networks used in our study. In addition, gamma irradiated PTMC contains non-crosslinked polymer chains as well which can be degraded relatively faster in enzyme solutions than the less mobile network chains ¹⁷.

Figure 5. Erosion of PTMC (A), PCL (B) in aqueous solutions of cholesterol esterase (CE), lipoprotein lipase (LPL) and esterase, and the relationship between the change in relative thickness and relative mass of PTMC films incubated in aqueous CE and LPL solutions (C).

Figure 5B shows that when PCL films were incubated in aqueous CE and esterase solutions, mass loss was insignificant whereas relatively rapid erosion was observed using LPL solutions. The LPL used in this study was from bacterial origin; therefore its substrate specificity might differ from LPL secreted by macrophages. In any case, as mentioned before, the more rapid erosion of PCL compared to PTMC when using LPL is not representative for the *in vivo* degradation. Although it was found previously that PCL can be eroded in aqueous solutions of CE from *Pseudomonas* species²⁹, the CE from porcine pancreas that we used in this study did not erode the polymer. This can be attributed to variations in the substrate-binding parts of enzymes from different origins, which might lead to different substrate specificities³⁰. We used CE from porcine pancreas, as it is known to be identical to human macrophage-derived CE³¹. The effectiveness of porcine pancreatic CE in eroding PTMC and not PCL suggests that in the *in vivo* erosion of PTMC this enzyme might also play an important role.

Figure 6. SEM micrographs showing the surface of PTMC films after incubation for 14 days in CE solutions (A, B), and in LPL solutions (C,D).

Erosion by Reactive Oxygen Species

The erosion of PTMC and PCL by superoxide anion radicals was studied using a buffer solution (pH=13) containing 0.25 M and 1.0 M KO_2 . Control experiments were done using the buffer solution without KO_2 . In these control experiments, mass loss for PTMC was 1.8, 3.0 and 3.8 % after 4, 6 and 8 weeks, respectively. In the case of PCL, mass loss was 1.4, 2.7 and 4.5 % after 4, 6 and 8 weeks, respectively.

When incubated in 0.25 M KO_2 solutions for eight weeks, the mass loss of PTMC and PCL films were 15.0 ± 0.4 % and 7.6 ± 0.2 %, respectively. When using solutions with a higher superoxide concentration (1.0 M) for eight weeks, mass losses of 51.8 ± 1.1 % and 12.3 ± 0.1 % were obtained for PTMC and PCL, respectively. The mass loss and the decrease in thickness of the films placed in 1.0 M superoxide solutions in time are shown in Figure 7A and 7B respectively. Up to week 6, the mass and thickness of PTMC and of PCL decreased linearly in time. In this time interval, the rates of mass loss were 0.20 ± 0.02 mg/(cm² x day) and 0.056 ± 0.012 mg/(cm² x day) for PTMC and PCL, respectively. The erosion rates were 2.18 ± 0.35 μm/day and 0.72 ± 0.27 μm/day for PTMC and PCL, respectively. SEM analyses clearly illustrate the surface erosion process with pitting on the surfaces of both materials

(Figure 8). On the surface of PCL films, spherulite-like structures were visible indicating that erosion mainly occurs in the amorphous parts of the polymer films (Figure 8E-H). In these parts the mobility of chain segments is higher than in crystalline domains, making the ester bonds more accessible. This also explains the slower erosion of the semi-crystalline PCL compared to the amorphous PTMC.

Figure 7. Erosion of PTMC films and PCL films in buffer (pH=13) containing 1.0 M KO₂ in time. The figure shows changes in mass (A), changes in thickness (B), and the relationship between the relative thickness and relative mass of PTMC and PCL films during erosion (C).

The mass loss of the PCL films was linear with the decrease in thickness (Figure 7C). For PTMC films, mass loss was slightly higher compared to the decrease in thickness (Figure 7C). For both polymers, the rates of mass loss increased after six weeks. Although a surface erosion process is clear from the data presented from Figures 7 and 8, bulk degradation of the polymers cannot be ruled out in these highly basic superoxide anion containing buffers. These erosion studies showed that, besides enzymatic hydrolysis, superoxide anion radicals might also be involved in the macrophage-mediated erosion of PTMC.

Figure 8. SEM micrographs showing the surface of PTMC (A-D), PCL (E-H) films after incubation in buffer (pH=13) containing 1M KO_2 for 2 weeks (A,E) 4 weeks (B,F), 8 weeks (C,D,G,H).

In the degradation of polyesters³⁴ and polycarbonates^{19, 23}, nucleophilic attack of the superoxide anion radical produces anionic end groups. In the case of PTMC, these react with

the carbonate carbonyl groups in the polymer backbone and H₂O to form 1,3-propane diol and carbon dioxide.

In aqueous media at physiological pH, superoxide ions immediately form H₂O₂ and O₂. To investigate the effect of superoxide anion radical on the degradation of biodegradable polymers, relatively stable superoxide solutions can be prepared using alkaline aqueous solutions³² or in organic solvents³³. Although these conditions are very different from *in vivo* conditions, it was previously postulated that macrophages can tightly adhere to polymer surfaces to create a confined environment where short-lived ROS can act on a polymer surface^{23,24}.

Earlier studies have shown that poly(D,L-lactide)³⁴ and photo-polymerized PTMC networks¹⁹ can be degraded in tetrahydrofuran (THF) containing KO₂/18-crown-6 ether. Stoll and co-workers have used alkaline aqueous superoxide solutions to investigate the degradation of poly(ethylene carbonate)²⁴. The mass loss observed during degradation of PTMC networks swollen in organic solvent/KO₂ systems might be partly due to the dissolution of formed oligomers or polymer chains. We carried out our experiments using alkaline aqueous KO₂ solutions. As the solutions remained clear during the degradation experiments, the mass loss of gamma irradiated PTMC and PCL in these solutions was due to water-soluble degradation products.

The effect of hydroxyl radicals on the erosion of gamma irradiated PTMC films was also studied using 20 wt % H₂O₂ solutions. CoCl₂ (0.1 M) was added to these solutions to catalyze the formation of hydroxyl radicals. However, no mass loss was observed when PTMC films were incubated in these solutions for 8 weeks.

Conclusions

Gamma irradiated PTMC films can be effectively eroded by J774A macrophages *in vitro*, provided that the cells are in contact with the polymer surface. Macrophage-mediated erosion occurs via a surface erosion process. Experiments with cholesterol esterase and superoxide anion radicals, which are both secreted by macrophages, reveal two different degradation pathways that could play a role in the macrophage-mediated erosion of PTMC. Both pathways also proceed via surface erosion. The presented macrophage culture model offers opportunities to study macrophage-biomaterial interactions in detail, and therewith can contribute to the better understanding of the resorption processes of degradable polymers as they occur *in vivo*. Future studies will focus on the characterization of the compounds secreted by macrophages upon contacting TMC-based material surfaces.

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Chapter 7

Biodegradable Elastomeric Networks Prepared by Gamma Irradiation of Poly(trimethylene carbonate) in the Presence of Pentaerythritol Triacrylate^{*}

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Abstract

The properties of networks formed by gamma irradiation of poly(trimethylene carbonate) (PTMC) polymer films containing (0, 1, 5 wt %) pentaerythritol triacrylate (PETA) as a crosslinking aid were investigated. The gel contents and network densities increased with increasing PETA contents, irradiation dose, and initial polymer molecular weights. At a dose of 25 kGy, networks with gel percentages up to 96 % could be obtained. The networks were non-cytotoxic, had elastic moduli below 10.7 MPa and high tensile strengths of up to 37.7 MPa. Incorporation of PETA also improved the resistance to creep and tear propagation significantly: permanent set values were as low as 0.9 % strain and tear strengths were up to 9.3 ± 2.0 N/mm. With the incorporation of PETA, the enzymatic erosion rates of the networks could be decreased from 12.0 ± 2.9 $\mu\text{m/day}$ to 3.0 ± 1.6 $\mu\text{m/day}$. These biodegradable PTMC networks have excellent elastomeric properties, allowing their utilization in a broad range of medical applications.

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Introduction

High energy radiation is known to cause chemical changes in the structure of polymers by the formation of free radicals, ions, and excited states ^{1, 2}. Upon irradiation crosslinking prevails for some polymers ^{1, 3-5}, whereas for other polymers chain scission is dominant ⁶⁻⁹. For biomedical applications, gamma- and electron beam irradiation have been widely used to modify the surface or bulk properties of polymeric biomaterials and to sterilise them in a cost-effective way ¹⁰⁻¹³.

Poly(trimethylene carbonate) (PTMC) is a biocompatible and biodegradable polymer which has a glass transition temperature (T_g) of approximately -17 °C ¹⁴. This amorphous and flexible polymer can be crosslinked by gamma irradiation in an inert atmosphere to form a creep resistant and form-stable network ^{4, 5}. Such elastomeric polymers are especially used as scaffolding materials for the engineering of soft tissues or as depots for controlled release systems ¹⁵⁻¹⁷. Also in the design of implants, such as anti-adhesion membranes or vascular prostheses, creep resistance and form-stability are desired properties ¹⁸. Gamma irradiation significantly improves the creep resistance and form-stability of PTMC, but the elastic modulus and the tensile strength decrease. This is mainly due to the chain scission that simultaneously occurs with crosslinking ^{4, 5}. *In vivo*, PTMC degrades relatively rapid by surface erosion without the release of acidic degradation products ^{4, 14}. To broaden the applicability of this polymer, TMC based materials having tuneable low erosion rates need to be developed. Previously, we reported that the *in vivo* erosion rates of gamma irradiated non-extracted PTMC films decrease with increasing gel contents ¹⁹.

By increasing the efficiency of crosslinking of PTMC by gamma irradiation, the adverse effects of irradiation on the tensile properties of the networks can be prevented. The higher gel contents and network densities obtained will further improve creep resistance and possibly also slow down erosion. It has been previously reported that polymers which did not crosslink upon irradiation, such as polylactides ^{20, 21} and poly(propylene carbonate) ⁶, or where the crosslinking is inefficient, such as is the case for poly(ϵ -caprolactone) ³, could be effectively crosslinked in the presence of multifunctional double bond containing monomers. These crosslinked polymers had higher thermal stabilities, improved mechanical properties and degraded at a lower rate than the non-crosslinked polymers.

In this study, PTMC homopolymers of different molecular weights were gamma irradiated in the presence of varying amounts of pentaerythritol triacrylate (PETA). The

effects of the initial molecular weight of PTMC and the PETA content on crosslinking, physical properties and erosion behaviour of the resulting PTMC networks were investigated.

Materials and Methods

Materials

Polymer grade 1,3-trimethylene carbonate (TMC, Boehringer Ingelheim, Germany), and stannous octoate (Sigma, U.S.A.) were used as received. Pentaerythritol triacrylate (PETA, Aldrich, U.S.A.) was used as received. Solvents (Merck, Germany or Biosolve, The Netherlands) were of analytical grade.

Polymer Synthesis

Poly(1,3-trimethylene carbonate) (PTMC) homopolymers were synthesized by ring opening polymerization of the TMC monomer under vacuum at 130 °C for three days using stannous octoate as catalyst. To control molecular weight, different amounts of hexanediol were used as initiator. The polymers were purified by dissolution in chloroform and precipitation into ethanol, washing with fresh ethanol and drying at room temperature under vacuum.

Polymer Characterisation

Monomer conversion was determined by proton nuclear magnetic resonance (^1H -NMR) spectroscopy (300 MHz, Varian Innova, U.S.A.) using CDCl_3 (Merck, Germany).

Number average- and weight average molecular weights ($\overline{M}_n$ and $\overline{M}_w$, respectively), polydispersity indices (PDI) and intrinsic viscosities ($[\eta]$) of the purified polymers were determined by gel permeation chromatography (GPC, Viscotek U.S.A.). The setup was equipped with ViscoGEL I-guard-0478, ViscoGEL I-MBHMW-3078, and ViscoGEL I-MBLMW-3078 columns placed in series and a TDA 302 Triple Detector Array with refractometer-, viscometer-, and light scattering detectors, allowing the determination of absolute molecular weights. All determinations were performed at 30 °C, using chloroform as the eluent at a flow rate of 1.0 ml/min.

Preparation of PTMC Films

Purified polymers were compression moulded at 140 °C using 500 μm thick stainless steel moulds using a laboratory press (Fonteijne THB008, The Netherlands). The films were moulded at approximately 25 kg/cm^2 and quenched to room temperature using cold water. To

crosslink PTMC films in the presence of PETA, purified polymers and PETA (1 or 5 wt % of the polymer) were dissolved in dichloromethane to achieve good mixing. After evaporation of the solvent, compression moulded films were prepared in the same way as the purified polymers. These PETA-containing PTMC films were completely soluble in chloroform, confirming that no crosslinking had occurred under these conditions.

Gamma Irradiation, Network Formation and Network Characterization

The compression moulded films were sealed under vacuum in laminated polyethylene/polyamide bags (Hevel Vacuum B.V., The Netherlands) and exposed to 25, 50 or 100 kGy gamma irradiation from a ^{60}Co source (Isotron B.V., Ede, The Netherlands).

To determine equilibrium swelling ratios and gel contents, disk-shaped specimens (500 μm thick, 10 mm in diameter) were punched out from the irradiated films and placed in 30 mL CHCl_3 for 1 week, the solvent was refreshed once after 3 days. This procedure ensured complete removal of the sol fraction. Then the swollen gels were weighed, dried to constant weight at room temperature in vacuo and weighed again. The gel and the sol fractions were calculated according to equations (1) and (2) respectively:

$$\text{Gel fraction (\%)} = \frac{m_d}{m_0} \times 100 \quad (1)$$

$$\text{Sol fraction (\%)} = \left(1 - \frac{m_d}{m_0}\right) \times 100 \quad (2)$$

where m_d is the mass of dried (extracted) samples and m_0 is the mass of the specimens before swelling.

The swelling ratio (q) was calculated according to equation (3).

$$q = 1 + \rho_p \times \left(\frac{m_s}{m_d \times \rho_s} - \frac{1}{\rho_s} \right) \quad (3)$$

where m_s is the mass of the extracted and swollen samples, and ρ_s and ρ_p are the densities of chloroform (1.48 g/cm³) and PTMC (1.31 g/cm³), respectively.

Thermal and Mechanical Properties

Glass transition temperatures (T_g) of the different PTMC films were determined by differential scanning calorimetry (DSC). The films containing 0 or 5 wt % PETA were irradiated at 0 or 25 kGy. T_g s of PETA containing irradiated films were also determined after

extraction of the sol fraction using ethanol. Samples (5-10 mg) were analyzed at a heating rate of 10 °C/min in a temperature range of -50 to 200 °C using a PerkinElmer Pyris 1 DSC. After the first scan, samples were quenched to -50 °C at 300 °C/min and a second scan was recorded after 5 minutes. The reported values were determined from the second scan. Indium, lead, and cyclohexane were used as standards for temperature calibration of the instrument.

The mechanical properties of melt pressed and irradiated PTMC films were determined in triplicate according to ASTM-D 882-91. A Zwick Z020 tensile tester (Ulm, Germany) equipped with a 500 N load cell was operated at a crosshead speed of 50 mm/min. The initial grip-to-grip separation was 50 mm and a preload of 0.01 N was applied. The specimen deformation was derived from the grip-to-grip separation; therefore the presented values of Young's modulus (calculated from the initial slope of the stress-strain curves) give only an indication of the stiffness of the polymers.

To assess their behaviour under dynamic loading conditions, the specimens (n=1) were repetitively (20x) elongated to 50 % strain at 50 mm/min in cyclic tests. After a 2 h recovery period, the permanent deformation was estimated from the stress-strain diagram of the 21st cycle. In these experiments a preload of 0.01 N was applied, the deformation was derived from the grip to grip separation. The error in the values is approximately 0.5 % strain.

The tear strength of films was determined according to ASTM 1938 using trouser shaped specimens (n=3). The dimensions of the specimens were 75×25×0.5 mm with a 50 mm long cut halfway through the width of the specimens. Measurements were performed in triplicate at a speed of 250mm/min. The reported maximum tear strengths are normalized to the thickness of the specimens and expressed in N/mm.

For comparison, the mechanical properties of silicone elastomer (Sylgard[®] 184, Dow Corning, U.S.A.) films were also determined as described above. Silicone elastomer films with a thickness of approximately 500 µm were cast and thermally cured according to the instructions of the manufacturer.

Cell Viability Assay

Possible cytotoxicity of the gamma irradiated and non-extracted PTMC films containing 5 wt % PETA was evaluated using a direct and an indirect MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay²². Briefly, mouse skin fibroblasts (NIH 3T3 cell line) were cultured using a 96 wells plate (5000 cells/well) and Dulbecco's modified Eagle's medium (DMEM). The cells were incubated at 37 °C and 5 % CO₂ for three days.

For the indirect cytotoxicity evaluations, non-extracted disks (n=3, diameter 8 mm, thickness 500 μ m) of each film were incubated at 37 °C and 5 % CO₂ with 500 μ l DMEM for 24 hours to extract any leachable components. After the three day period of cell culturing, the medium was replaced by the leachables-containing DMEM medium. In the direct cytotoxicity assays, non-extracted discs (n=3) were placed directly on the cells in culture. In both assays, the absorbance of formazan, which is soluble in the culture medium, was measured after another two days of culture. The mean value obtained for cell cultures incubated with DMEM medium only (negative control) was standardized as 100 % cell viability. Latex rubber (Hilversum Rubber Factory, Hilversum, The Netherlands) was used as positive control.

In Vitro Enzymatic Erosion Studies

Cholesterol esterase (CE) from porcine pancreas was used to study the enzymatic hydrolysis of gamma irradiated PTMC films. Aqueous CE enzyme solutions were prepared at a concentration of 20 μ g/mL using phosphate buffered saline (PBS, pH=7.4) containing 0.02 wt % NaN₃ (Sigma, U.S.A.) as a bactericide. Non-extracted, disk-shaped films (10 mm diameter, approximately 500 μ m thickness, n=3 per time point) were placed in vials containing 1 ml of enzyme solution and conditioned at 37 °C. The medium was refreshed once every two days. Control experiments without enzyme were performed using PBS (pH 7.4, n=1 per time point). At predetermined time points, the mass and thickness of wet specimens were determined after rinsing and blotting their surfaces. The measurements were performed again after drying the specimens to constant weight *in vacuo* at room temperature.

Results and Discussion

The effect of initial polymer molecular weight and pentaerythritol triacrylate content on gamma irradiation crosslinking behaviour and on the physical properties of the resulting PTMC networks was evaluated. By varying the initiator concentration from 0 to 0.1 mol %, PTMC polymers having molecular weights in the range of 80 - 450 kg/mol were synthesized (Table 1). Monomer conversions were higher than 99 % in all cases. The molecular weights of the obtained polymers were lower than could be expected from the monomer to initiator ratios assuming each hydroxyl group initiates a growing polymer chain. This is probably due to initiation of the polymerisation from hydroxyl group containing impurities such as water. After compression moulding, slightly lower $\overline{M}_n$ values and higher polydispersity indices were obtained.

Table 1. Characteristics of the synthesized PTMC homopolymers before and after compression moulding. The values for compression moulded polymers are given between brackets.

Polymer	Initiator content (mol %)	$\overline{M}_n^a$ (kg/mol)	$\overline{M}_w^a$ (kg/mol)	PDI ^a	$[\eta]^a$ (dl/g)
PTMC ₈₈	0.1	88 (79)	139 (143)	1.58 (1.81)	1.7 (1.8)
PTMC ₁₅₇	0.05	157 (144)	228 (217)	1.45 (1.51)	2.6 (2.6)
PTMC ₂₇₇	0.025	277 (230)	407 (382)	1.47 (1.66)	4.4 (4.3)
PTMC ₄₄₃	0	443 (436)	620 (698)	1.48 (1.60)	6.6 (7.1)

^a Determined by GPC at 30 °C using chloroform as the eluent.

Crosslinking of PTMC by Gamma Irradiation

Compression moulded PTMC films having different molecular weights were gamma irradiated under vacuum at doses of 25, 50 or 100 kGy. Gel contents and equilibrium swelling ratios in chloroform are given in Figure 1A and 1B. The figures show that at a given irradiation dose, networks with higher gel contents and lower swelling ratios are obtained with increasing initial PTMC molecular weight. At 25 kGy, PTMC₈₈ and PTMC₁₅₇ did not crosslink at all, whereas PTMC₂₇₇ and PTMC₄₄₃ had relatively low gel contents of 22±1 and 60±1 %, respectively. For the range of polymer molecular weights investigated the gel contents varied between 15±4 % and 73±1 %, with swelling ratios varying between 327±32 and 22±1 at an irradiation dose of 100 kGy.

In Figure 1A and 1B, it can also be seen that for a given molecular weight gel contents increased and swelling ratios decreased with increasing irradiation doses. Increasing the irradiation dose had a larger effect on PTMC of the lower molecular weights. For instance, increasing the dose from 25 to 100 kGy during irradiation of PTMC₄₄₃ increased the gel content from 60±1 to 73±1 %, whereas the gel content increased from 0 to 42±2 % for PTMC₁₅₇. At the same time swelling ratios decreased from 45±1 to 22±1 for PTMC₄₄₃ and from 537±9 (50 kGy) to 92±4 for PTMC₁₅₇. It can be concluded that the gel content and density of PTMC networks can be increased either by increasing the initial molecular weight of the polymer or by increasing the gamma irradiation dose.

In other work, we are investigating the network formation and properties of methacrylate end-functionalized three-armed PTMC macromers with molecular weights of up to 40.7 kg/mol. These photocrosslinked networks had a swelling ratio of 16.9±1 in chloroform. This suggests that networks formed by gamma irradiation of the linear PTMC chains (with swelling ratios of 22 to 327) described here have molecular weights between crosslinks that are much higher than that of the photocrosslinked networks.

Figure 1. Effect of initial polymer molecular weight and irradiation dose on gel content (A) and swelling ratio in chloroform (B) of PTMC films crosslinked by gamma irradiation in vacuo.

The crosslinking behaviour of PTMC polymers of different initial molecular weights was evaluated according to Charlesby and Pinner²³. Their equation (Equation 4) relates the irradiation dose (r) to the sol fraction (s) of the formed network assuming that both chain scission and crosslinking events occur at random and that their number is proportional to the irradiation dose:

$$s + \sqrt{s} = \frac{G(s)}{2G(x)} + \frac{1}{2.08 \times 10^{-6} \times G(x) \times \overline{M}_n \times r} \quad (4)$$

here $G(s)$ and $G(x)$ are defined as the number of scission and crosslinking events per 100 eV, respectively. (As each crosslink involves two chains, the ratio of chain scission density to crosslink density is $G(s)/2G(x)$ ²³.) For polymers with random, monomodal molecular weight distributions, a linear relationship between $s + \sqrt{s}$ and the reciprocal of the irradiation dose is obtained. In Figure 2A, such plots are given for the PTMC polymers used in this study. Extrapolation of $s + \sqrt{s}$ to infinite irradiation dose, allows determination of the ratios of the radiation chemical yield of scission to crosslinking given in equation 4. The ratios $G(s)/G(x)$ determined for PTMC₄₄₃, PTMC₂₇₇, and PTMC₁₅₇ are 2.1, 2.0, and 1.4, respectively.

By extrapolating the plots to zero gel contents ($s + \sqrt{s} = 2$), the minimum gelation dose can be estimated. The minimum gelation doses for PTMC₄₄₃, PTMC₂₇₇ and PTMC₁₈₇ were determined to be 6.3, 16.7 and 36.3 kGy, respectively.

The maximum gel fraction $((1-s)_{\max})$ that can be obtained, can be determined using the expression derived by Inokuti ²⁴:

$$(1-s)_{\max} = \frac{1}{2} \times \left[1 - \frac{G(s)}{G(x)} + \left(1 + \frac{2G(s)}{G(x)} \right)^{1/2} \right] \quad (5)$$

In Figure 2B, it can be clearly seen that the $G(s)/G(x)$ ratio decreased, whereas $(1-s)_{\max}$ increased with increasing initial molecular weight of the PTMC polymers. The polymer with the highest molecular weight, PTMC₄₄₃, had the lowest ratio of chain scission to crosslinking (1.4) and the highest obtainable gel content (77 %).

Figure 2. Charlesby-Pinner plots for networks prepared by gamma-irradiation of PTMC films of different initial molecular weights (A), and the effect of initial polymer molecular weight on the ratio of the radiation chemical yield of chain scission to crosslinking ($G(s)/G(x)$) and on the maximum gel percentage that can be obtained $(1-s)_{\max}$ (B).

The nature of radicals formed by irradiation of polyethylene and linear aliphatic polyesters has been investigated by electron spin resonance (ESR) ²⁵⁻²⁸. For polyethylene, alkyl radicals formed upon hydrogen abstraction can combine to form a crosslink²⁵ although main chain scission can also occur. Therefore, irradiation can simultaneously lead to the formation of polymer networks, branched structures and polymer chains of lower molecular weight. Alkyl radicals are also formed upon irradiation of poly(ϵ -caprolactone) and poly(glycolide) ^{26, 28}. However, the observed formation of gaseous products (carbon monoxide, carbon dioxide and hydrogen) suggests that C-O bonds are also broken upon irradiation ²⁹.

When irradiating PTMC₈₈ and PTMC₁₅₇ at 25 kGy, $\overline{M}_n$ values decreased to 45 and 127 kg/mol, while $\overline{M}_w$ values increased to 173 and 394 kg/mol, respectively. The respective polydispersity indices (PDI) increased from 1.81 and 1.51 to 3.84 and 3.10, which indicates the formation of branched PTMC structures. Upon irradiation at 50 kGy, the PDI value of PTMC₈₈ increased even further to 6.99.

Crosslinking of PTMC by Gamma Irradiation in the Presence of Pentaerythritol Triacrylate

PTMC crosslinks upon gamma irradiation, but to reach high gel contents polymers of very high molecular weight are required. In case of the highest molecular weight PTMC polymer we synthesized (443 kg/mol), gamma irradiation at 100 kGy resulted in a network with a gel content of 73 %. Even theoretically, our estimations show that a maximum gel percentage of 77 % can be expected (Figures 1A, 2B and Equation 5).

In general, the gel percentage of networks formed can be increased by irradiation in the presence of a crosslinking aid that reduces the ratio of chain scission to crosslinking events. To enhance network formation of PTMC upon gamma irradiation, we used pentaerythritol triacrylate (PETA) as a crosslinking aid. Gamma irradiation of PETA at 25 kGy yielded gel percentages of 99.8±0.1 % upon.

Figures 3A and 3B show the gel contents and the equilibrium swelling ratios of networks prepared by gamma-irradiating PTMC of different initial molecular weights containing different amounts of PETA (0, 1, 5 wt %) at 25 kGy. The effect of the incorporation of PETA can be clearly seen: The resulting PTMC networks had greatly increased gel contents and decreased swelling ratios. The effect was most pronounced when PETA was added to PTMC of relatively lower molecular weight. For PTMC₈₈ that contained 0, 1, and 5 wt % PETA, the gel content of the networks after irradiation at 25 kGy increased from 0 % to 31±1 % and 73±1 %, respectively. For PTMC₄₄₃ the respective values were 60±1 %, 76±1 %, and 96±1 %. Incorporation of PETA also resulted in more densely crosslinked PTMC networks. After irradiating PTMC₄₄₃ at 25 kGy the swelling ratios of networks were respectively 45±1, 22±1, and 4.6±0.2 for films that contained 0, 1, and 5 wt % PETA.

For the range of PTMC polymer molecular weights investigated, the gel contents of networks formed upon irradiation at 25 kGy ranged from 31±1 to 76±1 % and from 73±1 to 96±1 % for films that contained 1 and 5 % PETA respectively. The corresponding swelling ratios in chloroform ranged from 75±1 to 22±1 and from 7.1±0.1 to 4.6±0.2. This shows that by incorporating PETA, the extent of PTMC crosslinking by gamma irradiation was greatly

enhanced. The gel content and network density of the networks could readily be varied by adjusting the PETA content.

Figure 3. The effect of incorporating PETA on gel content (A) and equilibrium swelling ratio in chloroform (B) of PTMC networks formed upon irradiation of PTMC polymers of different initial molecular weights at 25 kGy.

Irradiating PTMC films containing 5 wt % PETA at higher irradiation doses of 50 or 100 kGy, led to lower gel contents of the formed networks. The gel content of networks formed using PTMC₄₄₃ decreased from 96 % to 87 % upon increasing the irradiation dose from 25 kGy to 100 kGy. It is likely that already at 25 kGy all PETA had been consumed and no further enhancement of the crosslinking efficiency can be expected. Indeed, NMR spectra of extracts of PETA-containing gamma irradiated PTMC films did not reveal the presence of compounds containing unreacted acrylate groups.

The swelling ratios of the different PTMC networks prepared by gamma irradiation in the presence of 5 wt % PETA ranged from 4.6 to 7.1. These values are comparable to those of the previously mentioned photocrosslinked networks prepared from three-armed PTMC macromers with molecular weights of 1500 and 17200 g/mol, which had swelling ratios of 3.3 ± 0.4 and 9.8 ± 0.4 , respectively. As a crude approximation, the molecular weight between crosslinks of networks prepared by irradiating PTMC in the presence of 5 wt % PETA can be expected to range from approximately 1500 to 3600 g/mol.

Thermal and Mechanical Properties of Gamma Irradiated PTMC Networks

All PTMC polymers were amorphous with glass transition temperatures (T_g) below room temperature. The T_g s of compression moulded, non-irradiated PTMC films were independent of polymer molecular weight and varied from -17.7 to -18.5 °C. When PETA

was not added to the polymer, gamma irradiation at 25 kGy did not have a significant effect on the T_g s of the films. Incorporation of 5wt % PETA did increase the glass transition temperature of gamma irradiated PTMC films. This could be due to denser network formation, as shown earlier. Before extraction, the T_g values of these films were between -16.2 and -16.7 °C, while the values were somewhat higher (-14.7 to 15.1 °C) after extraction with ethanol. Thermal analysis of the films only showed a single T_g in the investigated temperature range (-50 to 200 °C), indicating that PETA was homogeneously incorporated into the PTMC networks. Homogeneous mixing of PTMC and PETA can be expected as their solubility parameters determined using group contributions³⁰ are very close, 21.5 and 21.2 $J^{1/2}/cm^{3/2}$, respectively.

Figure 4. Effect of initial molecular weight, irradiation dose, and PETA content on elastic modulus (A), yield strength (B), stress at break (C), and permanent set (D) values of PTMC films.

The effect of initial molecular weight and network formation on elastic modulus (E-modulus), yield strength and stress at break values of PTMC films are given in Figures 4A, 4B and 4C, while representative stress-strain curves of PTMC₈₈ and PTMC₄₄₃ are given in Figures 5A and 5B, respectively.

In the non-crosslinked state, E-modulus, yield strength and stress at break values increased with increasing polymer molecular weight. All PTMC films were flexible; E-modulus and yield strength values for PTMC₈₈ were 6.3 ± 0.2 and 1.2 ± 0.1 MPa, respectively, whereas for PTMC₄₄₃ these values were 7.2 ± 0.1 and 2.8 MPa, respectively (Figures 4A and 4B). The stress at break increased from 0.7 ± 0.1 MPa for PTMC₈₈ to 25.2 ± 3.7 MPa for PTMC₄₄₃. This dependence of mechanical properties on molecular weight of PTMC has been observed before ^{4, 5}. Upon gamma-crosslinking the different PTMC films at 25 kGy, the E-modulus and yield strength values decreased to values ranging from 5.5 ± 0.1 to 6.7 ± 0.2 MPa and 0.9 ± 0.1 to 2.0 ± 0.1 MPa, respectively (Figures 4A and 4B). Similarly, the stress at break of these non-extracted networks were lower (values ranging from 0.7 ± 0.1 to 14 ± 0.4 MPa) than those of non-crosslinked films (Figure 4C and also Figure 5A, 5B). These values are close to those of silicone elastomer films which had an E-modulus of 2.6 ± 0.2 MPa and a stress at break tensile strength 7.3 ± 0.5 MPa.

Figure 5. Effect of gamma irradiation, PETA content, and ethanol extraction on stress-strain behaviour of PTMC₈₈ (A) and PTMC₄₄₃ (B) (curves are offset for clarity).

Increasing the irradiation dose further decreased the E-modulus and yield strength of the PTMC films. At 100 kGy, the E-modulus and yield strength of the films ranged from 2.9 ± 0.2 to 4.6 ± 0.2 MPa and from 0.5 to 1.2 MPa, respectively. The stress at break values were also lower ranging from 0.5 ± 0.1 to 2.7 ± 0.2 MPa. Although increasing the irradiation dose yields higher gel content and network density, it also leads to lower strength and

stiffness. This is likely due to the chain scission that simultaneously occurs with crosslinking during gamma-irradiation ^{4, 5}.

By incorporating PETA into the network structure, the decrease in E-modulus and stress at break can be prevented. In Figures 4 and 5 it can also be seen that all gamma irradiated (non-extracted) PTMC networks containing 5 wt % PETA were still very flexible and rubber-like. The E-modulus and yield strength values ranged from 9.5 ± 0.6 to 10.7 ± 0.2 MPa (Figure 4A) and from 3.1 ± 0.1 to 4.9 ± 0.1 (Figure 4B), respectively. Large increases in the stress at break of PTMC networks were observed upon irradiation in the presence of PETA. The stress at break of PTMC₁₅₇ increased very significantly from 1.4 ± 0.1 MPa to 25.0 ± 1.8 MPa upon irradiation at 25 kGy in the presence of 5 wt% PETA (Figure 4C). Films of PTMC₄₄₃ gamma irradiated in the presence of 5 wt % PETA had an exceptionally high maximum tensile strength of 35.3 ± 0.3 MPa. This allows its utilization in applications where high strengths are required, such as in the tissue engineering of ligaments ³¹.

Extraction of the sol fraction of PTMC networks prepared in the presence of 5 wt % PETA with ethanol further improved the maximum tensile strength. The stress at break of PTMC₈₈ increased from 5.5 ± 0.9 to 12.4 ± 2.9 MPa (Figures 4C and 5A) and that of PTMC₄₄₃ to 37.7 ± 1.3 MPa (Figure 4C and 5B). The E-modulus and yield strength values of extracted networks were comparable to those of non-extracted ones.

The creep resistance of PTMC films was evaluated in cyclic deformation experiments, as described in the experimental part. Figure 4D shows that the permanent set of non-crosslinked PTMC films is mainly determined by the polymer molecular weight and varied from 11.2 % to 1.2 %. The highest molecular weight polymers are the most resistant to creep. Gamma irradiation at 25 kGy lowered the permanent set of all polymers. Irradiated non-extracted films of PTMC₄₄₃, and PTMC₂₇₇ had permanent set values which were the same as that of silicone rubber (1.0 % strain), showing the excellent creep resistance of these biodegradable elastomers. When PTMC₈₈ was irradiated at 25kGy, the non-extracted films also had a much lower permanent set (3.2 %) than that of non-irradiated films (11.2 %), even though no gel formation was observed.

Upon gamma irradiation at 25 kGy in the presence of 5 wt % PETA, the permanent set values of non-extracted PTMC₈₈ and PTMC₁₅₇ further decreased to 1.4 and 1.6 % strain, respectively. This implies that even in the case of PTMC polymers of relatively low initial molecular weights, networks with excellent creep resistance can be obtained by gamma

irradiation. Upon extraction with ethanol, the permanent set of all PETA-containing networks was even lower (0.9-1.2 %).

The effect of gamma irradiation on the tear propagation resistance of PTMC₄₄₃ was evaluated. Non-irradiated PTMC₄₄₃ films had a maximum tear strength of 1.9 ± 0.2 N/mm, a value already was much higher than that of silicone elastomer films (0.21 ± 0.01 N/mm). Upon gamma irradiation in the presence of 5 wt % PETA, the tear resistance of the PTMC networks significantly increased to values of 4.2 ± 0.2 N/mm. After extraction with ethanol, the tear strength increased even further to 9.3 ± 2.0 N/mm.

Viability of Cells in Contact with Gamma Irradiated PTMC Networks

Previously we have shown that non-irradiated and gamma irradiated PTMC films are non-cytotoxic⁵. Here, we assessed possible cytotoxicity of (non-extracted) PTMC films gamma irradiated at 25 kGy in the presence of 5 wt % PETA by a direct and an indirect cell viability assay (Figure 6). In both assays, no adverse effect on the morphology of the cells was observed. When fibroblasts were in direct contact with the films, cell viability values were very high with values ranging from 87 ± 16 to 117 ± 26 %. Also in the indirect assay, cell viabilities were very high with values ranging from 91 ± 8 to 100 ± 7 %. It is therefore confirmed that crosslinking of PTMC in the presence of 5 wt % PETA did not have an adverse effect on cell viability.

Figure 6. The viability of fibroblasts cultured in direct- or indirect contact with PTMC networks. The networks were prepared by irradiating films of PTMC of different molecular weights in the presence of 5 wt % PETA. The first two bars indicate cell viability of the blank (culture medium only) and positive controls, respectively.

In vitro Enzymatic Erosion of Gamma Irradiated PTMC Networks

The *in vitro* enzymatic erosion of non-extracted PTMC₄₄₃ films irradiated at 25 kGy was investigated using cholesterol esterase (CE), as this enzyme probably plays an important role in the erosion of PTMC³². Figures 7A, 7B and 7C show the changes in mass and thickness of films incubated in aqueous CE solutions as a function of time. Irradiated PTMC films containing 5 wt % PETA eroded much slower than those that did not contain PETA. After three weeks the mass loss of films that did not contain PETA was approximately 64 %, whereas films containing 5 % PETA had lost approximately 13 % of their mass. The decrease in thickness of 5 wt % PETA containing films was also much lower than that of the films that did not contain PETA (Figure 7B). The corresponding erosion rates were respectively 12.0 ± 2.9 $\mu\text{m/day}$ and 3.0 ± 1.6 $\mu\text{m/day}$. The first value is comparable to our previously reported results (13.7 ± 3.3 $\mu\text{m/day}$,³²). For both polymer films, the decrease in mass occurred simultaneously with a decrease in thickness (Figure 7C), which implies a surface erosion process. Figure 7C shows that the decrease in thickness seems to be somewhat lower than the decrease in mass, especially at the later time points. This can be the result of errors in determining the thickness of the films due to roughening of the surface during degradation.

These results show that crosslinking of PTMC in the presence of PETA is an effective manner of reducing the erosion rate of PTMC networks. This can be due to the higher gel contents and network densities of PTMC₄₄₃ films containing PETA.

Figure 7. Erosion of PTMC₄₄₃ films gamma-irradiated in the presence (5 wt %) and absence of PETA in aqueous solutions of cholesterol esterase (CE). The figure shows the relative thickness (A) and relative mass (B) in time and the relationship between the relative thickness and relative mass (C).

Conclusions

Flexible PTMC networks with high gel contents and high network densities can be obtained by gamma irradiation of compression moulded PTMC films in the presence of pentaerythritol triacrylate (PETA). The incorporation of this crosslinking aid also leads to networks with high tensile strengths and excellent tear- and creep resistance. Moreover, the enzymatic erosion rates of PTMC networks can be decreased in this manner. Based on direct and indirect assays, these networks do not have any adverse effect on the viability of fibroblasts. It can therefore be concluded, that gamma irradiation in the presence of PETA is a very effective method to obtain biodegradable PTMC networks with elastomeric properties. These rubber-like biodegradable PTMC networks are potentially well-suited for medical applications such as the engineering of soft- and cardiovascular tissues, and controlled release.

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Chapter 8

Crosslinking of Trimethylene Carbonate and D,L-lactide (co)Polymers by Gamma Irradiation in the Presence of Pentaerythritol Triacrylate

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Abstract

High molecular weight (co)polymers of trimethylene carbonate and D,L-lactide have been crosslinked very efficiently using pentaerythritol triacrylate (PETA) as a crosslinking agent/aid during gamma irradiation. Upon irradiation at 25 kGy, form-stable networks with gel contents ranging from 86±5 to 96±1 were obtained from non-crystalline (co)polymers that otherwise either degrade or crosslink inefficiently during gamma irradiation. Glass transition temperatures and elastic modulus values of the (co)polymer networks could be varied by adjusting the copolymer composition. Based on direct- and indirect cell viability assays, the PETA-containing (co)polymer networks were found to be non-cytotoxic. Upon incubation in macrophage cultures for 14 days, all (co)polymer films and PETA-containing network films had eroded to varying extents showing that these materials can be degraded by cell-mediated erosion processes. This crosslinking method is very useful for the facile preparation of TMC and DLLA containing form-stable networks from high molecular weight polymers.

Furthermore, it can prevent damage to these polymers during sterilization by gamma irradiation.

Introduction

Gamma irradiation is a widely used cost-effective method for the sterilisation of (polymeric) biomaterials ^{1, 2}. The high energy gamma rays can initiate free radical- or ionic reactions, which result in changes in the surface- and bulk properties of the materials being sterilised ^{1, 3, 4}. For some polymers crosslinking is observed during gamma irradiation ^{3, 5-9}, whereas for others the irradiation leads to chain scission or unzipping reactions and a decrease in molecular weight ¹⁰⁻¹⁵. These decreased molecular weights can limit the useful life-time of resorbable polymeric implants like tissue engineering scaffolds, fracture fixation devices or sutures, due to rapid decrease in mechanical strength and disintegration of the device ^{10, 16}. Also upon implantation of drug releasing matrices prepared from biodegradable polymers, the lowering of the molecular weight can lead to early onset of mass loss and influence drug release characteristics ^{14, 15, 17}.

Poly(D,L-lactide) (PDLLA) and poly(trimethylene carbonate) (PTMC) are well known biocompatible and biodegradable polymers used in the preparation of resorbable sutures, controlled drug release systems, and tissue engineering scaffolds. PDLLA is a glassy, non-crystalline aliphatic polyester that degrades by bulk hydrolysis *in vivo* ¹⁸. Upon gamma irradiation, a reduction in molecular weight of PDLLA is observed ¹³⁻¹⁵. PTMC is a flexible, rubbery polymer that degrades by surface erosion *in vivo* ¹⁹. Unlike PDLLA, high molecular weight PTMC crosslinks upon gamma irradiation, forming networks with gel contents of up to 70 % ^{5, 7}. While at room temperature the non-crosslinked PTMC polymer tends to creep, owing to its amorphous nature and its low glass transition temperature ($T_g = -17\text{ }^{\circ}\text{C}$), network formation significantly improves the form-stability and elastomeric behaviour of PTMC.

To adjust the physical properties and the degradation behaviour of PTMC and PDLLA, copolymers of TMC and DLLA have been prepared ²⁰⁻²⁵. (Co)polymers having a range of glass transition temperatures, elastic modulus values, tensile strengths and degradation rates were obtained. This makes these polymers suitable for numerous biomedical applications. However, non-crosslinked (co)polymers with glass transition temperatures below physiological temperatures have poor form stability ^{22, 24}. We observed, for example, that scaffolds prepared from these amorphous copolymers had significantly shrunk upon incubation in PBS at 37 °C ²². This is undesired as the characteristics of the porous structure

are different than those designed and fabricated. Furthermore, these properties will continue to change in time.

Being able to crosslink (co)polymers prepared from TMC and DLLA monomers by gamma irradiation would be highly advantageous, as it would allow the facile preparation of form stable and creep resistant materials with low glass transition temperatures and tuneable degradation properties. Upon irradiation at 25 kGy (a commonly used sterilization dose), the average molecular weight of a TMC and DLLA copolymer containing 19 % TMC decreased from 220 kg/mol to 60 kg/mol ²³. And, just like in the case of PDLLA, no network formation could be observed.

Recently, we have shown that the efficiency of gamma irradiation crosslinking of high molecular weight PTMC can be significantly increased by using pentaerythritol triacrylate (PETA) as a crosslinking aid ²⁶. Therefore, in this study, we investigated the potential of crosslinking TMC and DLLA (co)polymer films containing pentaerythritol triacrylate as a crosslinking aid/agent by gamma irradiation.

Materials and Methods

Materials

Polymer grade 1,3-trimethylene carbonate (TMC, Boehringer Ingelheim, Germany), polymer grade D,L-lactide (DLLA, Purac Biochem, The Netherlands), stannous octoate (Sigma, U.S.A.), and pentaerythritol triacrylate (PETA, Aldrich, U.S.A.) were used as received. Solvents (Merck, Germany or Biosolve, The Netherlands) were of analytical grade. J774A macrophages (ATCC-TIB-67) were obtained from the American Type Culture Collection. NIH 3T3 mouse skin fibroblasts were used for *in vitro* cell viability assays. Culture media, fetal bovine serum, Glutamax™ and penicillin-streptomycin were obtained from Invitrogen (Gibco, U.S.A.). Culture disposables were from Nunc (U.S.A.) and Greiner (Germany).

Polymer Synthesis

Poly(1,3-trimethylene carbonate), poly(D,L-lactide) homopolymers and copolymers of TMC with DLLA were synthesized by ring opening polymerization of the corresponding monomers (50, 60, and 70 mol % TMC) under vacuum at 130 °C for three days using stannous octoate as catalyst. The polymers were purified by dissolution in chloroform and precipitation into ethanol or isopropanol, washing with fresh alcohol and drying at room

temperature under vacuum. (Co)polymers with DLLA were further dried at 80 °C under nitrogen until constant weight.

Polymer Characterisation

Monomer conversion and copolymer compositions were determined by proton nuclear magnetic resonance (^1H -NMR) spectroscopy (300 MHz, Varian Innova, U.S.A.) using CDCl_3 (Merck, Germany).

Number average- and weight average molecular weights ($\overline{M}_n$ and $\overline{M}_w$, respectively), polydispersity indices (PDI) and intrinsic viscosities ($[\eta]$) of the (co)polymers were determined by gel permeation chromatography (GPC, Viscotek U.S.A.). The setup was equipped with ViscoGEL I-guard-0478, ViscoGEL I-MBHMW-3078, and ViscoGEL I-MBLMW-3078 columns placed in series and a TDA 302 Triple Detector Array with refractometer-, viscometer-, and light scattering detectors, allowing the determination of absolute molecular weights. All determinations were performed at 30 °C, using chloroform as the eluent at a flow rate of 1.0 ml/min.

Preparation of Polymer Films

Purified polymers were compression moulded at 140 °C in 500 micrometer thick stainless steel moulds using a laboratory press (Fonteijne THB008, The Netherlands). The films were moulded at approximately 25 kg/cm² and quenched to room temperature using cold water. For the crosslinking experiments of polymer films containing PETA (see below), purified polymers and PETA (5 wt % of the polymer) were dissolved in dichloromethane to achieve homogeneous mixing. After evaporation of the solvent compression moulded films were prepared in the same way as the purified polymers.

Gamma Irradiation, Network Formation and Network Characterization

The compression moulded films were sealed under vacuum in laminated polyethylene/polyamide bags (Hevel Vacuum B.V., The Netherlands) and exposed to 25 kGy gamma irradiation from a ^{60}Co source (Isotron B.V., Ede, The Netherlands).

To determine equilibrium swelling ratios and gel contents, disk-shaped specimens (500 μm thick, 10 mm in diameter) were punched out from the irradiated films and placed in 30 mL CHCl_3 for 1 week, the solvent was refreshed once after 3 days. This procedure ensured complete removal of the sol fraction. Then the swollen gels were weighed, dried to

constant weight at room temperature *in vacuo* and weighed again. The gel and the sol fractions were calculated according to equations (1) and (2) respectively:

$$\text{Gel fraction (\%)} = \frac{m_d}{m_0} \times 100 \quad (1)$$

$$\text{Sol fraction (\%)} = \left(1 - \frac{m_d}{m_0} \right) \times 100 \quad (2)$$

where m_d is the mass of dried (extracted) samples and m_0 is the mass of the specimens before swelling. The volume degree of swelling (q) was calculated according to equation (3):

$$q = 1 + \rho_p \times \left(\frac{m_s}{m_d \times \rho_s} - \frac{1}{\rho_s} \right) \quad (3)$$

where m_s is the mass of the extracted and swollen samples and ρ_s and ρ_p are the densities of chloroform (1.48 g/cm³) and the (co)polymers respectively. The densities of the copolymers were determined by measuring the mass and dimensions of compression moulded films. The densities of TMC and DLLA copolymers containing 100 %, 72 %, 60 %, 48 %, and 0 % TMC were 1.31, 1.29, 1.27, 1.26, and 1.25 g/cm³, respectively.

Thermal and Mechanical Properties

The mechanical properties of melt pressed and irradiated (0 kGy, 25 kGy) (co)polymers containing PETA (0 wt %, 5 wt %) were determined in triplicate according to ASTM-D 882-91. The measurements were performed on extracted as well as on non-extracted specimens that measured 0.5×10×0.05 cm³. Extraction of the leachable components was performed with ethanol. A Zwick Z020 tensile tester (Ulm, Germany) equipped with a 500 N load cell was operated at a crosshead speed of 500 mm/min. The initial grip to grip separation was 50 mm and a preload of 0.01 N was applied. The specimen deformation was derived from the grip-to-grip separation; therefore the presented values of the elastic modulus (calculated from the initial slope of the stress-strain curves) give only an indication of the stiffness of the polymers.

The tear strength of films was determined according to ASTM 1938 using trouser shaped specimens (n=3). The dimensions of the specimens were 75×25×0.5 mm with a 50 mm long cut halfway through the width of the specimens. Measurements were performed in

triplicate at a speed of 250 mm/min. The reported maximum tear strengths are normalized to the thickness of the specimens and expressed in N/mm.

Glass transition temperatures (T_g) of purified polymers and irradiated polymer films were determined by differential scanning calorimetry (DSC). Samples (5-10 mg) were analyzed at a heating rate of 10 °C/min in a temperature range of -100 to 200 °C using a PerkinElmer Pyris 1 DSC. After the first scan, samples were quenched to -100 °C at 300 °C/min and a second scan was recorded after 5 minutes. The reported values were determined from the second heating scan. Indium, lead, and cyclohexane were used as standards for temperature calibration.

Cell viability assay

Possible cytotoxicities of gamma irradiated, non-extracted (co)polymer films containing 5 wt % PETA were evaluated using a direct and an indirect MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay²⁷. Briefly, mouse skin fibroblasts were cultured using a 96 wells plate (5000 cells/well) and Dulbecco's modified Eagle's medium (DMEM). The cells were incubated at 37 °C and 5 % CO₂ for three days.

For indirect cytotoxicity evaluations, non-extracted disks (n=3, diameter 8 mm, thickness 500 µm) of each film were incubated at 37 °C and 5 % CO₂ with 500 µl DMEM for 24 hours to extract any leachable components. After the three day period of cell culturing, the medium was replaced by the leachables-containing DMEM medium. In the direct cytotoxicity assays, non-extracted discs (n=3) were placed directly on the cells in culture. In both assays, the absorbance of formazan, which is soluble in the culture medium, was measured after another two days of culture. The mean value obtained for cell cultures incubated with DMEM medium only (negative control) was standardized as 100 % cell viability. Latex rubber (Hilversum Rubber Factory, Hilversum, The Netherlands) was used as positive cytotoxic control.

Macrophage-Mediated Erosion Studies

J774A macrophages were maintained in DMEM containing 4.5 g/L D-glucose, pyruvate, 10 % fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2mM Glutamax™. Cells were passaged every 4 to 7 days by scraping.

Macrophage-mediated erosion of non-irradiated (co)polymer films that did not contain PETA as well as gamma irradiated, non-extracted (co)polymer films that contained 5 wt %

PETA was investigated by directly culturing J774A macrophages on the surfaces of the films²⁸. The test specimens were 15 mm in diameter and approximately 500 μm in thickness. The seeding density was approximately 8×10^4 cells/ cm^2 . Fresh aliquots of cells were added to each well at days 7 and 10, and the medium was exchanged three times a week. Cells were cultured on six disks of each material.

After 14 days of culturing, three specimens of each material were first placed in Milli-Q water to lyse the cells, and then thoroughly rinsed and weighed. After drying the films to constant weight *in vacuo* at room temperature, the samples were weighed again. The specimens were sputter-coated with gold and then their surfaces were analysed by scanning electron microscopy (SEM, Philips XL 30 ESEM-FEG, The Netherlands) at an operating voltage of 5 kV.

The remaining disks of each material ($n=3$) were fixed with 3.7 % para-formaldehyde in cytoskeletal stabilizing (CS) buffer (0.1 M piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer, 1 mM ethylene glycol tetraacetic acid (EGTA), pH=6.9) for 15 min and then transferred to PBS. The specimens were used for fluorescence staining of cell nuclei using 4',6-diamidino-2-phenylindole (DAPI) and of the actin cytoskeleton using tetramethylrhodamine iso-thiocyanide-phalloidin (TRITC-phalloidin). These specimens were then analysed by confocal laser scanning microscopy (LEICA TCS SP2, with a fully water-immersed 40x objective (NA 0.80)).

Results and Discussion

High molecular weight copolymers of trimethylene carbonate (TMC) and D,L-lactide (DLLA), and PTMC and PDLLA homopolymers were synthesized by ring opening polymerization. The conversion of monomers was higher than 95 %, and (co)polymers having monomer compositions close to the charged compositions were obtained after purification by precipitation (Table 1). After compression moulding the molecular weights of the (co)polymers remained very high, although the molecular weights of (co)polymers with high DLLA contents had decreased somewhat upon compression moulding. Polymers with high molecular weights are desired, as it was shown that the efficiency of crosslinking by gamma irradiation increased with increasing polymer molecular weight²⁶. The synthesized TMC containing (co)polymers are rubbery at physiological temperatures, as their glass transition temperatures ranged from -17.9 to 16.5 $^{\circ}\text{C}$.

Table 1. Characteristics of the synthesized and purified *P*(TMC-DLLA) (co)polymers before- and after compression moulding. Values given between brackets are for compression moulded specimens.

TMC content ^a (mol %)	DLLA content ^a (%)	$\overline{M}_n$ ^b (kg/mol)	$\overline{M}_w$ ^b (kg/mol)	PDI ^b	$[\eta]$ ^b (dl/g)	T _g ^c (°C)
100 ^d	0	443 (436)	620 (698)	1.5 (1.6)	6.6 (7.1)	-17.9
72	28	399 (385)	624 (586)	1.6 (1.5)	5.0 (4.6)	0.1
60	40	404 (384)	633 (593)	1.6 (1.5)	4.7 (4.4)	10.4
48	52	285 (232)	429 (361)	1.5 (1.6)	2.8 (2.8)	16.5
0	100	283 (208)	375 (336)	1.3 (1.6)	3.2 (2.8)	52.1

^a Determined by ¹H-NMR on specimens purified by precipitation.^b Determined by GPC at 30 °C using chloroform as the eluent.^c Determined by DSC(second heating scan) using compression moulded films.^d Reference ²⁶.**Crosslinking of *P*(TMC-DLLA) (co)Polymers by Gamma Irradiation**

The crosslinking behaviour of compression moulded (co)polymer films by gamma irradiation under vacuum was first investigated in the absence of PETA. The gel contents and swelling ratios of the resulting (co)polymer networks are given in Figure 1. Upon irradiation at 25 kGy, the 48 % TMC-containing copolymer and PDLLA did not crosslink. This is in agreement with previous studies ^{13-15, 22}. In the case of PDLLA, $\overline{M}_n$, $\overline{M}_w$, values had decreased to 78 kg/mol and 117 kg/mol, respectively, whereas for the copolymer the respective values were 60 kg/mol and 111 kg/mol. The copolymers with higher TMC contents (60 % and 72 %), however, could be crosslinked by gamma irradiation to yield networks with 12±2 %, and 30±1 % gel contents. The very high swelling ratios in chloroform indicate the low crosslink densities of these copolymer networks. (The swelling ratios of 60 % TMC-containing networks could not be determined due the weakness of these highly swollen gels). Gamma irradiated PTMC films had the highest gel content of 60±1 %.

Figure 1 also clearly shows that incorporation of a small amount of PETA into the (co)polymer films significantly increased the gel percentage of the formed networks, likely by increasing the ratio of crosslinking- to chain scission events that occur during the gamma irradiation process. At 25 kGy, transparent and form-stable (co)polymer networks with high gel contents ranging from 86±5 to 96±1 % were obtained. Interestingly, even PDLLA could be crosslinked efficiently during gamma irradiation by using PETA as a crosslinking agent. The formed networks were densely crosslinked with swelling ratios in chloroform ranging from 4.6±0.2 to 11.0±2.5 vol/vol. This indicates the possibility of sterilizing these (co)polymers by gamma irradiation without significant chain scission and loss in mechanical properties.

Figure 1. Effect of the copolymer composition and the incorporation of PETA on the gel content (A) and swelling ratio (B) of networks formed by gamma irradiation (25 kGy) of P(TMC-DLLA) (co)polymer films.

To improve the thermal- and mechanical properties of aliphatic polyesters and polycarbonates, triallyl isocyanurate (TAIC) was mixed with these polymers in the melt to serve as a crosslinking agent upon high energy irradiation²⁹⁻³². Multi-acrylate based crosslinking agents were not efficient when mixed in the melt, as their use resulted in lower gel contents than when TAIC was used^{31, 32}. In biomedical applications, however, the use of TAIC might not be desired, as isocyanurate can decompose and form isocyanuric acid and allyl alcohol. In our experiments, we used a common solvent to mix PETA with the (co)polymers at ambient temperatures. This allowed us to obtain very high gel contents after evaporation of the solvent and gamma irradiation.

We also investigated the potential of PETA as a crosslinking aid in the network formation using (co)polymers of TMC and ϵ -caprolactone (five (co)polymers with ϵ -caprolactone contents of 41 to 100 mol %, all having $\overline{M}_n$ values above 200 kg/mol). PETA was also very effective in crosslinking these (co)polymers: At 25 kGy, networks with gel contents ranging from 88 to 93 % and swelling ratios ranging from 5.4 to 7.0 vol/vol were obtained with PETA contents of 5 wt %. This shows that this enhanced crosslinking method can be applied to other resorbable polymers as well.

Thermal- and Mechanical Properties of Gamma Irradiated P(TMC-DLLA) (co)Polymer Networks

In the non-crosslinked state, all P(TMC-DLLA) (co)polymers were amorphous (non-crystalline) materials with glass transition temperatures (T_g) ranging from -17.9 to 52.1 °C (Table 2). The T_g values decreased with increasing TMC content and could readily be tuned by adjusting the copolymer composition. Gamma irradiation in the absence of PETA seemed

to lower T_g values slightly, probably due to formation of relatively short chains by chain scission events. Upon irradiation in the presence of PETA, T_g values increased owing to the formation of a dense network (compare Figure 1B). The glass transition temperatures further increased upon removal of low molecular weight leachables from the networks using ethanol, and range from 14.9 to 53.7 °C. The PETA-containing networks showed a single T_g , indicating that the (co)polymers were homogeneously incorporated into the PETA network.

Table 2. Thermal- and mechanical properties of *P*(TMC-DLLA) (co)polymers before and after gamma irradiation in the absence or presence of PETA. Values are expressed as mean±standard deviation, ($n=3$).

TMC content (mol %)	Irradiation dose (kGy)	PETA content (wt %)	T_g (°C)	E (MPa)	σ_{yield} (MPa)	ϵ_{yield} (%)	σ_{break} (MPa)	ϵ_{break} (%)
100	0	0.0	-17.9	7.2±0.1	2.8±0.1	180±20	25.2±3.7	974±45
100	25	0.0	-18.1	6.7±0.2	2.0±0.1	190±15	14.0±0.4	1330±40
100	25	5.0	-16.4	10.7±0.2	4.9±0.1 ^a	110±10 ^a	35.3±0.3	580±30
100 ^b	25	5.0	-14.9	10.8±0.3	4.9±0.1 ^a	120±15 ^a	37.7±1.3	570±15
72	0	0.0	0.1	5.1±0.1	1.9±0.1	120±15	5.5±1.4	1323±108
72	25	0.0	-1.9	4.8±0.4	1.5±0.1	250±25	1.5±0.1	1330±174
72	25	5.0	1.8	32.4±0.5	2.1±0.1 ^a	25±10 ^a	12.5±1.2	523±18
72 ^b	25	5.0	3.2	35.6±0.2	2.2±0.1 ^a	25±10 ^a	15.4±0.8	588±36
60	0	0.0	10.4	14.5±1.1	2.0±0.1	130±15	21.8±2.0	1047±25
60	25	0.0	9.4	10.6±1.0	1.8±0.1 ^a	150±10 ^a	19.7±4.7	1384±28
60	25	5.0	11.7	34.8±2.8	2.5±0.1 ^a	40±10 ^a	20.2±2.0	593±36
60 ^b	25	5.0	13.1	40.2±2.8	2.4±0.1 ^a	40±10 ^a	18.5±1.3	535±24
48	0	0.0	16.5	195±17	2.0±0.1	55±15	13.4±1.7	804±100
48	25	0.0	13.8	61±11	1.6±0.3 ^a	100±25 ^a	6.2±1.6	733±111
48	25	5.0	20.0	630±120	5.8±1.1	4.2±2.7	18.8±3.6	378±111
48 ^b	25	5.0	21.9	936±88	10.3±1.2	3.6±2.1	23.8±2.6	419±26
0	0	0.0	52.1	2780±112	55.5±1.1	2.6±0.1	48.8±1.6	5.5±0.8
0	25	0.0	51.1	2610±101	49.9±1.4	2.4±0.1	42.2±1.3	5.9±1.6
0	25	5.0	52.6	2860±84	59.1±1.5	2.6±0.1	58.1±2.2	2.7±0.2
0 ^b	25	5.0	53.7	2970±117	59.9±2.7	2.5±0.1	59.3±2.2	2.6±0.2

^a Estimated from the intersection of tangents to stress-strain diagrams as a distinct yield point could not be observed.

^b Measurements were performed on gamma irradiated specimens after extraction of leachable components with ethanol.

Table 2 also gives an overview of the tensile properties of P(TMC-DLLA) (co)polymers and networks. It can be seen that by adjusting the copolymer composition, non-irradiated films having elastic modulus values ranging from 5.1 ± 0.1 to 2780 ± 112 MPa can be obtained. Upon gamma irradiation in the absence of PETA, the elastic modulus and stress at break values of the (co)polymer films decreased somewhat due to chain scission. The deterioration the mechanical properties of the (co)polymers during gamma irradiation could be prevented by irradiating in the presence of PETA. Network films with higher elastic modulus, yield strength, and stress at break values are obtained, probably due to the prevention of chain scission and dense network formation. For the same reasons, the elongation at break values and yield strain values were lower for PETA-containing networks than for irradiated films that did not contain PETA. It was confirmed, that after tensile testing, the combined lengths of the fractured specimens were essentially the same as that of the original crosslinked specimens. Considering their relatively low glass transition temperatures, these amorphous networks might be useful in minimally invasive surgery where use of materials with shape memory properties is advantageous³³.

Removal of the low molecular weight compounds that might have formed during gamma irradiation by extraction with ethanol further increased the elastic modulus and stress at break values of the network films. While retaining their high tensile strength, the elastic modulus values of PETA-containing gamma irradiated films were also found to depend on the copolymer composition. This indicates that the mechanical properties of these (co)polymer networks can be tailored to suit any intended application.

An important characteristic of the network films is their resistance to tearing, as sutured polymeric biomaterials usually experience dynamic environments *in vivo*. The determined maximum tear strength of linear PTMC films was 1.9 ± 0.2 N/mm. By gamma irradiation in the presence of PETA and by increasing the DLLA content of the copolymers, the tear propagation resistance of the formed networks could significantly be improved. The tear strengths of PETA-containing networks prepared from 100, 72, 60, and 48 % TMC containing (co)polymers were respectively 4.2 ± 0.2 , 10.6 ± 1.0 , 13.7 ± 1.2 and 38.9 ± 6.4 N/mm.

Compatibility of Gamma Irradiated (co)Polymer Network Films with Fibroblasts

We investigated the compatibility of PETA containing gamma irradiated non-extracted networks with fibroblasts using a direct and an indirect assay. Figure 2 shows the percentage of viable fibroblasts when incubated with different (co)polymer network films. For all (co)polymer networks the percentage of viable fibroblasts was comparable to that obtained

for negative controls ($100 \pm 13.5\%$). The cell viabilities in the direct assay ranged from 88 ± 18 to $108 \pm 1\%$ whereas in the indirect assay the values ranged from 90 ± 5 to 102 ± 10 . Furthermore, no abnormalities in the morphology of the cells were observed during culture. The results of both assays confirmed that these PETA-containing networks are not cytotoxic. In addition, these results showed that the solvents used for purification and for mixing PETA could be effectively removed from the polymer matrix.

Figure 2. The viability of fibroblasts cultured in direct- or indirect contact with non-extracted (co)polymer networks. The networks were prepared by irradiating films of (co)polymers in the presence of 5 wt % PETA at 25 kGy. The first two bars indicate the cell viability of the blanks (culture medium only, negative controls) and positive controls, respectively.

In Vitro Erosion of (co)Polymer Films and Networks in Macrophage Cultures

In vivo, macrophages play an important role in the tissue response to biomaterials and in their degradation by secreting numerous substances. Superoxide anion radicals that can be secreted by macrophages can degrade linear PDLLA polymer and PTMC networks^{28, 34, 35}. Cholesterol esterase, a hydrolytic enzyme that is also secreted by macrophages, has been shown to erode PTMC networks and P(TMC-DLLA) copolymers as well^{25, 28, 35}.

As an initial assessment of the effect of macrophages on their erosion behaviour *in vivo* and of their biocompatibility^{28, 36, 37}, we cultured macrophages directly on gamma irradiated and on non-irradiated (co)polymer films. The cells generally were present as isolated cells, without signs of foreign body giant cell formation or formation of cell aggregates. During 14 days of culturing no adverse effects on the viability of macrophages due to the P(TMC-DLLA) (co)polymer networks or their degradation products were observed. In addition, the macrophages were not activated during culturing on these surfaces as their morphology was still spherical after 14 days of culturing. This suggests that these PETA-containing P(TMC-DLLA) networks induce a mild macrophage response.

We investigated the effect of copolymer composition and of crosslinking in the presence of PETA on the macrophage-mediated erosion of the films. Figure 3 shows the surfaces of (co)polymer films after 14 days of macrophage culturing; the corresponding erosion rates are given in Table 3. It can be seen from Figure 3 that all non-irradiated (co)polymer films had eroded during macrophage culturing. With decreasing TMC content, the extent of surface erosion seemed to decrease, this observation can be corroborated with the mass loss and erosion rate values given in Table 3. The mass loss values in 14 days and the erosion rates ranged from 5.35 ± 0.49 to 0 % and from 276 ± 23 to 0 $\mu\text{g}/(\text{cm}^2 \times \text{day})$, respectively. This shows that copolymers containing higher amounts of TMC are most prone to surface erosion by macrophage activity. This could be due to the differences in chemical structure or due to their lower glass transition temperatures. Interestingly, even on PDLLA films signs of erosion were seen. Apparently, macrophage-derived compounds are able to degrade PDLLA to some extent. (Although mass loss could not be detected, signs of erosion were not detected on the sides of the films that were not in contact with the cells).

Table 3. Effect of (co)polymer composition on the surface erosion rates and mass loss of non-irradiated P(TMC-DLLA) (co)polymer films and of P(TMC-DLLA) (co)polymer films containing 5 % PETA gamma irradiated at 25 kGy after 14 days of macrophage culture. Values are expressed as mean $\pm$ standard deviation ($n=3$).

TMC content (mol %)	DLLA content (mol%)	0 kGy, 0 % PETA		25 kGy, 5 % PETA	
		Erosion rate ($\mu\text{g}/(\text{cm}^2 \times \text{day})$)	Mass loss (wt %)	Erosion rate ($\mu\text{g}/(\text{cm}^2 \times \text{day})$)	Mass loss (wt %)
100	0	276 ± 23	5.35 ± 0.49	31 ± 12	0.60 ± 0.20
72	28	74 ± 2	1.46 ± 0.04	13 ± 2	0.26 ± 0.04
60	40	14 ± 2	0.28 ± 0.05	12 ± 1	0.25 ± 0.01
48	52	18 ± 12	0.40 ± 0.26	- ^a	- ^a
0	100	- ^a	- ^a	- ^a	- ^a

^a Mass loss could not be detected.

By irradiating (co)polymer films to which 5 wt% PETA was added, the erosion rates of (co)polymer films could be reduced significantly. For the films prepared from PTMC homopolymer, the erosion rate decreased from 276 ± 23 to 31 ± 12 $\mu\text{g}/(\text{cm}^2 \times \text{day})$ (see also Figures 3A and 3B). This is probably due to the formation of a dense network.

Figure 3. SEM micrographs showing the macrophage-mediated erosion of non-irradiated *P*(TMC-DLLA) (co)polymer films (A,C,E,G,I), and non-extracted network films prepared by gamma irradiating (co)polymer films containing 5 % PETA (B,D,F,H,J) after 14 days of culture.

The erosion rate of gamma irradiated PTMC that did not contain PETA has a relatively low crosslink density (Figure 1B) and showed erosion rates of $284 \pm 34 \mu\text{g}/(\text{cm}^2 \times \text{day})$, this is comparable to the erosion rates of non-irradiated PTMC shown in Table 3. Also upon subcutaneous implantation in rats, we have shown that *in vivo* erosion rates of gamma irradiated PTMC network films are comparable to those of non-irradiated linear PTMC homopolymer³⁸. This similarity indicates that the macrophage culturing assay is a suitable model for the initial assessment of the effect of structural variables on the *in vivo* erosion behavior of degradable polymeric biomaterials. Erosion was also observed for copolymer network films containing 72 % and 60 % TMC (Figures 3D and 3F), while for 48 % and 0 % TMC-containing (co)polymer networks no mass loss could be detected. Only few eroded spots were observed on these network films (Figure 3H and 3J).

Figure 4. Density of macrophages on different non-irradiated P(TMC-DLLA) (co)polymer films (A), and on non-extracted network films prepared by gamma irradiating (co)polymer films containing 5 % PETA (B) after 14 days of culture.

The differences in erosion behaviour of different materials could also be related to the number of macrophages present on the surfaces of the films. Macrophage densities on non-irradiated and gamma irradiated P(TMC-DLLA) films are presented in Figure 4. For the non-irradiated copolymer films, the cell numbers were quite similar and ranged from 3537 ± 137 to $4770 \pm 612 \text{ cells/mm}^2$. Only the 72 % TMC containing copolymer film had somewhat higher cells numbers ($6010 \pm 405 \text{ cells/mm}^2$). This similarity is reasonable, as these (co)polymers are all hydrophobic materials with contact angles that do not differ much with composition²³. In the case of gamma irradiated networks, the values were even more comparable, and ranged from 3317 ± 815 to $4254 \pm 215 \text{ cells/mm}^2$. The cell numbers were not much affected by the crosslinking process. These results suggests that structural effects like polymer composition,

glass transition temperature, and crosslinking density are the decisive factors in determining the macrophage-mediated erosion of these materials.

Copolymers of TMC and DLLA, and PDLLA homopolymers also degrade by non-enzymatic bulk hydrolysis, and the effect of copolymer composition on their degradation behaviour has been investigated previously ^{21, 24}. Although we did not perform non-enzymatic hydrolytic degradation experiments on networks prepared by irradiating TMC and DLLA (co)polymer films containing 5 wt% PETA, other experiments have shown that the onset of mass loss of photocrosslinked acrylate-functionalized DLLA oligomers is delayed when compared to linear high molecular weight PDLLA ³⁹. We can therefore expect that the non-enzymatic hydrolysis of our (co)polymer networks will also be slower than that of the linear (co)polymers.

Conclusions

Pentaerythritol triacrylate can be used as a crosslinking agent/aid to efficiently crosslink P(TMC-DLLA) (co)polymers by gamma irradiation. By adjusting the copolymer composition, the thermal- and mechanical properties of the networks can readily be tuned. *In vitro* assays showed that these PETA-containing (co)polymer networks and their degradation products are compatible with cells. The macrophage-mediated erosion of the (co)polymer films could significantly be reduced by increasing the DLLA contents of the (co)polymers. Erosion was also much reduced by the crosslinking process. This crosslinking method can be used to minimize damage during sterilization of TMC and DLLA-based materials by gamma irradiation and it allows for the preparation of polymeric materials with widely tuneable mechanical properties and erosion rates.

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Chapter 9

Ultraviolet Light Crosslinking of Poly(trimethylene carbonate) and its Application in Preparing Elastomeric Tissue Engineering Scaffolds^{*}

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Abstract

A practical method of photocrosslinking high molecular weight poly(trimethylene carbonate) (PTMC) is presented. Flexible, elastomeric and biodegradable networks could be readily prepared by UV irradiating PTMC films containing pentaerythritol triacrylate (PETA) and a photoinitiator. The network characteristics, mechanical properties, wettability, and in vitro enzymatic erosion of the photocrosslinked PTMC films were investigated. Densely crosslinked networks with gel contents up to 98 % could be obtained in this manner. Upon photocrosslinking, flexible and tough networks with excellent elastomeric properties were obtained. To illustrate the ease with which the properties of the networks can be tailored,

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blends of PTMC with mPEG-PTMC or with PTMC-PCL-PTMC were also photocrosslinked. The wettability and the enzymatic erosion rate of the networks could be tuned by blending with block copolymers. Tissue engineering scaffolds were also fabricated using these flexible photocrosslinkable materials. After crosslinking, the fabricated PTMC-based scaffolds showed interconnected pores and extensive microporosity. Human mesenchymal stem cell (hMSC) culturing studies showed that the photocrosslinked scaffolds prepared from PTMC and PTMC/PTMC-PCL-PTMC blends are well-suited for tissue engineering applications.

Introduction

The physical, chemical and biological properties of scaffold materials are very important in tissue engineering. For instance, cell behaviour is influenced by the rigidity of the substrate on which the cells are cultured^{1, 2}. In soft tissue engineering, the scaffold should transmit mechanical stimuli to the cells and tissues, and withstand repeated dynamic loadings *in vivo*. Also *in vitro* this can be required, as mechanical stimulation of cell-seeded tissue engineering scaffolds during cell culture enhances the development of engineered tissues and their function^{3, 4}. In this respect, flexible, form-stable and resorbable elastomeric polymer networks that allow cell adhesion and proliferation are of great interest⁵⁻¹¹.

Photoinduced polymerization and crosslinking has been used to prepare resorbable polymer networks efficiently at ambient temperatures¹²⁻¹⁷. To prepare elastomeric resorbable networks, amorphous oligomers with low glass transition temperatures (T_g s) based on trimethylene carbonate (TMC), D,L-lactide (DLLA), and ϵ -caprolactone (CL) monomers and poly(glycerol sebacate) have been developed by several groups¹⁸⁻²⁴. End-functionalization of these relatively low molecular weight polymers with (meth)acrylate- or fumarate groups allows network formation upon UV irradiation in the presence of a photoinitiator. The ultimate tensile strengths and elongation at break values of flexible networks obtained in this manner significantly increase with increasing macromer molecular weights and molecular weights between crosslinks²².

High molecular weight poly(trimethylene carbonate) is a flexible, biocompatible and biodegradable polymer^{25, 26}. Owing to its amorphous structure and low glass transition temperature, linear PTMC has low form stability and tends to creep at room temperature. Therefore, crosslinking is necessary to obtain form-stable PTMC materials with elastomeric properties. Previously, we have prepared elastomeric networks by gamma irradiation of high molecular weight trimethylene carbonate based (co)polymers^{22, 25, 27}. These polymers erode relatively rapidly *in vivo* by enzymatic surface erosion²⁸.

The ratio of crosslinking to chain scission events that occur during the gamma irradiation process, could be significantly increased by irradiating PTMC films that contain pentaerythritol triacrylate as a crosslinking aid ²⁹. The PTMC networks formed in this way did not only have very high gel contents, but also had excellent elastomeric properties: in tensile tests, the materials showed low modulus values, and high strength and elongation at break. The permanent deformation upon cyclic loading was very low. In addition, crosslinking in this manner also resulted in reduced enzymatic erosion rates of the networks.

It would be advantageous to crosslink the high molecular weight polymer in-house in a more practical manner than by using gamma irradiation. For example, the possibility of crosslinking PTMC polymer during or immediately after (melt) processing would allow the facile preparation of form-stable flexible porous tissue engineering scaffolds in the laboratory. In this study, we aimed at photocrosslinking of polymer films of high molecular weight PTMC containing a photoinitiator and pentaerythritol triacrylate by ultraviolet light irradiation instead of using gamma irradiation. Fused deposition modelling was used to fabricate porous flexible scaffolds, which were then also crosslinked by UV irradiation. The *in vitro* biocompatibility of the prepared scaffolds was assessed by human mesenchymal stem cell culturing experiments.

Materials and Methods

Materials

Polymer grade 1,3-trimethylene carbonate (TMC) (Boehringer Ingelheim, Germany) and the catalyst, stannous octoate (Sigma, U.S.A.), were used as received. 1,6-Hexanediol (Aldrich, Germany) was used as initiator in the PTMC synthesis. Also commercially available α,ω -dihydroxy poly(ϵ -caprolactone) (PCL) (Aldrich, Germany) and poly(ethylene glycol) monomethoxy ether (mPEG) (Fluka, Germany) having number average molecular weights ($\overline{M}_n$) of 10000 g/mol and of 5800 g/mol, respectively, were used as initiators to synthesize TMC block copolymers. A PCL polymer (Aldrich, Germany) having a molecular weight of 46000 g/mol was used for crosslinking studies. Pentaerythritol triacrylate (PETA) (Aldrich, Germany) and Irgacure® 369 (2-Benzyl-2-dimethylamino-1-(4-morpholinophenyl)-butanone-1) (Ciba®, Switzerland) were used as the crosslinking agent and photoinitiator, respectively.

Cholesterol esterase (CE) from porcine pancreas (Sigma, U.K., 56.2 U/mg) in phosphate buffered saline (PBS, pH=7.4, B. Braun Melsungen A.G., Germany) was used for the enzymatic erosion studies. Solvents (Merck, Germany or Biosolve, The Netherlands) were

of analytical grade. For cell culturing, α -minimum essential proliferation medium (α -MEM, Gibco U.S.A.) was used. This medium contained fetal bovine serum (10 %, Biowhitaker, Belgium), ascorbic acid-2-phosphate (0.2 mM, Sigma, U.S.A.), penicillin G (100 Units/mL, Invitrogen U.S.A.) and streptomycin (100 μ g/mL, Invitrogen U.S.A.), L-glutamine (2 mM, Sigma, U.S.A.), and basic fibroblast growth factor (1 ng/mL, Instruchemie, The Netherlands).

Polymer Synthesis

Poly(1,3-trimethylene carbonate) (PTMC) homopolymers were synthesized by ring opening polymerization of the TMC monomer under vacuum at 130 °C for three days using stannous octoate as catalyst. To control molecular weight, varying amounts of hexanediol were used as initiator.

Monomethoxy poly(ethylene glycol)-*block*-poly(trimethylene carbonate) (mPEG-PTMC) diblock copolymers and poly(trimethylene carbonate)-*block*-poly(ϵ -caprolactone)-*block*-poly(trimethylene carbonate) (PTMC-PCL-PTMC) triblock copolymers were also prepared by ring opening polymerization of TMC at 130 °C for three days. The mPEG and PCL initiators were dried under vacuum at 130 °C for 90 min. In both cases, the charged TMC contents were 60 mole %.

All polymers were purified by dissolution in chloroform and precipitation into ethanol or hexane, washing with fresh non-solvent and drying at room temperature under vacuum.

Polymer Characterisation

Residual monomer contents and compositions of the (co)polymers were determined by proton nuclear magnetic resonance (^1H -NMR) spectroscopy (300 MHz, Varian Innova, U.S.A.) using CDCl_3 (Merck, Germany).

Number average- and weight average molecular weights ($\overline{M}_n$ and $\overline{M}_w$, respectively), polydispersity indices (PDI) and intrinsic viscosities ($[\eta]$) of the PTMC homopolymers were determined by gel permeation chromatography (GPC, Viscotek U.S.A.) using chloroform as the eluent at a flow rate of 1.0 mL/min. The setup was equipped with ViscoGEL I-guard-0478, ViscoGEL I-MBHMW-3078, and ViscoGEL I-MBLMW-3078 columns placed in series and a TDA 302 Triple Detector Array with refractometer-, viscometer-, and light scattering detectors, allowing the determination of absolute molecular weights. Both GPC and NMR were used for the molecular weight determination of the PTMC-PCL-PTMC block copolymer, whereas the molecular weight of mPEG-PTMC was only determined using NMR.

Preparation of Polymer Films

Purified PTMC polymers were compression moulded at 140 °C using 500 µm thick stainless steel moulds (Fonteijne THB008 laboratory press, The Netherlands). The films were moulded at approximately 25 kg/cm² and quenched to room temperature using cold water.

Films of PTMC and blends of PTMC with the block copolymers (at a 9:1 weight ratio) containing PETA (5 wt %) and Irgacure® 369 (0.025 wt %) were prepared by dissolving the components in DCM, casting the solutions in Petri dishes and drying. These steps were performed in the dark, and compression moulding was then done as described above.

Photocrosslinking and Network Characterization

Compression moulded films containing PETA and photoinitiator were vacuum sealed in laminated polyethylene/polyamide bags (Hevel Vacuum B.V., The Netherlands) and exposed to short wave UV light (UltraLum crosslinking cabinet, U.S.A., wavelength 254 nm) at a distance of 7 cm. Both sides of the specimens were illuminated at room temperature, for different time periods. The light intensity at this distance was 10-14 mW/cm², the polyethylene/polyamide bags reduced the intensity to 5-7 mW/cm² as measured with an optical power meter (Newport 1916-C, U.S.A.).

The equilibrium swelling ratios and gel contents of the photocrosslinked films were determined using chloroform as previously described ^{8, 27}

Mechanical Properties

The tensile properties of melt pressed and photocrosslinked PTMC-based networks films before and after extraction with ethanol were determined in triplicate according to ASTM-D 882-91. A Zwick Z020 tensile tester (Germany) equipped with a 500 N load cell was operated at a crosshead speed of 50 mm/min. The initial grip-to-grip separation was 50 mm and a preload of 0.01 N was applied. The specimen deformation was derived from the grip-to-grip separation; therefore the presented values of Young's modulus (calculated from the initial slope of the stress-strain curves) give only an indication of the stiffness of the polymers.

To assess their behaviour under dynamic loading conditions, the specimens (n=1) were repetitively (20x) elongated to 50 % strain at 50 mm/min in cyclic tests. After a 2 h recovery period, the permanent deformation was estimated from the stress-strain diagram of the 21st cycle. In these experiments a preload of 0.01 N was applied, the deformation was derived from the grip to grip separation. The error in the values is approximately 0.5 % strain.

Wettability and Water Uptake

Water contact angles were measured of films prepared by casting polymer solutions in dichloromethane (approximately 1 wt %) on glass discs (n=8 per material), drying under vacuum, and photocrosslinking in an inert atmosphere. Measurements were done after extracting the photocrosslinked films with ethanol. Static, advancing and receding contact angles were determined using ultra-pure water (MilliQ Plus-Millipore, France) a video-based system (OCA 20 DataPhysics Instruments GmbH, Germany) equipped with an electronic syringe module.

The equilibrium water uptake of compression moulded and photocrosslinked films was determined after extraction with ethanol. Specimens (n=4) were conditioned in PBS (pH=7.4) at 37 °C for one week. Water uptake was defined as the mass increase of the specimens.

In Vitro Enzymatic Erosion

Cholesterol esterase (CE) from porcine pancreas was used to study the enzymatic hydrolysis of PTMC-based photocrosslinked films. Aqueous CE enzyme solutions were prepared at a concentration of 20 µg/mL using phosphate buffered saline (PBS, pH=7.4) containing 0.02 wt % NaN₃ (Sigma, U.S.A.) as a bactericide. Ethanol extracted, disk-shaped films (8 mm diameter, approximately 500 µm thickness, n=3 per time point) were placed in vials containing 1 mL of enzyme solution and conditioned at 37 °C. The medium was refreshed once every two days. Control experiments without enzyme were performed using PBS (pH 7.4, n=1 per time point). At predetermined time points, the mass and thickness of wet specimens were determined after rinsing and blotting their surfaces. The measurements were performed again after drying the specimens to constant weight *in vacuo* at room temperature.

Fabrication of Tissue Engineering Scaffolds

Fused deposition modelling was used to fabricate three-dimensional (3D) scaffolds with interconnected pores. Solutions were prepared by dissolving the PTMC homopolymer or the PTMC blends together with PETA and the photoinitiator in dichloromethane in the dark, and adding 80 wt % (based on the total polymer mass) of ethylene carbonate (melting temperature 35-38 °C). After evaporation of dichloromethane, the ethylene carbonate solution (containing polymer(s), PETA, and photoinitiator) was extruded at 100-120 °C and 4 bar nitrogen pressure using a Bioplotter device (Envisiontec GmbH, Germany).

Three dimensional scaffolds were plotted by deposition of up to 20 layers (each layer measured 12×12 mm) of fibres extruded through a stainless steel needle with internal diameter of 260 μm . The plotting parameters were: distance between the centres of neighbouring fibres: 500 μm , layer thickness: 130 μm . After each successive layer the plotting direction was changed by 90°. The fibre deposition speed was 100-200 mm/min. Cold air was blown over the built structure to crystallize ethylene carbonate. After plotting, the scaffolds were kept at 4 °C until further use.

The scaffolds were crosslinked by UV irradiation for 300 min. During irradiation, the temperature was maintained at approximately 20 °C by flowing cold nitrogen through the crosslinking chamber. This prevents melting of ethylene carbonate and provides an inert atmosphere. Subsequently, the scaffolds were placed in gently stirred Milli-Q water at 4 °C for 5 days to leach out ethylene carbonate (the water was refreshed twice a day). The scaffolds were then placed in ethanol to remove other possible leachables and dried to constant weight under vacuum. The porosity of the scaffolds was determined using the known densities of the polymers (densities were 1.31 g/cm³ and 1.29 g/cm³ for PTMC homopolymer and the blends, respectively.) and by measuring the density of the fabricated scaffolds.

The surface and the cross-section of the scaffolds were analysed by scanning electron microscopy (SEM, Philips XL 30 ESEM-FEG, The Netherlands) after sputter coating the specimens with gold, at an operating voltage of 5 kV.

Human Mesenchymal Stem Cell (hMSC) Seeding and Culturing in 3D Fabricated Tissue Engineering Scaffolds

Photocrosslinked and ethanol-extracted scaffolds (approximately 1 mm thick) prepared from the PTMC and the blends were sterilized by immersing in 70 % ethanol for 15 min. Then, the scaffolds were washed and incubated three times at room temperature for two hours with sterile PBS. Prior to cell culturing, the scaffolds were incubated in α MEM proliferation media overnight at 37 °C.

The isolation, culture and cryopreservation of hMSCs were as described previously³⁰. hMSCs were obtained from a donor undergoing total hip replacement surgery, who gave informed consent. Approval was obtained from the local medical ethical committee.

The thawed cells (passage 2) were plated in 300 cm² T-flasks (T-300 flasks) at 1000 cells/cm² in α MEM proliferation medium. hMSCs were expanded for one week with one refreshment of the α MEM proliferation medium. Cell numbers were determined with a

particle count and size analyzer (Z2, Beckman Coulter, Fullerton, CA). A volume from the cell suspension equivalent to 5×10^5 hMSCs - passage 3 – was placed on each scaffold with a pipette; these were then placed in wells of 25-well non-tissue-culture treated plates (Nunc) for four hours in an incubator. After this time period, the medium in the wells was replaced with 2 mL fresh α MEM proliferation medium. The cell-seeded scaffolds were cultured for time periods of 5 or 10 days. In the 10 day culturing period the medium was refreshed at day 5. Directly after seeding, and at day 5 and 10, the cell-containing constructs were washed with PBS and the cells were fixated with 1.5 % glutaraldehyde/0.14 M cacodylate buffer for 15 min. Methylene blue was used to stain the viable cells, which were observed using a stereomicroscope.

Samples of medium were drawn from different wells ($n=3$) to obtain metabolic profiles of the cells. Glucose and lactate concentrations were determined using the Vitros DT60 II system (Ortho-Clinical Diagnostics, Tilburg, The Netherlands).

Results and Discussion

To prepare elastomeric photocrosslinked PTMC-based network films and scaffolds, PTMC homopolymers and block copolymers of PTMC with mPEG and PCL were synthesized.

Characteristics of PTMC-based Polymers

The characteristics of the synthesized high molecular weight PTMC homopolymers after purification and compression moulding are given in Table 1. The polymer molecular weights could be adjusted by varying the amount of hexanediol used as initiator in the polymerisation reaction. The molecular weights of the PTMC polymers after compression moulding did not differ much from the purified polymers.

Table 1 also shows the characteristics of the PTMC-PCL-PTMC and mPEG-PTMC block copolymers after purification. The monomer conversions in the block copolymer synthesis were higher than 99 %. The TMC contents in the block copolymers were very close to the charged amounts.

Table 1. Characteristics of the purified TMC-based polymers used to prepare photocrosslinked networks.

Polymer	Hexanediol content (mol %)	TMC content (mol %) ^a	$\overline{M}_n$ ^a (kg/mol)	$\overline{M}_n$ ^b (kg/mol)	$\overline{M}_w$ ^b (kg/mol)	PDI ^b	$[\eta]$ ^b (dl/g)
PTMC ₄₅	0.2	100	-	45 (47)	75 (80)	1.7 (1.7)	1.3 (1.2)
PTMC ₁₈₃	0.03	100	-	183 (159)	227 (196)	1.2 (1.2)	2.6 (2.4)
PTMC ₃₇₃	0	100	-	373 (366)	474 (471)	1.3 (1.3)	5.7 (5.2)
PTMC ₄₄₃ ^c	0	100	-	443 (436)	620 (698)	1.5 (1.6)	6.6 (7.1)
PTMC-PCL-PTMC	0	60.4	24	15	26	1.7	0.5
mPEG-PTMC	0	60.7	27	n.d.	n.d.	n.d.	n.d.

^a Determined after purification by ¹H NMR using CDCl₃ as the solvent.

^b Determined after purification by GPC at 30 °C using chloroform as the eluent. The values for compression moulded polymers are given in brackets.

^c Reference ²⁹.

n.d.: not determined.

UV Crosslinking of PTMC-based Films

To photocrosslink high molecular weight PTMC, compression moulded films containing PETA and a photoinitiator were irradiated with short-wave UV (254 nm). Upon irradiation with UV light, PTMC could be effectively crosslinked with relative ease, resulting in transparent films. Figures 1A and 1B show the gel contents and swelling ratios determined using chloroform of the photocrosslinked PTMC networks as a function of irradiation time. The gel contents initially increased rapidly with increasing irradiation time and then increased slowly. In accordance with the increasing gel contents the swelling ratios of the formed networks in chloroform decreased with increasing irradiation time, indicating that denser networks were formed at longer irradiation times.

At 300 minutes of UV exposure, depending on the initial molecular weight of the polymer the gel contents of the networks ranged from 57±1 to 98±1 % and the swelling ratios ranged from 11.3±0.8 to 3.7±0.1.

Adjusting the PETA content in the PTMC films also allowed preparation of networks with varying gel contents and swelling ratios. For instance, irradiating the PTMC₁₈₃ polymer containing 1, 3, 5 % PETA for 180 minutes lead to networks with gel contents of 41, 71, and

91 % and with swelling ratios of 65, 14, 7 vol/vol, respectively. These results demonstrate that PETA content, UV irradiation time, and the initial polymer molecular weight can be varied to adjust the gel content of the networks and the network density.

Figure 1. Effect of UV irradiation time and initial polymer molecular weight on gel content (A) and swelling ratio in chloroform (B) of PTMC films that contain 5 wt % PETA and 0.025 wt % photoinitiator and exposed to 254 nm UV light. Values are expressed as mean $\pm$ standard deviation, ($n=3$).

In other work done in our laboratory, photocrosslinked PTMC networks were prepared from three-armed PTMC-methacrylate macromers having molecular weights from 0.7 to 40.7 kg/mol (to be published). The swelling ratios of these networks in chloroform ranged from 3.1 to 16.9. By comparing our results with the swelling ratios of these PTMC –macromer- based networks, it can be estimated that the molecular weights between the crosslinks of the PTMC networks (containing PETA and photoinitiator and irradiated with UV light for 300 min) range to a first approximation from 1100 to 7600 kg/mol depending on the initial molecular weight of the polymer.

Photocrosslinking PTMC using only a crosslinking aid and a photoinitiator is more practical than by using end-functionalized macromers of different arm lengths and to prepare networks of varying crosslink densities. Furthermore, we have observed that (meth)acrylate end-functionalized PTMC oligomers can crosslink prematurely before processing even in the presence of radical scavengers (unpublished data). The PETA containing polymer films that we used in this study were still soluble in chloroform, even after compression moulding implying that no crosslinking had occurred prior to UV exposure.

Interestingly, when PTMC homopolymer films that did not contain PETA and Irgacure® 369 were exposed to UV light, the number and weight average molecular weights

of the polymers increased. After 300 minutes of irradiation, the $\overline{M}_n$ values of PTMC₄₅, PTMC₁₈₃, and PTMC₃₇₃ were 49, 244, and 435 kg/mol, respectively. The corresponding PDI values were 2.2, 1.9, and 1.3. We observed that, prolonged irradiation of very high molecular weight PTMC with short wave UV light, lead to crosslinking of the polymer. Upon 48 hours of irradiation of compression moulded films of PTMC₄₄₃, networks with gel contents of 53±13 %, and swelling ratios of 84±10 vol/vol in chloroform were formed.

Irradiation of PTMC films which contained only Irgacure® 369 and no PETA resulted in network formation, but the gel contents were much lower than that of the films which contained both PETA and Irgacure® 369. Upon 300 minutes of UV irradiation, PTMC₁₈₃ and PTMC₃₇₃ films that contained 0.025 wt % photoinitiator only had gel contents of 25±3 and 50±2 % and swelling ratios of 253±6 and 72±4 vol/vol, respectively.

This difference between the gel contents of films that contained only photoinitiator or both photoinitiator and PETA could be due to efficient incorporation of the acrylate network to PTMC chains. Such semi-interpenetrating networks of multifunctional acrylates and several linear polymers were proposed to be formed by hydrogen abstraction from linear polymer chains by the acrylic radicals ^{31, 32}. ¹H-NMR analysis of the sol fraction of photocrosslinked PTMC films did not reveal any acrylate related peaks. This implies that all PETA had been incorporated in the formed networks.

In addition to PTMC polymers and blends, we investigated the photocrosslinking of poly (D,L-lactide) (PDLLA) and PCL films that contained the same amount of PETA and photoinitiator as the PTMC films. Initial results showed that PCL and PDLLA can also be photocrosslinked in this manner. A relatively low molecular weight ($\overline{M}_n$ =46 kg/mol) PCL homopolymer film could be crosslinked in to a network with a gel percentage of 41±1 % and swelling ratio of 11.3±0.7 vol/vol in chloroform. In the case of PDLLA ($\overline{M}_n$ =289 kg/mol), a gel content of 71±1 and a swelling ratio of 28.6±1.3 vol/vol could be obtained.

Also blends of PTMC₃₇₃ with PTMC-PCL-PTMC or with mPEG-PTMC block copolymers which contained 5 wt % PETA and photoinitiator could be photocrosslinked efficiently. Upon 300 minutes of UV irradiation the gel contents of the PTMC₃₇₃/PTMC-PCL-PTMC blend and the PTMC₃₇₃/mPEG-PTMC blend were 96±1 and 93±1, respectively. The corresponding swelling ratios in chloroform were 6.3±0.1 and 6.1±0.6 vol/vol. Prior to UV exposure, the blends contained 90 wt % PTMC₃₇₃. ¹H-NMR analyses of the sol fractions of the networks revealed that the block copolymer contents were higher than in the initial film. It

could be shown that approximately 75-80 % of the block copolymers had been incorporated into the networks upon exposing the films to UV irradiation.

Mechanical Properties of Photocrosslinked PTMC-based Networks

To evaluate the effect of photocrosslinking on the tensile properties of PTMC polymers, measurements were performed on non-irradiated compression moulded PTMC films and on PTMC films that contained 5 wt % PETA and 0.025 wt % photoinitiator and irradiated with UV light for 300 min (the films irradiated by UV light for 300 min are abbreviated as PTMC_x-300). An overview of the tensile properties of non-crosslinked and photocrosslinked PTMC homopolymers, as well as photocrosslinked PTMC₃₇₃/PTMC-PCL-PTMC and PTMC₃₇₃/mPEG-PTMC blends are given in Table 2.

All PTMC homopolymers are flexible materials with elastic moduli ranging from 3.7±0.1 to 7.7±0.3 MPa. Their yield strength, stress at break, and strain at break values increased with increasing polymer molecular weight. The photocrosslinked PTMC network films were also flexible materials, although their elastic moduli were somewhat higher than of non-crosslinked PTMC films. The values ranged from 7.2±0.2 to 9.6 ±0.3 MPa. The yield strength and the ultimate tensile strength of PTMC homopolymers increased significantly upon photocrosslinking. Depending on the initial polymer molecular weight, the stress at break values of the non-crosslinked films ranged from 0.2±0.1 MPa to 6.7±2.8 MPa. Upon crosslinking, the stress at break values of the films increased and ranged from 2.7±0.1 to 30±9 MPa. Moreover, the energy needed to break the photocrosslinked PTMC network films was 3 to 9 times higher than for the non-crosslinked PTMC films, implying that the toughness of PTMC films can be improved significantly by photocrosslinking in the presence of PETA. Table 2 clearly illustrates that, depending on the initial PTMC molecular weight, PTMC networks having a range of yield strength, ultimate tensile strength and toughness can be obtained.

The tensile measurements were also performed after extracting the sol fraction of the PTMC network films with ethanol, a relatively poor solvent for PTMC. The enhancement of properties was most pronounced for the networks prepared from PTMC₄₅ as the extracted films had higher elastic moduli, yield strength, stress at break, and energy to break values. In the case of the networks prepared from PTMC₁₈₃ and PTMC₃₇₃, these values also seemed to increase slightly.

Table 2. The effect of photocrosslinking of PTMC-based polymers in the presence of PETA on the mechanical properties. Values are expressed as mean $\pm$ standard deviation, ($n=3$).

Polymer-Irradiation time (min)	PETA content (wt %)	Ethanol extraction	E (MPa)	σ_{yield} (MPa)	ϵ_{yield} (%)	σ_{break} (MPa)	ϵ_{break} (%)	Energy to break (Joules)	Permanent Set ^a (%)
PTMC ₄₅ -0	0.0	No	3.7 $\pm$ 0.1	0.5 $\pm$ 0.1	71 $\pm$ 3	0.2 $\pm$ 0.1	479 $\pm$ 165	0.2 $\pm$ 0.05	22.0
PTMC ₄₅ -300	5.0	No	7.2 $\pm$ 0.2	2.1 $\pm$ 0.1 ^b	107 $\pm$ 3 ^b	2.7 $\pm$ 0.1	530 $\pm$ 79	1.4 $\pm$ 0.3	3.0
PTMC ₄₅ -300	5.0	Yes	9.8 $\pm$ 0.3	2.7 $\pm$ 0.1 ^b	93 $\pm$ 3 ^b	4.4 $\pm$ 0.5	515 $\pm$ 29	2.1 $\pm$ 0.2	2.0
PTMC ₁₈₃ -0	0.0	No	6.9 $\pm$ 0.5	1.4 $\pm$ 0.1	116 $\pm$ 5	1.1 $\pm$ 0.1	532 $\pm$ 44	0.7 $\pm$ 0.1	8.0
PTMC ₁₈₃ -300	5.0	No	9.2 $\pm$ 0.1	3.6 $\pm$ 0.2 ^b	98 $\pm$ 3 ^b	19 $\pm$ 5	706 $\pm$ 61	6.6 $\pm$ 1.6	1.5
PTMC ₁₈₃ -300	5.0	Yes	9.5 $\pm$ 0.2	3.9 $\pm$ 0.1 ^b	97 $\pm$ 3 ^b	21 $\pm$ 3	610 $\pm$ 47	6.7 $\pm$ 0.3	1.3
PTMC ₃₇₃ -0	0.0	No	7.7 $\pm$ 0.3	2.2 $\pm$ 0.1	126 $\pm$ 10	6.7 $\pm$ 2.8	847 $\pm$ 73	2.5 $\pm$ 0.6	2.2
PTMC ₃₇₃ -300	5.0	No	9.6 $\pm$ 0.3	4.4 $\pm$ 0.3 ^b	93 $\pm$ 6 ^b	30 $\pm$ 9	560 $\pm$ 79	7.7 $\pm$ 2.7	1.5
PTMC ₃₇₃ -300	5.0	Yes	10.5 $\pm$ 0.2	4.6 $\pm$ 0.2 ^b	102 $\pm$ 10 ^b	35 $\pm$ 4	622 $\pm$ 26	9.5 $\pm$ 1.4	1.0
PTMC ₃₇₃ /PTMC-PCL-PTMC-300	5.0	No	9.9 $\pm$ 0.3	3.9 $\pm$ 0.1 ^b	87 $\pm$ 6 ^b	19 $\pm$ 2	513 $\pm$ 3	4.1 $\pm$ 0.4	1.1
PTMC ₃₇₃ /PTMC-PCL-PTMC-300	5.0	Yes	10.3 $\pm$ 0.1	3.9 $\pm$ 0.1 ^b	95 $\pm$ 8 ^b	17 $\pm$ 3	608 $\pm$ 65	4.1 $\pm$ 0.9	1.1
PTMC ₃₇₃ /mPEG-PTMC-300	5.0	No	8.8 $\pm$ 0.5	4.0 $\pm$ 0.1 ^b	103 $\pm$ 3 ^b	30 $\pm$ 6	630 $\pm$ 39	8.4 $\pm$ 1.9	1.2
PTMC ₃₇₃ /mPEG-PTMC-300	5.0	Yes	10.0 $\pm$ 0.3	4.1 $\pm$ 0.2 ^b	96 $\pm$ 1 ^b	34 $\pm$ 1	645 $\pm$ 22	9.4 $\pm$ 0.3	1.3

^a Single measurements. The permanent set is estimated from the 21st cycle, performed after a two hours recovery period. The error is approximately 0.5 % strain.

^b Estimated from the intersection of tangents to stress-strain diagrams as a distinct yield point could not be observed.

Photocrosslinked films prepared from blends of PTMC₃₇₃ with the relatively low molecular weight mPEG-PTMC block copolymer had only slightly lower elastic moduli and yield strength values than those of non-extracted networks prepared from PTMC₃₇₃ while their stress at break and energy to break values were comparable. This shows that blending PTMC₃₇₃ with up to 10 % mPEG-PTMC does not deteriorate the tensile properties significantly in the dry state. Networks obtained from the blends with PTMC-PCL-PTMC block copolymer however had lower yield strength, ultimate tensile strength and toughness than networks prepared from PTMC₃₇₃ only. The tensile properties of the networks prepared from the blends were not affected much by extraction of the sol fraction with ethanol.

As these PTMC networks are intended to withstand repeated dynamic loadings *in vitro* as well as *in vivo*, cyclic tests were performed to assess the effect of photocrosslinking on their creep behaviour under dynamic conditions. Figures 2A, 2B, and 2C show the hysteresis behaviour of PTMC polymers before and after photocrosslinking. These figures clearly demonstrate that in all cases photocrosslinking of PTMC in the presence of PETA and photoinitiator significantly reduces hysteresis with reduced extent of creep observed in each successive cycle. In the non-crosslinked state, PTMC₄₅, PTMC₁₈₃ and PTMC₃₇₃ had creep values of 45, 29, 15 %, respectively at the end of the 20th cycle. Upon photocrosslinking in the presence of PETA and photoinitiator, the values significantly decreased: PTMC₄₅-300, PTMC₁₈₃-300 and PTMC₃₇₃-300 had creep values of 29, 12, and 8 %, respectively. Moreover, the stress relaxation (the decrease in maximum stress reached in each cycle) of the PTMC films was also very much reduced upon photocrosslinking. Table 2 shows the permanent deformation of these films after applying 20 cycles of 50 % strain and a 2 h recovery period. It can be seen that the permanent deformation of PTMC homopolymers significantly decreased upon photocrosslinking: Ethanol extracted PTMC networks showed comparable creep behaviour with the non-extracted networks in the cyclic tests, although permanent deformation seemed to be slightly lower for the extracted network films. Permanent deformation values were as low as 1.0 % strain after photocrosslinking and extraction of PTMC₃₇₃. Comparison of Figures 2A, 2B, 2C and the permanent set values in Table 2, shows that the photocrosslinked networks prepared from PTMC₃₇₃ showed the least hysteresis, the least stress relaxation and the least permanent deformation in cyclic tests. This implies that high initial PTMC molecular weights allows preparation of networks with better elastomeric properties than those prepared from polymers of relatively lower initial molecular weights.

Figure 2D and the permanent set values in Table 2 show that the photocrosslinked blends also have excellent elastomeric properties. It can be seen that blending PTMC₃₇₃ with

PTMC-PCL-PTMC or mPEG-PTMC does not have an adverse effect on the cyclic creep behaviour of the networks.

Figure 2. Effect of photocrosslinking on the hysteresis behaviour of PTMC polymers and networks in cyclic tensile tests. The curves show repetitive (20 cycles) application of 50 % strain on PTMC homopolymers of different molecular weights before ($PTMC_x$) and after photocrosslinking in the presence of PETA and photoinitiator by 300 minutes of UV exposure (A,B,C). Figure D shows the behaviour of photocrosslinked blends under dynamic loading.

In Vitro Enzymatic Erosion and Wettability of Photocrosslinked PTMC-based Networks

The enzymatic erosion of photocrosslinked networks based on $PTMC_{373}$ was investigated using aqueous cholesterol esterase solutions. This enzyme is one of the secretory products of macrophages, and previously has been shown to effectively degrade gamma irradiated and photocrosslinked PTMC networks^{19, 29, 33}. The changes in relative mass and thickness of the incubated polymer films in time is given in Figure 3. The erosion of linear $PTMC_{373}$ homopolymer was relatively fast in aqueous cholesterol esterase solutions. Upon 11 days of incubation, the mass loss of the $PTMC_{373}$ specimens was 84 ± 2.5 % corresponding to a rate of mass loss of 2.48 ± 0.45 mg/(cm²×day).

Figure 3. Effect of photocrosslinking and blending with block copolymers on in vitro enzymatic erosion of PTMC₃₇₃-based networks: Decrease in relative mass in time (A): PTMC₃₇₃ (■), PTMC₃₇₃-300 (●), PTMC₃₇₃/PTMC-PCL-PTMC-300 (□), PTMC₃₇₃/mPEG-PTMC-300 (○).

The mass loss of photocrosslinked films of PTMC₃₇₃-300, PTMC₃₇₃/PTMC-PCL-PTMC-300, and PTMC₃₇₃/mPEG-PTMC-300 after 28 days of incubation in aqueous enzyme solution were 24 ± 2.7 , 16 ± 1.8 , and 1.5 ± 0.4 %, respectively (Figure 3A). The corresponding rates of mass loss were 0.41 ± 0.11 , 0.27 ± 0.07 , and 0.017 ± 0.01 mg/(cm²×day). Photocrosslinking of PTMC₃₇₃ films resulted in networks with much reduced erosion rates allowing their use in long-term biomedical applications. The decrease in erosion rates could be due to hindered mobility of the chains as a result of dense network formation as the chains need to adopt the correct conformation for enzymatic attack. These results also show that the enzymatic erosion rates of PTMC networks can be tuned by blending and photocrosslinking with block copolymers. Especially, when blended with the mPEG-PTMC block copolymer, enzymatic erosion was dramatically reduced. It is likely that the surface characteristics of mPEG-PTMC containing blends did not allow binding of the enzyme to the surface of the polymers, as it was suggested that this enzyme is most active at a hydrophilic-hydrophobic interface³⁴. Indeed in Table 3 it can be seen that the photocrosslinked networks which contain mPEG-PTMC block copolymer have significantly lower contact angles and somewhat higher water uptake values than the photocrosslinked networks prepared from PTMC₃₇₃ homopolymer or from its blends with the PTMC-PCL-PTMC block copolymer. It is clear that the surface characteristics of photocrosslinked PTMC networks can be easily tailored by blending with a hydrophilic-hydrophobic block copolymer.

Table 3. Effect of photocrosslinking and blending with block copolymers on water contact angles and water uptake values of PTMC₃₇₃-based films. Values are expressed as mean $\pm$ standard deviation.

Polymer	Water contact angle (°) ^a			Water uptake (%) ^b
	Static	Advancing	Receding	
PTMC ₃₇₃	77.4 $\pm$ 0.8	80.4 $\pm$ 0.4	48.6 $\pm$ 3.5	1.4 $\pm$ 0.3
PTMC ₃₇₃ -300	98.9 $\pm$ 3.4	101.5 $\pm$ 2.6	65.1 $\pm$ 1.8	1.2 $\pm$ 0.2
PTMC ₃₇₃ /PTMC-PCL-PTMC-300	94.6 $\pm$ 2.8	95.9 $\pm$ 2.8	63.6 $\pm$ 2.3	1.3 $\pm$ 0.2
PTMC ₃₇₃ /PTMC-mPEG-300	60.6 $\pm$ 1.0	63.1 $\pm$ 1.0	30.4 $\pm$ 1.2	1.8 $\pm$ 0.2

^a Performed on polymer films cast on glass discs (n=8).

^b Compression moulded, photocrosslinked, and extracted films (n=4) were conditioned in PBS at 37 °C for one week.

The non-irradiated PTMC₃₇₃ films had an average static contact angle of 77.4 $\pm$ 0.8 which is comparable to previously reported values ³⁵. Interestingly, the contact angles of UV irradiated PTMC₃₇₃ films which contained PETA and photoinitiator (PTMC₃₇₃-300) were significantly higher (98.9 $\pm$ 3.4) than those of the non-irradiated films. To assess the sole effect of UV irradiation, we irradiated PTMC₃₇₃ films which did not contain PETA or photoinitiator. After 300 min of irradiation, the static, advancing, and receding contact angles of PTMC₃₇₃ were 85.3 $\pm$ 1.1°, 87.5 $\pm$ 0.9°, and 52.0 $\pm$ 1.4°, respectively, which were also higher than for non-irradiated PTMC. This confirms that the crosslinking was done under inert conditions, as short wavelength ultraviolet irradiation in air would lead to oxidation of the surface of the polymer and increase hydrophilicity (lower contact angles). It was reported that, the contact angle of PMMA also increases approximately 20° upon vacuum UV irradiation (126 nm) at a reduced pressure of 2 $\times$ 10⁻⁴ Pa ³⁶. This was related to an increase in the percentage of carbon at the polymer surface.

Figure 3B shows that the thickness of non-crosslinked PTMC₃₇₃ films and of the photocrosslinked PTMC₃₇₃-300 and PTMC₃₇₃/PTMC-PCL-PTMC-300 films also decreased in time during enzymatic degradation. This indicates that these polymer and network films eroded by a surface erosion process. No significant decrease in thickness of the PTMC₃₇₃/mPEG-PTMC-300 specimens could be determined but SEM analysis revealed surface pitting of these films to some extent, confirming their slow surface erosion (data not shown).

Photocrosslinked PTMC-based Tissue Engineering Scaffolds

For the fabrication of TE scaffolds, solutions of the polymer (PTMC₃₇₃ homopolymer, PTMC₃₇₃/PTMC-PCL-PTMC or PTMC₃₇₃/mPEG-PTMC blend), PETA, photoinitiator, and

ethylene carbonate were prepared. Photocrosslinked three-dimensional PTMC-based scaffolds were prepared by computer controlled extrusion of the polymer solutions using a bioplotter, photocrosslinking, and extraction of ethylene carbonate and other possible leachables in water and ethanol.

The scaffolds prepared from flexible PTMC-based polymers had high porosities of $70 \pm 6\%$ with interconnected pores. Figure 4 shows SEM micrographs of the photocrosslinked scaffolds prepared from PTMC₃₇₃ polymer. Similar structures were also obtained when PTMC₃₇₃/PTMC-PCL-PTMC or PTMC₃₇₃/mPEG-PTMC blends were used. It can be seen from Figures 4A and 4B that the distance between the fibres plotted in the x or y directions was approximately 200-250 μm and the diameter of the fibres ranged between 100-150 μm . The height of the pores ranged from 50 to 100 μm (Figure 4D).

Figure 4. SEM micrographs of the photocrosslinked porous scaffolds prepared from PTMC₃₇₃. A and B show the top view of a scaffold. Microporosity of the fibres is illustrated in C. A cross section of a scaffold is given in D.

Use of ethylene carbonate (EC), as a solvent allowed the extrusion of high molecular weight PTMC polymers at relatively low temperatures of 100-120 °C. Without the use of EC, temperatures higher than 220 °C are required even for the extrusion of the relatively low molecular weight PTMC₄₅ polymer. Moreover, cooling the extruded fibres with cold nitrogen

induced crystallisation of ethylene carbonate. Crystallisation of EC not only provided the required form-stability to the extruded fibres of amorphous PTMC polymers, but also led to formation of fibres with microporosity. These micropores (ranging from few microns to few tens of microns) in the fibres and the roughness of the surface of the fibres can be seen especially clearly in Figure 4C. Microporosity of these PTMC scaffolds can be advantageous in tissue engineering as the attachment of cells to substrates and their viability has been shown to improve with surface roughness^{37, 38}. This microporosity could also lead to an enhancement in the transport of nutrients to the cells and removal of waste products from the cells.

Human mesenchymal stem cells (hMSCs) were seeded in photocrosslinked (300 min) PTMC-based scaffolds and cultured for 10 days. Figure 5 shows the viable cells that were stained with methylene blue. It can be seen that the number of cells that are present on and in scaffolds prepared from PTMC₃₇₃ and PTMC₃₇₃/PTMC-PCL-PTMC increased from the day of seeding (Figures 5A, 5D) to day 5 (Figures 5B, 5E). After 10 days of culturing, these scaffolds contained very high numbers of viable cells as can be seen from extensive (blue) staining in Figures 5C and 5F. The amount of cells on and in the scaffolds prepared from PTMC₃₇₃/mPEG-PTMC blends was much less than for the other two materials. The hydrophilic nature of this material probably did not allow adsorption of cell binding proteins that are present in the cell culture medium which hindered hMSC adhesion to these surfaces.

Figure 5. Micrographs showing viable cells on/in 3D fabricated and photocrosslinked scaffolds prepared from PTMC₃₇₃ (A,B,C), PTMC₃₇₃/PTMC-PCL-PTMC (D,E,F), PTMC₃₇₃/mPEG-PTMC (G,H,I) after cell seeding (A, D, G), and after 5 days (B, E, H) and 10 days (C, F, I) of hMSC culturing. A cross-section of each scaffold is shown in insets.

These observations were corroborated by SEM (Figure 6). After 5 days of culturing, hMSCs with stretched fibroblast-like morphology were observed on the fibres (Figures 6A, 6D, 6G). Figures 6B and 6E show that the hMSCs proliferated and that the surface of the photocrosslinked scaffolds prepared from PTMC₃₇₃ and PTMC₃₇₃/PTMC-PCL-PTMC were almost completely covered with cells. It can be seen from the cross sections in Figures 6C, and 6F that cells were also present inside the scaffolds and had produced extracellular matrix. This indicates that these materials were compatible with hMSCs. Also with SEM, low numbers of cells were seen on scaffolds prepared from PTMC₃₇₃/mPEG-PTMC blends.

Figure 6. SEM micrographs showing hMSCs on/in the 3D fabricated and photocrosslinked scaffolds prepared from PTMC₃₇₃ (A,B,C), PTMC₃₇₃/PTMC-PCL-PTMC (D,E,F), PTMC₃₇₃/mPEG-PTMC (G,H,I) after 5 days (A,D,G) and 10 days (top-view B,E,H, cross section (C,F,I) of hMSC culturing.

During hMSC culturing, the metabolic profile of the cells were monitored by determining the glucose and lactate concentrations in the culture media. Figures 7A and 7B show the glucose and lactate concentrations on day 5, 8 and 10. (Note that the medium was refreshed on day 5.) In all cultures, glucose concentrations decreased whereas lactate concentrations increased in time. As higher numbers of cells were present in photocrosslinked scaffolds prepared from PTMC₃₇₃ and PTMC₃₇₃/PTMC-PCL-PTMC, glucose consumption and lactate production were high in the wells containing these cell-seeded scaffolds. In the wells that contained cell-seeded scaffolds prepared from PTMC₃₇₃/mPEG-PTMC blends glucose consumption and lactate production were low, as there were significantly less cells in these scaffolds. The rates of glucose consumption for cultures using scaffolds of PTMC₃₇₃-300, PTMC₃₇₃/PTMC-PCL-PTMC-300, and PTMC₃₇₃/mPEG-PTMC-300 were 23.3 ± 1.7 , 25.0 ± 2.4 , and 9.8 ± 1.5 $\mu\text{M/h}$, respectively. The corresponding lactate production rates were 60.8 ± 6.5 , 63.2 ± 8.1 , and 34.1 ± 3.9 $\mu\text{M/h}$. These results suggest that the scaffolds of PTMC₃₇₃-

300 and PTMC₃₇₃/PTMC-PCL-PTMC-300 were more suited for tissue engineering applications than those prepared from PTMC₃₇₃/mPEG-PTMC. (However, the networks containing mPEG-PTMC block copolymer might be used to improve the blood compatibility of biomaterials such as vascular grafts.)

Figure 7. Glucose consumption (A) and lactate production (B) of hMSCs cultured in 3D fabricated and photocrosslinked PTMC-based scaffolds: PTMC₃₇₃-300 (■), PTMC₃₇₃/PTMC-PCL-PTMC-300 (●), PTMC₃₇₃/mPEG-PTMC-300 (▲).

Conclusion

This study shows that flexible and tough PTMC networks with excellent elastomeric properties and reduced enzymatic erosion rates can be obtained by efficient photocrosslinking of PTMC films containing PETA and photoinitiator. The properties of these PTMC networks such as wettability and enzymatic erosion can be easily tailored by blending with PCL or PEG containing block copolymers. Tissue engineering scaffolds with interconnected pores and extensive microporosity can be obtained by fused deposition modelling of thermoplastic PTMC polymer and subsequent UV photocrosslinking of the scaffolds. Photocrosslinked scaffolds prepared from PTMC₃₇₃ polymer and PTMC₃₇₃/PTMC-PCL-PTMC blends are suitable for tissue engineering applications as they allowed adhesion and proliferation of human mesenchymal stem cells. This versatile crosslinking method offers interesting opportunities for the facile crosslinking of high molecular weight polymers and modification of their properties in this manner.

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Chapter 10

Resorbable Elastomeric Networks Prepared by Photocrosslinking of High Molecular Weight Poly(trimethylene carbonate) with Photoinitiators and Poly(trimethylene carbonate) Macromers as Crosslinking Aids^{*}

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Abstract

Resorbable and elastomeric poly(trimethylene carbonate) (PTMC) networks were efficiently prepared by photoinitiated crosslinking of linear high molecular weight PTMC. To crosslink PTMC films, low molecular weight PTMC macromers with methacrylate endgroups were synthesized and used as crosslinking aids. By exposing PTMC films containing only photoinitiator (Irgacure® 2959) or both photoinitiator and PTMC macromers to ultraviolet light, PTMC networks with high gel contents (87-95 %) could be obtained. The network density could readily be varied by adjusting the irradiation time or the amount of crosslinking aid used. The formed networks were flexible with low elastic modulus values ranging from 7.1 to 7.5 MPa, and also showed excellent resistance to creep in cyclic tests. In vitro experiments showed that the photocrosslinked PTMC networks could be eroded by

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macrophages, and upon incubation in aqueous cholesterol esterase enzyme- or potassium dioxide solutions. The rate of surface erosion of photocrosslinked PTMC networks was significantly lower than that observed for films prepared from linear PTMC. These resorbable PTMC elastomeric networks are compatible with mesenchymal stem cells and may find application in tissue engineering and controlled release.

Introduction

Synthetic resorbable elastomers are very well suited for controlled release applications and soft-tissue engineering, as their flexible and elastic nature closely mimics that of tissues such as arteries, muscles and cartilage ¹. In the ideal case, they should degrade by a surface erosion process. This would allow (sequential) zero-order release profiles of drugs ²⁻⁴ and the maintenance of structural integrity of tissue engineering scaffolds during degradation ⁵.

To obtain elastomeric properties, degradable polymer networks with physical or chemical crosslinks have been prepared. Physical crosslinks are formed by (micro)phase separation of hard- and soft segments of a block copolymer (a thermoplastic elastomer) ⁶⁻⁹. Chemically crosslinked networks have been obtained by ring opening polymerization ¹⁰⁻¹³, polycondensation ^{14, 15}, thermally-induced ^{16, 17} or light-induced (photocrosslinking) ¹⁸⁻²⁴ free radical polymerization reactions. High energy irradiation of linear polymers can also lead to crosslinking ^{25, 26}. To meet specific requirements of different tissue engineering or controlled release applications, the development of resorbable elastomers with tuneable properties is essential.

Poly(trimethylene carbonate) (PTMC) is a resorbable, amorphous, and flexible polymer with a glass transition temperature of approximately -17 °C ²⁷⁻²⁹. *In vivo* PTMC degrades rapidly by surface erosion ²⁹. Crosslinking of PTMC is required to obtain form-stable elastomeric PTMC networks. We have prepared flexible elastomeric PTMC-based networks by gamma irradiation under vacuum ^{25, 26}. As chain scission and crosslinking events occur simultaneously, gel contents were lower than 70 % and degrees of swelling in chloroform were rather high, indicating that the formed networks were also not densely crosslinked. Therefore, *in vivo* erosion of these networks was not significantly slower than that of linear PTMC ³⁰. It was possible, however, to obtain PTMC networks with very high gel contents and network densities by gamma irradiating PTMC films that contained small amounts of pentaerythritol triacrylate (PETA) ³¹. The enzymatic erosion rates of these PTMC networks were significantly reduced.

Photocrosslinking by visible light or ultraviolet (UV) irradiation is highly efficient, and has been utilized in preparing elastomeric PTMC networks at ambient temperatures^{18, 19, 21, 22}. For this, PTMC macromers were obtained by end-functionalisation of PTMC oligomers with photocrosslinkable groups such as (meth)acrylates or fumarates. As it was shown that the toughness of the networks improved with increasing macromer molecular weight²², we investigated the photocrosslinking behaviour and properties of networks prepared by UV photocrosslinking films of linear (non-functionalized) high molecular weight PTMC that contained PETA and a photoinitiator³². Using this facile crosslinking method, high molecular weight PTMC could first be processed into cytocompatible tissue engineering scaffolds by fused deposition modelling and subsequently crosslinked efficiently by exposure to UV light. (Interestingly, high molecular weight PTMC films that only contained photoinitiator were also found to crosslink, although less efficiently than films that contained both PETA and photoinitiator.)

In this study, we aimed at using a compatible TMC-based macromer as a crosslinking aid for the photocrosslinking of linear high molecular weight PTMC. Poly(trimethylene carbonate) macromers were synthesized by functionalization of the oligomers with methacrylate end-groups. Films prepared from mixtures of linear high molecular weight PTMC, the PTMC macromer crosslinking aid and Irgacure® 2959 as a cytocompatible^{33, 34} photoinitiator were photocrosslinked by UV irradiation. The characteristics of the formed networks were evaluated.

Materials and Methods

Materials

Polymer grade 1,3-trimethylene carbonate (TMC, Boehringer Ingelheim, Germany), 1,3-propanediol, trimethylol propane, and stannous octoate (all from Sigma, U.S.A. or Germany) were used as received. Methacrylic anhydride and hydroquinone were purchased from Aldrich (Germany). (±)- α -tocopherol was obtained from Fluka (Switzerland). Solvents (Merck, Germany or Biosolve, The Netherlands) were of analytical grade. Photoinitiators Irgacure® 2959 (1-[4[(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one) Irgacure® 369 (2-Benzyl-2-dimethylamino-1-(4-morpholinophenyl)-butanone-1), Irgacure® 500 (50 % 1-hydroxy-cyclohexyl-phenyl-ketone, 50 % benzophenone) (Ciba®, Switzerland), benzophenone (Aldrich, Germany), and DMPA (2,2-dimethoxy-2-phenyl acetophenone) (Aldrich, Germany) were used as received.

J774A macrophages (ATCC-TIB-67) were obtained from the American Type Culture Collection. Culture media, fetal bovine serum, Glutamax™ and penicillin-streptomycin were obtained from Invitrogen (Gibco, U.S.A.). Culture disposables were from Nunc (U.S.A.) and Greiner (Germany). For human mesenchymal stem cell culturing, α -minimum essential medium (α -MEM, Gibco U.S.A.) was used. This medium also contained fetal bovine serum (10 %, Biowhitaker, Belgium), penicillin G (100 Units/ml, Invitrogen U.S.A.) and streptomycin (100 μ g/ml, Invitrogen U.S.A.), L-glutamine (2 mM, Sigma, U.S.A.). Cholesterol esterase (CE) from porcine pancreas (Sigma, U.K., 56.2 U/mg) and potassium dioxide (KO₂, Sigma, U.S.A.) were dissolved in phosphate buffered saline (PBS, pH=7.4, B. Braun Melsungen A.G., Germany) and NaOH/NaCl buffer solution (pH=13, Scharlau Chemie, Spain), respectively prior to use.

Synthesis of PTMC Polymer and PTMC Macromer Crosslinking Aids

Linear high molecular weight poly(trimethylene carbonate) (PTMC) was synthesized by ring opening polymerization of the TMC monomer under vacuum at 130 °C for three days using stannous octoate as catalyst. The polymer was purified by dissolution in chloroform and precipitation into ethanol, washing with fresh ethanol and drying at room temperature under vacuum.

By using 1,3-propanediol (20 mol %) or trimethylol propane (14.3 mol %) as initiators, two-armed and three-armed hydroxyl-group terminated PTMC oligomers were synthesized by ring-opening polymerization (oligomerization). The syntheses were conducted under argon at 130°C for 48 h, using stannous octoate as catalyst. To obtain methacrylate-functionalized PTMC oligomers (macromers), the hydroxyl-group terminated oligomers were reacted with a 30 mol % excess of methacrylic anhydride at 120 °C for 8 h. To prevent premature crosslinking during this step, hydroquinone and ($\pm$)- α -tocopherol (0.06 wt %) were used as inhibitors. The excess methacrylic anhydride and the formed methacrylic acid were removed by distillation under reduced pressure. The PTMC macromers were further purified by dissolution in acetone, precipitation into water and freeze drying.

Scheme 1. Chemical structures of the synthesized two -armed PTMC macromer (DMAC) (A), three-armed PTMC macromer (TMAC) (B), and linear PTMC polymer (C).

Characterisation

Conversion of TMC monomer was determined by proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy (300 MHz, Varian Innova, U.S.A.) using CDCl_3 .

Number average- and weight average molecular weights ($\overline{M}_n$ and $\overline{M}_w$, respectively), polydispersity index (PDI) and intrinsic viscosity ($[\eta]$) of PTMC was determined by gel permeation chromatography (GPC, Viscotek, U.S.A.). The setup was equipped with ViscoGEL I-guard-0478, ViscoGEL I-MBHMW-3078, and ViscoGEL I-MBLMW-3078 columns placed in series and a TDA 302 Triple Detector Array with refractometer-, viscometer-, and light scattering detectors, allowing the determination of absolute molecular weights. All determinations were performed at 30 °C, using chloroform as the eluent at a flow rate of 1.0 ml/min.

Molecular weights of the two- and three-armed macromers (dimethacrylate oligocarbonate, DMAC, and trimethacrylate oligocarbonate, TMAC, respectively) were determined by ^1H -NMR spectroscopy.

Preparation of PTMC Films

The PTMC homopolymer was mixed with different amounts of photoinitiator and macromer crosslinking aids by dissolving and mixing the compounds in dichloromethane in the dark. After evaporation of the solvent, compression moulded films were prepared using 500 μm thick stainless steel moulds and a laboratory press (Fonteijne THB008, The Netherlands). The films were moulded at approximately 25 kg/cm^2 , at $140\text{ }^\circ\text{C}$ and quenched to room temperature using cold water.

Photocrosslinking and Network Characterization

Compression moulded films containing macromers (DMAC or TMAC) and photoinitiator were vacuum sealed in laminated polyethylene/polyamide bags (Hevel Vacuum B.V., The Netherlands) and exposed to short wave UV light (UltraLum crosslinking cabinet, U.S.A., wavelength 254 nm) at a distance of 7 cm. Both sides of the specimens were illuminated at room temperature for different time periods. The light intensity at this distance was $10\text{--}14\text{ mW/cm}^2$, the polyethylene/polyamide bags reduced the intensity to $5\text{--}7\text{ mW/cm}^2$ as measured with an optical power meter (Newport 1916-C, U.S.A.).

To determine equilibrium swelling ratios and gel contents, disk-shaped specimens (500 μm thick, 10 mm in diameter) were punched out from the irradiated films and placed in 30 mL CHCl_3 for 1 week, the solvent was refreshed once after 3 days. This procedure ensured complete removal of the sol fraction. Then the swollen gels were weighed, dried to constant weight at room temperature *in vacuo* and weighed again. The gel and the sol fractions were calculated according to equations (1) and (2) respectively:

$$\text{Gel fraction (\%)} = \frac{m_d}{m_0} \times 100 \quad (1)$$

$$\text{Sol fraction (\%)} = \left(1 - \frac{m_d}{m_0}\right) \times 100 \quad (2)$$

where m_d is the mass of dried (extracted) samples and m_0 is the mass of the specimens before swelling.

The volume swelling ratio (q) was calculated according to equation (3).

$$q = 1 + \rho_p \times \left(\frac{m_s}{m_d \times \rho_s} - \frac{1}{\rho_s} \right) \quad (3)$$

where m_s is the mass of the extracted and swollen samples, and ρ_s and ρ_p are the densities of chloroform (1.48 g/cm³) and PTMC (1.31 g/cm³), respectively.

Mechanical Properties

The tensile properties of photocrosslinked PTMC films measuring approximately 100x5x0.5 mm³ were determined in triplicate after extraction in ethanol according to ASTM-D 882-91. A Zwick Z020 tensile tester (Ulm, Germany) equipped with a 500 N load cell was operated at a crosshead speed of 50 mm/min. The initial grip-to-grip separation was 50 mm and a preload of 0.01 N was applied. The specimen deformation was derived from the grip-to-grip separation; therefore the presented values of Young's modulus (calculated from the initial slope of the stress-strain curves) give only an indication of the stiffness of the polymers.

To assess their behaviour under dynamic loading conditions, the specimens (n=1) were repeatedly (20x) elongated to 50 % strain at 50 mm/min in cyclic tests. After a 2 h recovery period, the permanent deformation was estimated from the stress-strain diagram of the 21st cycle. In these experiments a preload of 0.01 N was applied, the deformation was derived from the grip to grip separation. The error in the values is approximately 0.5 % strain.

Human Mesenchymal Stem Cell Culturing

Human mesenchymal stem cells (hMSCs) were cultured on disk-shaped, photocrosslinked and ethanol-extracted PTMC films (15 mm in diameter and approximately 200 µm thick). Prior to cell culturing, the films were sterilized by immersing in 70 % ethanol for 15 min and then washing with sterile PBS. hMSCs were obtained from a donor undergoing total hip replacement surgery, who gave informed consent. Approval was obtained from the local medical ethical committee. The cells (passage 2) were cultured at an initial seeding density of 5 x10⁴ cells/cm² using αMEM. The medium was refreshed twice every week.

After four weeks of culturing, the cells on the films were fixed with 3.7 % paraformaldehyde. Half the specimens were sputter-coated with gold and their surfaces were analysed by scanning electron microscopy (SEM, Philips XL 30 ESEM-FEG, The Netherlands) at an operating voltage of 5 kV. The remaining half was used for fluorescence

staining of cell nuclei using 4',6-diamidino-2-phenylindole (DAPI). The actin cytoskeleton of the cells was stained using mouse monoclonal α -sarcomeric actin as primary antibody and fluorescein isothiocyanate (FITC) labelled isotype-specific goat anti-mouse secondary antibody. These specimens were then analysed by confocal laser scanning microscopy (CLSM, Zeiss LSM 510 equipped with a 20X objective).

In Vitro Erosion Studies

J774A macrophages that were used in macrophage mediated erosion studies were maintained in DMEM containing 4.5 g/L D-glucose, pyruvate, 10 % fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin and 2mM GlutamaxTM. Cells were passaged every 4 to 7 days by scraping.

The erosion of photocrosslinked and ethanol-extracted polymer films in macrophage cultures was investigated by directly culturing J774A macrophages on the surface of the films. The test specimens were 15 mm in diameter and approximately 500 μ m in thickness. The seeding density was approximately 8×10^4 cells/cm². After 8 days of culturing cells on the different surfaces, fresh aliquots of cells were added to the culture. Cells were cultured on six disks of each material. Medium was refreshed once a week.

After 14 days of culturing, three specimens of each material were first placed in Milli-Q water to lyse the cells, and then thoroughly rinsed and weighed. After drying the films to constant weight *in vacuo* at room temperature, the samples were weighed again. Evaluation of the surfaces of the specimens was performed by SEM as described earlier.

The remaining disks of each material (n=3) were fixed with 3.7 % para-formaldehyde in cytoskeletal stabilizing (CS) buffer (0.1 M piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer, 1 mM ethylene glycol tetraacetic acid (EGTA), pH=6.9) for 15 min and then transferred to PBS. The specimens were used for fluorescence staining of cell nuclei using DAPI and of the actin cytoskeleton using tetramethyl rhodamine iso-thiocyanide-phalloidin (TRITC-phalloidin). Samples were analysed using CLSM (Leica TCS SP2 equipped with a 40X NA0.80 full immersion water lens) to allow in depth visualisation of adhered cells.

Cholesterol esterase (CE) from porcine pancreas was used to study the enzymatic hydrolysis of non-crosslinked and photocrosslinked PTMC films. Aqueous CE enzyme solutions were prepared at a concentration of 20 μ g/mL using phosphate buffered saline (PBS, pH=7.4) containing 0.02 wt % NaN₃ (Sigma, U.S.A.) as a bactericide. Ethanol extracted, disk-shaped films (8 mm diameter, approximately 500 μ m thickness, n=8) were placed in vials containing 1 ml of freshly prepared enzyme solution and conditioned at 37 °C. The medium

was refreshed once every two days. After 14 days of incubation, the mass of the wet specimens was determined after rinsing and blotting their surfaces. Measurements were performed again after drying the specimens to constant weight *in vacuo* at room temperature.

In vitro erosion of PTMC (network) films having the same dimensions was also investigated by incubation in aqueous KO₂ solutions [5M] that were prepared using a NaOH/NaCl buffer (pH=13). Specimens (n=4) were placed in vials containing 1 mL of the freshly prepared superoxide solutions. The solutions were refreshed daily. After one week incubation at 37 °C, mass loss was determined as described earlier.

Wettability and Water Uptake

Contact angle measurements were performed on films prepared by casting polymer solutions in dichloromethane (approximately 1 wt %) on glass discs (n=6 per material), drying under vacuum, and photocrosslinking in an inert atmosphere. At room temperature, the static, advancing and receding contact angles of ultra-pure water (MilliQ Plus-Millipore, France) on the different surfaces were determined using a video-based system (OCA 20 DataPhysics Instruments GmbH, Germany) equipped with an electronic syringe module.

The equilibrium water uptake of the compression moulded and photocrosslinked films was determined after extraction with ethanol. The specimens (n=4) were conditioned in PBS (pH=7.4) at 37 °C for one week. Water uptake was defined as the mass increase of the specimens.

Results and Discussion

Linear high molecular weight PTMC with $\overline{M}_n = 291$ kg/mol was synthesized by ring opening polymerisation of TMC. PTMC macromers were prepared by initiating the ring opening polymerization of TMC with small amounts of 1,3-propanediol or trimethylol propane, and subsequent reaction of the two-armed and three-armed oligomers with methacrylic anhydride. The methacrylate-functionalized DMAC- and TMAC macromers were used as crosslinking aids during the irradiation of high molecular weight PTMC with UV light.

¹H NMR analyses showed that in the ring opening polymerization (oligomerization) reactions the conversion of TMC was higher than 95 %, and that in the macromers more than 99 % of the hydroxyl endgroups had been converted to methacrylates. The number average molecular weights of the purified DMAC and TMAC macromers were also determined by NMR, the respective values were 810 g/mol and 1100 g/mol (or 2.9 and 2.5 TMC units/arm).

Photocrosslinking of PTMC Films Using Photoinitiators

In preliminary experiments, films of linear high molecular weight PTMC containing different photoinitiators (PI) were prepared and irradiated with short wave UV light for 30 to 120 min. (The PI to TMC repeating unit in the polymer molar ratios were 1:1000). It was surprisingly found that when Irgacure® 2959, Irgacure® 500, or benzophenone was used as PI, PTMC networks with high gel contents were obtained. With these PIs, networks with 84-87 % gel were obtained after 120 min irradiation. When PTMC films that contained DMPA, or Irgacure® 369 were irradiated for 120 min, the gel contents were much lower: 62 and 0 %, respectively.

Polyethylene and poly(ethylene oxide) also have been shown to crosslink upon UV irradiation in the presence of a photoinitiator that undergoes Norrish type II photolysis, like benzophenone, and acts as a hydrogen abstracting agent ^{35, 36}. It was proposed for both polymers that crosslinking occurs by combination of two macroradicals to form H-type crosslinks. In addition, poly(ethylene oxide) could also be crosslinked in the presence of photoinitiators that undergo Norrish type I photolysis (α -cleavage), less efficiently. This was also the case in our experiments with PTMC, where photoinitiators that undergo Norrish type I photolysis (DMPA and Irgacure® 369) gave lowest network gel contents.

Irgacure® 2959 was as efficient as benzophenone, and considering its good compatibility with a broad range of mammalian cell types ^{33, 34}, it was used as PI in further experiments.

The effect of the PI concentration on the gel content and network density was assessed by irradiating PTMC films containing varying amounts of Irgacure® 2959 for durations ranging from 30 min up to 300 min. For all irradiation times (30, 60, 120, 180, 240, and 300 min), increasing the PI content of the PTMC films initially increased the gel contents and decreased the swelling ratios of the formed PTMC networks. Figure 1 illustrates this for an irradiation time of 120 min. For all irradiation times, the optimal PI/TMC ratio (where the highest gel contents and lowest swelling ratios were obtained) was found to be 1/1000. The decrease in gel contents at PI concentrations higher than 1/1000 is probably due to a decrease in the quantum yield. This can be due to competing reactions: the photoinitiating radicals can either react with PTMC chains or the photoinitiating radicals can react with each other or with PTMC macroradicals ³⁷.

Figure 1. Effect of Irgacure® 2959 photoinitiator concentration on the gel content and swelling ratio of networks obtained by UV irradiation of PTMC films for 120 min.

For all PI concentrations, irradiating the films for less than 120 min resulted in lower network gel contents and higher swelling ratios. Increasing the irradiation time beyond 120 min did not yield networks with higher gel contents or higher network densities.

Photocrosslinking of PTMC Films Using Irgacure® 2959 Photoinitiator and PTMC Macromers as Crosslinking Aids

To investigate their crosslinking properties, high molecular weight PTMC films containing varying amounts of Irgacure® 2959 photoinitiator and varying amounts of PTMC macromers were exposed to UV light. In all cases, upon UV irradiation, transparent PTMC networks were formed.

At a constant concentration of PTMC macromers (1/50 methacrylate/TMC repeating unit in the polymer), the gel contents of the formed networks increased and the swelling ratios decreased with increasing PI concentration (1/8000, 1/4000, 1/2000, 1/1000 PI/TMC repeating unit in the polymer). This was observed when both DMAC and TMAC were used as the crosslinking aid. For instance when DMAC containing films were irradiated for 30 min, the gel content of the formed networks increased from 53 ± 2 % to 89 ± 1 % as the PI/TMC ratio was increased from 1/8000 to 1/1000. The corresponding swelling ratios in chloroform were 23.2 ± 0.6 and 13.9 ± 0.2 vol/vol. Therefore, the highest PI/TMC ratio of 1/1000 was used in further experiments. When the PTMC films were irradiated for 60 min or 120 min, the same trend was observed: increasing the irradiation time also yielded networks with higher gel contents and lower swelling ratios.

The effect of irradiation time and the crosslinking aid used on the gel content and swelling ratio of the PTMC network films is shown in Figure 2. The PTMC films had

PI/TMC ratios of 1/1000, and methacrylate/TMC ratios of 1/50 for DMAC or TMAC containing films. Depending on the UV irradiation time, the gel contents and swelling ratios of networks prepared from PTMC films that did not contain a PTMC macromer (PTMC-PI) ranged from 64 ± 3 to 87 ± 2 % and from 48 ± 1 to 14.6 ± 1.2 vol/vol, respectively. For the networks prepared from films that contained PTMC macromers, the gel contents ranged from 89 ± 1 to 95 ± 1 % for PTMC-PI-DMAC and from 91 ± 1 to 94 ± 1 % for PTMC-PI-TMAC. Importantly, the density of these networks could be tuned by adjusting the irradiation time while maintaining high gel contents. The swelling ratios in chloroform ranged from 13.9 ± 0.2 to 8.5 ± 0.1 vol/vol.

Figure 2. Effect of irradiation time and crosslinking aid used on gel content (A), and swelling ratio (B) of photocrosslinked PTMC networks. In all PTMC films, the PI/(TMC repeating unit in the polymer) ratio was 1/1000. PTMC-PI films contain only photoinitiator and no PTMC macromer. For PTMC films containing DMAC and TMAC, the methacrylate/(TMC repeating unit in the polymer) ratio was 1/50.

It can also be seen in Figure 2 that using a PTMC macromer in the films as a crosslinking aid significantly increases the rate of crosslinking, yielding high gel contents and low swelling ratios much faster than PTMC films containing only photoinitiator: After 30 min UV irradiation, PTMC-PI, PTMC-PI-DMAC, and PTMC-PI-TMAC had gel contents of 64 ± 3 , 89 ± 1 , 91 ± 1 %, respectively. The corresponding swelling ratios were 48 ± 1 , 13.9 ± 0.2 , 12.2 ± 0.3 vol/vol. Differences in characteristics of networks prepared from PTMC films containing a dimethacrylate- or a trimethacrylate crosslinking aid were minimal, although the TMAC network films seemed to be slightly more densely crosslinked.

We also investigated the effect of macromer content in the films on PTMC network formation. At a constant PI/TMC ratio (1/1000), we varied the TMAC content of the films.

We could readily tune the network density of these films in this manner as well: The swelling ratios of the networks were 14.6 ± 1.2 , 12.4 ± 0.3 , 11.4 ± 0.3 , 8.5 ± 0.1 vol/vol for ratios of methacrylate/TMC of 0, 1/200, 1/100, 1/50, respectively. All abovementioned networks had high gel contents ranging from 87 ± 2 to 94 ± 1 %.

The high gel contents and network densities observed, suggest that the macromer is incorporated into the PTMC network. Earlier studies on the photopolymerization of multifunctional methacrylates mixed into different polymer matrices, have shown that semi-interpenetrating networks are formed^{38, 39}. Growing methacrylate chains can be terminated by combination with other growing methacrylate chains or with formed polymer macroradicals. In addition, chain transfer to the polymer can occur.

Mechanical Properties of Photocrosslinked PTMC Networks

The tensile properties of compression moulded films prepared from linear PTMC and from photocrosslinked and extracted PTMC network films are given in Table 1, while representative stress-strain curves are shown in Figure 3. It can be seen from the Figure and the Table that photocrosslinking PTMC has led to significantly higher stress at break values, while the strain at break values remained very high. Photocrosslinking results in films with much higher toughness. PTMC networks prepared from high molecular weight PTMC films containing TMAC as crosslinking aid had stress at break values as high as 24 ± 3 MPa. Moreover, the flexible character of PTMC was not influenced much by the crosslinking. The E-moduli of films prepared from linear PTMC were 6.6 ± 0.5 MPa, whereas the values ranged from 7.1 ± 0.1 to 7.5 ± 0.2 for the photocrosslinked PTMC films. This is most important, as these networks are intended to be used in medical applications involving soft tissues.

Figure 3. Representative curves showing the stress-strain behaviour of linear and photocrosslinked extracted PTMC network films. In all PTMC films, the PI/(TMC repeating unit in the polymer) ratio was 1/1000. PTMC-PI films contain only photoinitiator and no PTMC macromer. For PTMC films containing DMAC or TMAC, the methacrylate/(TMC repeating unit in the polymer) ratio was 1/50. Curves are offset for clarity.

When culturing cells in tissue engineering scaffolds bioreactors are often used, as this allows to closely match the dynamic conditions *in vivo*. Therefore, the materials to be used in such applications should be able to withstand and recover from applied repetitive cyclic stresses. We evaluated the creep resistance of our network films by applying 20 cycles of extension to 50 % strain. The stress-strain curves showing these 20 cycles for different materials are given in Figure 4. Non-irradiated PTMC films had the highest creep after 20 cycles (15.3 %). After a 2 h recovery period, their permanent set was 1.6 % strain (Table 1). The photocrosslinked network films showed significantly improved elastic behaviour with much less creep in the cyclic tests. After the 20th cycle, PTMC-PI, PTMC-PI-DMAC, and PTMC-PI-TMAC had creep values of 9.9, 7.5, and 7.6 % strain, respectively. The permanent set values for these networks were as low as 0.6 % strain.

Another phenomenon observed in the cyclic testing, was decrease in the maximum stress value that is reached at the end of the loading cycle due to stress relaxation. Stress relaxation is undesired, as it would lead to dilatation and failure of constructs such as blood vessel tissue engineering scaffolds and grafts when cyclic stresses are applied. By photocrosslinking, the stress relaxation of PTMC could also be reduced. For non-crosslinked PTMC, the maximum stress decreased from 3.2 to 2.4 MPa, while in the case of photocrosslinked films this decrease was only 0.3-0.4 MPa.

Figure 4. Hysteresis behaviour of linear PTMC and UV-crosslinked (120 min) extracted PTMC network films containing only photoinitiator or photoinitiator and PTMC macromers. In all PTMC network films, the PI/(TMC repeating unit in the polymer) ratio was 1/1000. PTMC-PI network films contain only photoinitiator and no PTMC macromer. For PTMC network films containing DMAC or TMAC, the methacrylate/(TMC repeating unit in the polymer) ratio was 1/50. Curves are offset for clarity.

In preliminary experiments, we applied cyclic biaxial deformations (1 Hz, 10 % strain) to one of the networks prepared using a Flexercell™ bioreactor set-up. After four weeks of incubation in culture medium at 37 °C, these networks did not show signs of permanent deformation under the applied conditions, implying that these materials can be used for the long term culturing of cells under cyclic stresses and strains.

Table 1. Mechanical properties of PTMC and of PTMC networks prepared by UV photocrosslinking of PTMC films containing only photoinitiator or photoinitiator and PTMC macromers. Values are expressed as mean $\pm$ standard deviation, ($n=3$).

Network	Irradiation time (min)	PI/PTMC ^a (mol/mol)	Methacrylate/TMC ^a (mol/mol)	E (MPa)	σ_{yield} (MPa)	ϵ_{yield} (%)	σ_{break} (MPa)	ϵ_{break} (%)	Energy to break (Joules)	Permanent Set ^b (%)
PTMC ^c	non-irradiated		0	6.6 $\pm$ 0.5	2.1 $\pm$ 0.1	143 $\pm$ 5	4.6 $\pm$ 0.7	892 $\pm$ 47	2.2 $\pm$ 0.2	1.6
PTMC-PI ^d	120	1/1000	0	7.1 $\pm$ 0.1	2.2 $\pm$ 0.1 ^e	95 $\pm$ 7 ^e	16 $\pm$ 1	878 $\pm$ 7	4.0 $\pm$ 0.1	0.8
PTMC-PI- DMAC ^d	120	1/1000	1/50	7.1 $\pm$ 0.6	2.3 $\pm$ 0.1 ^e	90 $\pm$ 5 ^e	22 $\pm$ 2	749 $\pm$ 28	4.7 $\pm$ 0.6	1.0
PTMC-PI- TMAC ^d	120	1/1000	1/50	7.5 $\pm$ 0.2	2.3 $\pm$ 0.1 ^e	90 $\pm$ 8 ^e	24 $\pm$ 3	731 $\pm$ 28	5.2 $\pm$ 0.7	0.6

^a Based on TMC repeating units in the polymer.

^b Single measurements. The permanent set is estimated from the 21st cycle, after a two hours recovery period. The error is approximately 0.5 % strain.

^c Linear PTMC polymer, purified by precipitation and drying *in vacuo*, and subsequently compression moulded

^d Networks were extracted with ethanol and dried *in vacuo*.

^e Estimated from the intersection of tangents to stress-strain diagrams, as a distinct yield point could not be observed.

Human Mesenchymal Stem Cell Culturing

To assess the compatibility of photocrosslinked and extracted PTMC networks with cells, human mesenchymal stem cells (hMSCs) were cultured on PTMC-PI-TMAC (1/50 methacrylate/TMC repeating unit in the polymer) surfaces. The hMSCs remained viable during long-term culturing; Figure 5 shows hMSCs on the surface of the films after four weeks. At this time point, a dense population of hMSCs having an elongated morphology was present on the network films. These cells stained positive for α -sarcomeric actin (Figure 5) indicating their differentiation towards a myogenic phenotype, although this topic needs further investigation with cells from different donors to confirm this observation. The cell culturing experiments showed that these photocrosslinked PTMC networks are suitable materials for the preparation of tissue engineering scaffolds, as the hMSCs display a healthy morphology on these surfaces and remain viable for a long period of time.

Figure 5. SEM (A) and CLSM (B) micrographs showing hMSCs after 28 days of culturing on PTMC-PI-TMAC (1/50 methacrylate/TMC repeating unit in the polymer) films crosslinked by UV-irradiation for 120 min. The ratio of PI to TMC repeating unit in the polymer was 1/1000. In the figure, the nuclei are stained blue and the actin cytoskeleton is stained green.

In Vitro Erosion and Wettability of Photocrosslinked PTMC Networks

Soon after implantation of a resorbable polymer, macrophages become the predominant cells at the tissue-biomaterial interface. Macrophages are involved in events of inflammation that include the foreign body reaction, as well as in biomaterial degradation and erosion⁴⁰. Macrophage culturing has been used as an initial *in vitro* assay for assessing the biocompatibility and biodegradation of degradable polymers⁴¹⁻⁴³. To assess the erosion behaviour of photocrosslinked PTMC networks, we cultured macrophages on extracted network films and compared their erosion with that of linear PTMC films. All network films

contained 1/1000 PI/TMC repeating unit with varying amounts of TMAC (methacrylate/TMC repeating unit 0, 1/200, 1/100, 1/50) and were photocrosslinked by 120 min UV-irradiation.

Figure 6. SEM micrographs showing the macrophage-mediated erosion of films prepared from linear PTMC (A,B), and photocrosslinked (120 min) extracted films having different ratios of methacrylate/(TMC repeating unit in the polymer): PTMC-PI (C,D), PTMC-PI-TMAC (1/200) (E,F), PTMC-PI-TMAC (1/100) (G,H), PTMC-PI-TMAC (1/50) (I,J) network films. In all PTMC network films, the ratio of PI/(TMC repeating unit in the polymer) was 1/1000. PTMC-PI network films contain only photoinitiator and no PTMC macromer.

Macrophages adhered to all surfaces as isolated cells without the formation of giant cells or significant cell groups. After 14 days of macrophage culturing, all PTMC films were found to have eroded. Their mass had decreased and the formation of pits on the surface of the films was observed, indicating erosion of the surfaces during macrophage culture (Figure 6). An overview of the erosion rates of the films in macrophage cultures is given in Table 2. It is worth noting that the PTMC materials and their degradation products were not cytotoxic and did not induce activation of the macrophages. This is an indication of the biocompatibility of these materials, and corroborates with our observations using mesenchymal stem cells.

Table 2. Surface erosion rates of non-crosslinked and photocrosslinked PTMC films in macrophage (MQ) culture, or incubated in aqueous cholesterol esterase or potassium dioxide solution.

Network	PI/TMC ^a (mol/mol)	MA/TMC ^a (mol/mol)	Erosion rate in MQ cultures ($\mu\text{g}/(\text{cm}^2 \times \text{day})$)	Erosion rate in CE solutions ($\mu\text{g}/(\text{cm}^2 \times \text{day})$)	Erosion rate in KO ₂ solutions ($\mu\text{g}/(\text{cm}^2 \times \text{day})$)
PTMC ^b	Non-irradiated		159±8	2160±610	2040±360
PTMC-PI	1/1000	0	28±4	137±38	1280±130
PTMC-PI- TMAC ^c	1/1000	1/200	16±4	20±5	670±50
PTMC-PI- TMAC ^c	1/1000	1/100	12±3	13±4	670±15
PTMC-PI- TMAC ^c	1/1000	1/50	11±3	15±3	740±50

^a Based on TMC repeating units in the polymer

^b Linear PTMC polymer, purified by precipitation and drying *in vacuo*.

^c Networks were extracted with ethanol and dried *in vacuo*.

Macrophages were most effective in eroding the films prepared from linear PTMC. The erosion rate of these films was 159±8 ($\mu\text{g}/(\text{cm}^2 \times \text{day})$), which corresponds to a mass loss of 3.8±0.2 %. SEM revealed that the surfaces had been extensively eroded (Figures 6A and 6B). Erosion of PTMC films could be reduced by photocrosslinking, as erosion of the PTMC-PI network films was much less than that of the non-crosslinked PTMC films. This is of great importance, as linear or lightly crosslinked PTMC films erode relatively rapidly *in vivo*^{29, 30}, hindering their use in long term biomedical applications. The erosion rate could be reduced further by using PTMC macromers as crosslinking aids. The erosion rate of films having methacrylate to TMC ratios of 0, 1/200, 1/100, and 1/50 were respectively 28±4, 16±4, 12±1, 11±3 ($\mu\text{g}/\text{cm}^2 \times \text{day}$) (corresponding to mass losses ranging from 0.7 to 0.3 %). In Figures 6C

to 6J, the reduction in the extent of erosion of the films with increasing amounts of crosslinking aid is very clear; the pits on the surface of the films had become much shallower.

The observed reduction in macrophage-mediated erosion rate of photocrosslinked films could be due to the formation of a (denser) network that makes the carbonate bonds less accessible for (enzymatic) hydrolysis. As mentioned earlier the swelling ratios of these networks in chloroform ranged from 14.6 ± 1.2 to 8.5 ± 0.1 vol/vol. Another reason for the reduction in erosion rate could be due to differences in adhesion and proliferation of macrophages on the different surfaces. We determined the number of cells on the different network films after 14 days of culturing. It appears from Figure 7 that the network films containing TMAC as a crosslinking aid had lower numbers of macrophages (which ranged from 2607 ± 1033 to 4082 ± 681) than on non-crosslinked PTMC films (5657 ± 755 cell/mm²) or on PTMC-PI network films (6574 ± 1433 cell/mm²). This reduction in cell numbers on the different materials is, however, much less than the reduction in erosion rate of the different films.

Figure 7. Density of macrophages after 14 days of culturing on films prepared from linear PTMC, and photocrosslinked (120 min) extracted network films having different ratios of methacrylate/(TMC repeating unit in the polymer). In all PTMC network films, the ratio of PI/(TMC repeating unit in the polymer) was 1/1000. PTMC-PI films contain only photoinitiator and no PTMC macromer.

The water in air contact angles and the water uptake of non-crosslinked and of photocrosslinked PTMC films are given in Table 3. The values indicate that PTMC and its networks are relatively hydrophobic. All films gained approximately 1 % weight upon incubation in PBS for one week. Upon crosslinking and with increasing amounts of TMAC, the contact angles increased from 80° to 97°. The adhesion of cells on biomaterials depends to a large extent on protein adsorption which is dictated by the surface properties of biomaterials^{44, 45}. As cell adhesion to very hydrophobic or very hydrophilic surfaces is usually low, this

may have led to the adhesion of lower numbers of macrophages on TMAC containing network films when compared to the other PTMC films. Less strong adhesion of individual macrophages to the TMAC containing network films may result in a reduced effectiveness of released hydrolytic enzymes and reactive oxygen species in degrading the polymer ⁴⁶.

Table 3. Effect of photocrosslinking and macromer content on water contact angles and water uptake values of PTMC films. Values are expressed as mean $\pm$ standard deviation.

				Water contact angle (°) ^b		
Network	PI/TMC (mol/mol)	MA/TMC (mol/mol)	Water uptake (%) ^a	Static	Advancing	Receding
PTMC	Non-irradiated		1.2±0.3	80.1±2.7	83.3±1.4	48.6±1.7
PTMC-PI	1/1000	0	0.9±0.2	87.9±2.0	89.7±2.3	51.8±1.3
PTMC-PI-TMAC	1/1000	1/200	1.3±0.3	91.1±0.7	93.1±0.6	53.8±0.4
PTMC-PI-TMAC	1/1000	1/100	0.9±0.2	97.3±1.6	99.9±1.6	60.9±0.7
PTMC-PI-TMAC	1/1000	1/50	0.8±0.2	97.2±2.1	99.4±1.32	60.5±1.2

^a Compression moulded films were conditioned in PBS at 37 °C for one week (n=4).

^b Performed on polymer films cast on glass discs (n=6).

Previously, hydrolytic enzymes and oxidative species that can be secreted by macrophages have been shown to erode PTMC networks ^{21, 43}. We investigated the erosion of our photocrosslinked PTMC networks using aqueous cholesterol esterase (CE) and aqueous KO₂ solutions. Table 2 shows erosion rates of non-crosslinked and photocrosslinked PTMC films determined upon incubation in these solutions.

Upon incubation in aqueous cholesterol esterase solutions, non-crosslinked PTMC films eroded linearly in time. Surface erosion of non-crosslinked PTMC specimens proceeded at a rate of 2160 $\pm$ 600 ($\mu\text{g}/\text{cm}^2 \times \text{day}$) leading to a mass loss of 87.9 $\pm$ 1.6 % in 14 days. In this case, photocrosslinking had a more dramatic effect on enzymatic erosion of PTMC than that observed in the macrophage-mediated erosion. Upon 15 days of incubation in CE solutions, mass loss of the network films having 0 to 1/50 methacrylate to TMC ratios ranged from 6.6 $\pm$ 1.8 % to 0.6 $\pm$ 0.2 % (corresponding to rates from 137 $\pm$ 38 to 15 $\pm$ 3 ($\mu\text{g}/\text{cm}^2 \times \text{day}$)).

Upon incubation in superoxide anion radical containing buffers, the mass of non-crosslinked and crosslinked PTMC films decreased at a constant rate, implying their surface erosion in these media. The erosion rate of non-crosslinked PTMC was 2040 $\pm$ 360

($\mu\text{g}/\text{cm}^2 \times \text{day}$), which is comparable to that observed when these films were incubated in CE solutions (2160 ± 610 ($\mu\text{g}/\text{cm}^2 \times \text{day}$)). Upon one week incubation in aqueous KO_2 solutions, the mass loss of non-crosslinked films was 48.7 ± 8.5 %. The degradation of PTMC by oxidative species was also reduced by photocrosslinking in the presence of photoinitiator only. Upon one week incubation, the mass loss of PTMC-PI networks was 30.8 ± 3.7 , whereas PTMC-PI-TMAC networks had a mass loss of approximately 16 ± 0.5 %.

Conclusions

High molecular weight PTMC can be photocrosslinked using low molecular weight PTMC macromers and Irgacure® 2959 photoinitiator as crosslinking aids. The characteristics (network properties, mechanical properties, *in vitro* erosion behaviour, water contact angles and wettability) of the obtained networks can be tuned by adjusting the amounts of the crosslinking aid incorporated. These surface eroding elastomeric PTMC networks have excellent mechanical properties. Moreover, initial *in vitro* assays showed that these materials and their degradation products are compatible with (stem)cells. These materials are most suited for biomedical applications such as (soft-) tissue engineering and controlled release of biologically active compounds.

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PART IV

APPENDICES

Appendix A

Effect of Crystallinity on Biodegradation and Mechanical Properties of Gamma Irradiated Poly(trimethylene carbonate-co- ϵ -caprolactone) Films^{*}

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Abstract

A series of trimethylene carbonate (TMC) and ϵ -caprolactone (CL) (co)polymers were synthesized. By adjusting the copolymer composition, amorphous to semi-crystalline (co)polymers were obtained. Compression moulded films were gamma irradiated under vacuum, yielding networks with gel contents ranging from 59 to 67 %. The effect of crystallinity on physical properties and on erosion behaviour of these (co)polymer network films was investigated. Homopolymer and copolymer networks that were amorphous at room temperature were flexible and rubbery with elastic moduli ranging from 1.8 ± 0.3 to 5.2 ± 0.4 MPa, while the elastic moduli of the semi-crystalline networks were higher and ranged from 61 ± 3 to 484 ± 34 MPa. Upon being subjected to repetitive cyclic strains, the amorphous

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network films had permanent deformations as low as 0.9 % strain, whereas the values were higher than 9 % strain for the semi-crystalline networks. The erosion behaviour of (co)polymer networks was investigated *in vitro* using macrophage cultures, and *in vivo* by subcutaneous implantation in rats. In 15 days, amorphous network films had lost approximately 4 % mass in macrophage cultures, whereas the semi-crystalline ones had hardly eroded. Also in implantation studies, the amorphous networks eroded much faster than the semi-crystalline PCL networks. In macrophage cultures, as well as upon implantation, a transition from a surface erosion process to a bulk erosion process was observed with increasing crystallinity. This study shows that the physical properties and the erosion behaviour of TMC and CL (co)polymer networks largely depend on the crystallinity of the (co)polymer which can be tuned by adjusting the copolymer composition.

Introduction

Synthetic resorbable polymers are used in a growing number of biomedical applications such as temporary fracture fixation devices, tissue engineering, controlled release of drugs and proteins, or delivery of genes ¹⁻³. The degradation behaviour and the physical properties of resorbable polymers are of great importance in the successful application of these materials. For instance, when cells are to be cultured on these materials, the stiffness of the matrix, which depends on parameters such as chemical structure, glass transition temperature and crystallinity of the polymer, plays an important role in determining the behaviour of cells. It influences the spreading, motility ⁴, proliferation ⁵ and differentiation of (stem) cells ^{6, 7}. Also the level of gene transfer to cells and the expression of genes was found to depend on the properties of the substratum the cells were cultured on ⁸. Furthermore, surface characteristics such as wettability and charge influence the adsorption of proteins which subsequently governs the cell adhesion ⁹.

The degradation rate of the material and the manner in which the degradation progresses can also affect the response of cells and tissues to polymers ¹⁰⁻¹³ and the extent of new tissue formation ^{14, 15}. Most resorbable polymers degrade passively *in vivo* by non-enzymatic bulk hydrolysis ^{2, 16, 17}. During degradation of these polymers, molecular weight and mechanical strength rapidly decrease due to random cleavage of main chain bonds throughout the bulk of the implant. In this case, no mass loss is observed until a critical low molecular weight is reached. This results in fragmentation of the matrix and burst release of degradation products (and loaded drugs) which could be harmful for the cells and tissues. In the case of a surface erosion process degradation occurs mainly at the surface of the implant

¹⁶. This is advantageous in tissue engineering applications, as the scaffold does not fragment prematurely and its mechanical strength does not deteriorate very much during mass loss ¹⁸.

Poly(trimethylene carbonate) (PTMC) is an amorphous polymer with a low glass transition temperature ¹⁹ that can be crosslinked into a flexible, form-stable and creep-resistant network by gamma irradiation ²⁰. Such properties are desired in the engineering of soft tissues such as cardiovascular tissues. However, the relatively rapid surface erosion of PTMC *in vivo* ²¹ only allows its use in short term applications. Also poly(ϵ -caprolactone) (PCL), which is a more rigid semi-crystalline polymer, crosslinks upon gamma irradiation. The *in vivo* degradation of PCL occurs by bulk hydrolysis with complete resorption taking years ¹⁷.

Previously, we investigated the mechanical properties and the erosion behaviour of amorphous poly(trimethylene carbonate-co- ϵ -caprolactone) networks which contained 70 to 89 % TMC ^{22, 23}. Upon subcutaneous implantation in rats, however, these copolymer networks eroded at rates that were similar to those of the rapidly eroding PTMC homopolymer networks ²². In the current study, we evaluated the physical properties and investigated the erosion behaviour of both amorphous and semi-crystalline TMC and ϵ -caprolactone (co)polymer networks *in vitro* using macrophage cultures and *in vivo* by subcutaneous implantation in rats.

Materials and Methods

Materials

Polymer grade 1,3-trimethylene carbonate (Boehringer Ingelheim, Germany) and stannous octoate (Sigma, U.S.A.) were used as received. ϵ -Caprolactone (Aldrich, U.K.) was dried over CaH₂ and purified by distillation under reduced argon atmosphere. Solvents (Merck, Germany) were of analytical grade. J774A macrophages (ATCC-TIB-67) were obtained from the American Type Culture Collection. Culture media, fetal bovine serum, Glutamax™ and penicillin-streptomycin were obtained from Invitrogen (Gibco, U.S.A.). Culture disposables were from Nunc (U.S.A.) and Greiner (Germany).

Preparation of Polymer Films and Network Formation by Gamma Irradiation

Poly(trimethylene carbonate) and poly(trimethylene carbonate-co- ϵ -caprolactone) copolymers were synthesized by ring opening polymerization of the corresponding monomers under vacuum at 130 °C for three days using stannous octoate as a catalyst. The synthesized polymers were purified by dissolution in chloroform and precipitation into ethanol. The

precipitated polymers were washed with fresh ethanol and dried at room temperature under vacuum.

Monomer conversion and copolymer composition were determined by proton nuclear magnetic resonance (^1H NMR) spectroscopy (300 MHz, Varian Innova, U.S.A.) using CDCl_3 . Number average- and weight average molecular weights ($\overline{M}_n$ and $\overline{M}_w$, respectively) and polydispersity indices (PDI) of the polymers were determined by gel permeation chromatography (GPC, Viscotek U.S.A.) using chloroform as the eluent at a flow rate of 1.0 ml/min. The setup was equipped with ViscoGEL I-guard-0478, ViscoGEL I-MBHMW-3078, and ViscoGEL I-MBLMW-3078 columns placed in series and a TDA 302 Triple Detector Array with refractometer-, viscometer- and light scattering detectors, allowing the determination of absolute molecular weights. Glass transition temperatures (T_g), peak melting temperatures, and melting enthalpies of (co)polymers were determined by differential scanning calorimetry (DSC). Samples (5-10 mg) were analyzed at a heating rate of 10 $^\circ\text{C}/\text{min}$ in a temperature range of -100 to 100 $^\circ\text{C}$ using a Perkin Elmer Pyris 1 DSC. After the first scan, samples were quenched to -100 $^\circ\text{C}$ at 300 $^\circ\text{C}/\text{min}$ and a second scan was recorded after 5 minutes. Indium, lead, and cyclohexane were used as standards for temperature calibration. Unless mentioned otherwise, the presented data were obtained from the second heating scan.

The purified polymers were compression moulded at 140 $^\circ\text{C}$ using a laboratory press (Fontejne THB008, The Netherlands) and 500 μm thick stainless steel moulds. The polymers were moulded at approximately 25 kg/cm^2 and quenched to room temperature using cold water. The compression moulded films were then sealed under vacuum in laminated polyethylene/polyamide bags (Hevel Vacuum B.V., The Netherlands) and exposed to 50 kGy gamma irradiation from a ^{60}Co source (Isotron B.V., Ede, The Netherlands). This sterilization method leads to simultaneous crosslinking of the polymers^{20, 23}. The equilibrium swelling ratios and gel contents of the gamma irradiated films were determined as previously described using chloroform²³.

Mechanical Properties

The tensile properties of compression moulded and irradiated (co)polymer films were determined in triplicate according to ASTM-D 882-91. The networks formed upon irradiation were not extracted prior to tensile testing. A Zwick Z020 tensile tester (Ulm, Germany) equipped with a 500 N load cell was operated at a crosshead speed of 50 mm/min. The initial grip-to-grip separation was 50 mm and a preload of 0.01 N was applied. The specimen

deformation was derived from the grip-to-grip separation; therefore the presented values of elastic modulus (calculated from the initial slope of the stress-strain curves) give only an indication of the stiffness of the polymers.

To assess their behaviour under dynamic loading conditions, the specimens (n=1) were repetitively (20x) elongated to 50 % strain at 50 mm/min in cyclic tests. A preload of 0.01 N was applied. After a 2 h recovery period, the permanent deformation was estimated from the stress-strain diagram of the 21st cycle. The error in the values is approximately 0.5 % strain.

Cell Viability Assay

Possible cytotoxicity of the gamma irradiated (50 kGy) (co)polymer films was evaluated using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay ²⁴ as described previously ²³. Briefly, human skin fibroblasts (PK 84 cell line) were cultured in 96 wells plates (5000 cells/well) in RPMI medium (Roswell Park Memorial Institute medium). The cells were incubated at 37 °C and 5 % CO₂ for three days. Of each film, two disks (diameter 6 mm, thickness 500 µm) were incubated at 37 °C and 5 % CO₂ with 400 µl RPMI for 24 hours to extract any leachable components. The cell culture medium was then replaced by the medium containing leachables. After two days, the absorbance of formazan, which is soluble in the culture medium, was measured. The mean value obtained for cell cultures incubated with RPMI medium only was standardized as 100 % cell viability.

A medical grade polyurethane (2363-55D-Pellethane[®] resin from Dow Chemical, Midland, U.S.A.) and latex rubber (Hilversum Rubber Factory, Hilversum, The Netherlands) were used as negative and positive controls, respectively. Prior to the MTS assays and implantations, it was confirmed that the controls and the polymer films were essentially endotoxin-free.

Macrophage-Mediated Erosion Studies

J774A macrophages were maintained in DMEM containing 4.5 g/L D-glucose, pyruvate, 10 % fetal bovine serum, 100 U/mL penicillin, 100 µg/mL and 100 µg/mL 2mM Glutamax[™]. Cells were passaged every 4 to 7 days by scraping.

The erosion of gamma irradiated, non-extracted (co)polymer films in macrophage cultures was investigated by directly culturing J774A macrophages on the surface of the films. The test specimens were 15 mm in diameter and approximately 500 µm in thickness.

The seeding density was approximately 8×10^4 cells/cm². After 7 days of culturing cells on the different surfaces, fresh aliquots of cells were added to the culture. Cells were cultured on six disks of each material.

After 15 days of culturing, three specimens of each material were first placed in Milli-Q water to lyse the cells, and then thoroughly rinsed and weighed. After drying the films to constant weight *in vacuo* at room temperature, the samples were weighed again. The specimens were sputter-coated with gold and their surfaces were analysed by scanning electron microscopy (SEM, Philips XL 30 ESEM-FEG, The Netherlands) at an operating voltage of 5 kV.

The remaining disks of each material (n=3) were fixed with 3.7 % para-formaldehyde in cytoskeletal stabilizing (CS) buffer (0.1 M piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer, 1 mM ethylene glycol tetraacetic acid (EGTA), pH=6.9) for 15 min and then transferred to PBS. One of the disks was processed for SEM by further fixation with 2 % glutaraldehyde in 0.1 M cacodylate buffer (pH=7.4), dehydration in graded alcohols, and critical point drying. The rest of the specimens were used for fluorescence staining of cell nuclei using 4',6-diamidino-2-phenylindole (DAPI) and of the actin cytoskeleton using tetramethylrhodamine iso-thiocyanide-phalloidin (TRITC-phalloidin). These specimens were then analysed by confocal laser scanning microscopy (LEICA TCS SP2, with a fully water-immersed 40x objective (NA 0.80)).

Subcutaneous Implantations in Rats

The tissue response to gamma irradiated (co)polymer films containing 39 %, 18 %, and 0 % TMC was investigated using the subcutaneous rat implantation model. All procedures performed on the animals were done according to international guidelines on animal experiments and approved by the local committee for care and use of laboratory animals. Approximately three months old AO rats were housed individually in a humidity- and temperature-controlled room with 12 hours light/dark cycles. The rats received water and pelleted diet *ad libitum*. The operations were performed under general isoflurane, N₂O, and O₂ anaesthesia. After shaving and disinfection, subcutaneous pockets were made to the right and left of three midline incisions on the back of the rats. Gamma-irradiated, non-extracted disks (n=12 per polymer material) measuring 8 mm in diameter and approximately 500 µm in thickness were individually placed in each pocket.

The discs (n=6) with surrounding tissue were explanted from the subcutaneous site after 5, 28 and 60 days of implantation. Immediately after explantation, half of each disk was

fixed in 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH=7.4) for light microscopy while the other half was snap-frozen in liquid nitrogen for immunohistochemical analyses.

To assess the extent of erosion of the polymers, the thickness of the discs was measured with a digital micrometer (Mitutoyo IP65, U.S.A.) before and after implantation. Their surfaces were analysed by SEM as described before.

Histology

The explants were fixated in glutaraldehyde, dehydrated in graded alcohol solutions and then embedded in glycol methacrylate (Kulzer Histo-Technik Heraeus Kulzer, Germany). Histological sections (2 μm) were stained with toluidine blue. The tissue response was independently evaluated by two persons.

Immunohistochemistry

For immunohistochemical evaluation, cryo-sections (5-7 μm) were cut from snap-frozen explants. Acetone-fixed sections were incubated in PBS containing 10 % serum. They were then incubated with monoclonal mouse anti-rat ED1 (Serotec, U.K.) for staining of macrophages, R73 (Serotec Ltd, U.K.) for staining of T-lymphocytes, and with goat- α -human collagen IV (Southern Biotech, U.S.A.) antibodies for assessing vasculature. The endogenous peroxidase activity was blocked with 1% H_2O_2 in PBS, and colouring was performed with 3-amino-9-ethyl carbazole. Counterstaining was done with hematoxiline. Quantification was done by counting the number of positive cells on micrographs (magnification 400X for ED-1, and magnification 200X for R73). The micrographs were taken from eight different equally distributed areas. The percentage area stained for Coll IV was determined by computerized morphometry using micrographs taken at a magnification of 200X.

Statistical Analysis

The data are presented as mean $\pm$ standard deviation. The statistical significance of differences in the means was evaluated by one-way analysis of variance, followed by post-hoc Tukey's test. A p-value of less than 0.05 was considered to be statistically significant.

Results and Discussion

By adjusting the copolymer composition, a series of amorphous to semi-crystalline (co)polymers of TMC and CL were prepared. The effect of copolymer composition on physical properties and on *in vitro* and *in vivo* degradation behaviour was investigated.

Compression moulded (co)polymer films were simultaneously sterilized and crosslinked by gamma irradiation under vacuum.

Preparation of (co)Polymer Films and Network Formation by Gamma Irradiation

High molecular weight (co)polymers were synthesized by ring opening polymerization of TMC and CL (Table 1). By varying the ratio of the monomers, copolymers having TMC contents from 59 to 13 mol %, and PTMC and PCL homopolymers were synthesized. The conversion of the monomers was higher than 99 % in all cases and the number average molecular weights ($\overline{M}_n$) of the obtained (co)polymers ranged from 208 to 500 kg/mol. After compression moulding, changes in the molecular weight of the (co)polymers were minimal.

Table 1. Characteristics of TMC and CL (co)polymers after compression moulding.^a

TMC content ^b (mol %)	CL content ^b (mol %)	T _g ^c (°C)	T _{m-peak} ^c (°C)	ΔH ^c (J/g)	$\overline{M}_n$ ^d (kg/mol)	PDI ^d	[η] ^d (dl/g)
100 ^e	0	-18.6	-	-	500	1.51	7.5
59	41	-43.4	-	-	202 (238)	1.47 (1.37)	3.8 (3.8)
39	61	-52.6	-	-	214 (208)	1.44 (1.48)	4.1 (4.2)
18	82	-61.5	19.3	27.8	201 (211)	1.41 (1.37)	4.0 (4.2)
13	87	-60.4	34.4	37.3	239 (234)	1.38 (1.38)	4.6 (4.8)
0 ^f	100	-64.3	55.6	53.4	228 (234)	1.37 (1.34)	4.6 (4.7)

^a Before compression moulding, polymers were purified by precipitation.

^b Determined by ¹H-NMR on specimens purified by precipitation.

^c Determined by DSC in the second heating scan of compression moulded films.

^d Determined by GPC at 30 °C using chloroform as the eluent. The values for purified polymers before compression moulding are given in brackets.

^e Reference ²⁵.

^f Reference ²³.

The glass transition temperatures (T_g) of the (co)polymers decreased from -18.6 to -64.3 °C with decreasing TMC content. These low T_g values indicate that all amorphous (co)polymers are in the rubbery state at physiological temperatures. The PTMC homopolymer and the copolymers containing 59 % and 39 % TMC were amorphous, whereas the

copolymers with 18 % and 13 % TMC and the PCL homopolymer were semi-crystalline. With decreasing TMC content, the peak melting temperatures (T_m) and the melting enthalpies of the semi-crystalline (co)polymers increased.

Upon gamma irradiation at 50 kGy all (co)polymers were crosslinked. The gel contents of (co)polymers were similar, with values ranging from 59 to 67 % (Table 2). The swelling ratios in chloroform were relatively high, ranging from 33 to 52. This indicates that the formed (co)polymer networks were not densely crosslinked.

Table 2. Characteristics of networks formed by gamma irradiating TMC-CL (co)polymers at 50 kGy in vacuo. Values are expressed as mean $\pm$ standard deviation, ($n=3$).

TMC content (mol %)	100	59	39	18	13	0
Gel content (%)	65 $\pm$ 1	63 $\pm$ 1	62 $\pm$ 1	59 $\pm$ 3	67 $\pm$ 1	63 $\pm$ 1
Swelling ratio (vol/vol) ^a	33 $\pm$ 2	43 $\pm$ 1	48 $\pm$ 1	52 $\pm$ 2	33 $\pm$ 1	36 $\pm$ 1

^a Determined using chloroform at room temperature

Mechanical Properties of Gamma Irradiated (co)Polymer Films

The mechanical properties of compression moulded TMC-CL (co)polymer films determined before and after gamma irradiation at 50 kGy are given in Table 3, and representative stress-strain curves in Figure 1. It can be seen that the crystallinity has a significant effect on the tensile behaviour of the (co)polymers.

Figure 1. The effect of composition on the stress-strain behaviour of compression moulded TMC-CL (co)polymers before (A) and after gamma irradiation at 50 kGy (B). The measurements were performed at room temperature. (The stress-strain curves are offset for clarity).

Table 3. Effect of composition and gamma irradiation at 50 kGy on the mechanical properties of compression moulded TMC-CL (co)polymers. Values are expressed as mean $\pm$ standard deviation, ($n=3$).

TMC content (mol %)	Irradiation Dose (kGy)	E (MPa)	σ_{yield} (MPa)	ϵ_{yield} (%)	σ_{break} (MPa)	ϵ_{break} (%)	Permanent Set ^a (%)
100	0	7.3 $\pm$ 0.3	2.5 $\pm$ 0.1	171 $\pm$ 7	20 $\pm$ 3	883 $\pm$ 34	1.6
100	50	5.2 $\pm$ 0.4	1.4 $\pm$ 0.1 ^b	110 $\pm$ 15 ^b	5.7 $\pm$ 1.7	1263 $\pm$ 66	0.9
59	0	3.6 $\pm$ 0.3	0.7 $\pm$ 0.1	90 $\pm$ 3	0.7 $\pm$ 0.1	153 $\pm$ 20	12.3
59	50	2.4 $\pm$ 0.1	0.7 $\pm$ 0.1 ^b	100 $\pm$ 5 ^b	9.7 $\pm$ 0.1 ^d	2745 $\pm$ 62 ^d	1.2
39	0	2.6 $\pm$ 0.1	0.4 $\pm$ 0.1	72 $\pm$ 4	0.06 $\pm$ 0.03	896 $\pm$ 321	14.0
39	50	1.8 $\pm$ 0.3	0.5 $\pm$ 0.1 ^b	80 $\pm$ 10 ^b	>8.1 ^{c,d}	>2612 ^{c,d}	1.0
18	0	54 $\pm$ 4	2.9 $\pm$ 0.2	24 $\pm$ 2	24 $\pm$ 4	1286 $\pm$ 71	8.8
18	50	61 $\pm$ 3	3.0 $\pm$ 0.1	17 $\pm$ 2	>23 ^{c,d}	>2162 ^{c,d}	9.1
13	0	173 $\pm$ 1	8.0 $\pm$ 0.1	16 $\pm$ 1	32 $\pm$ 1	1239 $\pm$ 25	19.0
13	50	207 $\pm$ 5	9.2 $\pm$ 0.2	14 $\pm$ 1	31 $\pm$ 1	1682 $\pm$ 13	21.4
0	0	330 $\pm$ 22	15 $\pm$ 1	15 $\pm$ 2	39 $\pm$ 1	1032 $\pm$ 64	34.3
0	50	484 $\pm$ 34	20 $\pm$ 1	14 $\pm$ 1	36 $\pm$ 4	1078 $\pm$ 129	36.4

^a Single measurements. The permanent set is estimated from the 21st cycle, performed after a two hours recovery period. The error is approximately 0.5 % strain.

^b Estimated from the intersection of tangents to stress-strain diagrams as a distinct yield point could not be observed.

^c Specimens slipped from the grips.

^d A grip-to-grip separation of 25 mm was used.

Before gamma irradiation, the elastic moduli, yield strength, and stress at break values of 59 % and 39 % TMC containing copolymers were lower than those of PTMC. These amorphous polymers were all very flexible with elastic moduli ranging from 2.6 to 7.3 MPa. In contrast to PTMC, the copolymers had very low tensile strengths (less than 1 MPa) and high permanent deformations (more than 12.3 % strain) when subjected to cyclic tensile strains. It has been shown before that linear amorphous CL copolymers with low glass transition temperatures have low tensile strengths, and poor form-stability and creep resistance^{17, 23, 26}.

Upon crosslinking these (co)polymers by gamma irradiation, resistance to creep is very much improved. The amorphous PTMC and copolymer networks were much more stable than the starting polymers, with permanent set values as low as 0.9 % strain after subjecting the specimens to cyclic deformations. The values of the elastic moduli and yield strengths slightly decreased, whereas their elongation at break increased. This can be due to the

presence of non-load bearing chains, which are formed as a result of chain scission events during the gamma irradiation crosslinking process.

The stress at break values of the amorphous copolymers (containing 59 % and 39 % TMC) increased to values up to 9.7 MPa upon gamma irradiation. As a result of the crosslinks, the stress-strain diagrams of these copolymer networks show an upturn at high elongations (Figure 1). The maximum tensile strength of PTMC networks was lower than that of the starting polymer. In this case, strain-induced crystallisation of the PTMC polymer is hindered^{20, 23}.

For the linear semi-crystalline copolymers, which contained 18 % and 13 % TMC, the elastic modulus, yield strength, and stress at break values were higher than those of PTMC. These values increased with increasing crystallinity. The PCL homopolymer which had the highest melting point and crystallinity of the polymers investigated, had the highest elastic modulus, yield strength, and stress at break.

Unlike the amorphous materials, the elastic modulus and yield strength of the semi-crystalline copolymers and PCL increased upon gamma irradiation. Thermal analysis showed that the crystallinity of the (co)polymers was not much affected by gamma irradiation. After gamma irradiation, films prepared from polymers containing 18 %, 13 %, 0 % TMC had melting enthalpies of 31.1, 38.2, and 56.5 J/g, respectively and corresponding peak melting temperatures of 22.6, 39.2 and 55.9 °C. These values are somewhat higher than those of the non-irradiated films (Table 1).

The semi-crystalline copolymers and PCL show high permanent deformations after being subjected to repeated cyclic strains. Both for irradiated and non-irradiated specimens, the permanent set increased with decreasing TMC content and increasing crystallinity. Plastic deformation of these semi-crystalline (co)polymers and networks can be seen Figure 1 as well, where all specimens show pronounced yield points in the stress-strain curves. It should be noted that the copolymer network containing 18 % TMC which has a melting temperature of 22.6 °C is amorphous at physiological temperatures and will have rubber-like properties upon implantation.

Macrophage-Mediated Erosion of Gamma Irradiated (co)Polymer Films

The erosion behaviour of gamma irradiated (50 kGy) films prepared from (co)polymers containing 100 (PTMC), 39, 18, and 13, 0 (PCL) % TMC by J774A macrophages was assessed. Macrophages secrete numerous compounds, including hydrolytic enzymes and reactive oxygen species, which can affect the degradation of implanted polymers

^{27, 28}. We and others have shown that cholesterol esterase and superoxide anion radicals may play an important role in the macrophage-mediated erosion of PTMC networks ^{25, 29}.

The macrophages were cultured on the crosslinked films for 15 days at 37 °C. After this time period, the mass of the films prepared from PTMC and from amorphous copolymers containing 39 and 18 % TMC had decreased by 4.5 ± 0.5 , 4.3 ± 0.6 , and 3.6 ± 0.7 %, respectively. (It should be noted that under the macrophage culturing conditions the copolymer network containing 18 % TMC is amorphous, as its melting temperature is below 37 °C.) The mass of network films prepared from the semi-crystalline copolymer containing 13 % TMC decreased only by 0.3 ± 0.1 %, while the mass of the PCL network films remained unchanged. This is in agreement with the very slow erosion of linear highly crystalline PCL *in vivo* ¹⁷.

The average rates of mass loss as a function of copolymer composition (and of melting enthalpy) are given in Figure 2. Between 18 and 13 % TMC, the copolymer networks show a clear reduction in erosion rate. While the amorphous copolymer network containing 18 % TMC eroded at a rate of 0.12 ± 0.02 mg/(cm²×day), the semi-crystalline copolymer network containing 13 % TMC eroded at a significantly lower rate of 0.009 ± 0.002 mg/(cm²×day). This implies that the crystallinity of the (co)polymer at the culturing conditions significantly influences the erosion behaviour and -rate of these network films in macrophage culture. The erosion rates of amorphous (co)polymer networks also seemed to decrease with increasing TMC content, although this was only statistically significant when the TMC content was decreased from 100 to 18 %.

Figure 2. Effect of copolymer composition (crystallinity) on the average rates of mass loss of gamma irradiated TMC and CL (co)polymer films used as substrates in macrophage cultures.

The micrographs given in Figure 3 show that on all crosslinked films the macrophages adhered well and formed clusters. No giant cell formation was observed. The cross-sections of the amorphous films in Figures 3A, 3B and 3C, show pitting of the surface by macrophages. Semi-crystalline networks that contain 0 % (PCL) and 13 % TMC do not seem to be affected by macrophages (Figures 3D and 3E) despite adhesion of macrophages to their surfaces. This suggests that the crystallinity of the networks limits their erosion by the enzymes and reactive species secreted by macrophages.

The extent of erosion of the (co)polymer network films in macrophage cultures can also be seen in the SEM micrographs given in Figure 4. After 15 days of culturing, large and deep pits were observed on the surfaces of amorphous (co)polymer networks which were smooth before the cell culturing, implying surface erosion by macrophages (Figures 4A-4F). The pits formed on the surface of amorphous copolymer networks (Figures 4C and 4E) were deeper than those on amorphous PTMC networks (Figure 4A). Figures 4B, 4D, and 4F show macrophages that had tightly adhered to the films. The semi-crystalline copolymer network containing 13 % TMC (melting point 39.2 °C) had eroded to a much lesser extent (Figure 4G) than the amorphous network films (Figures 4C and 4E). In the case of PCL network films, no signs of erosion by macrophages could be observed at all (Figure 4H), this is likely due to much higher crystallinity and melting temperature of PCL.

Figure 3. Confocal laser scanning micrographs of J774 macrophages on gamma irradiated TMC and CL (co)polymer films containing 100 % TMC (PTMC) (A), 39 % TMC (B), 18 % TMC (C), 13 % TMC (D) and 0 % TMC (PCL) (E). The figures represent single optical sections of the films, measuring 375 μm by 375 μm . Below each micrograph a cross-section showing the extent of erosion of the film surface are given.

Figure 4. SEM micrographs showing the surfaces of (co)polymer network films containing 100 % TMC (A,B), 39 % TMC (C,D), 18 % TMC (E,F), 13 % TMC (G), 0 % TMC (H), after detachment of the cells (A,C,E,G,H) and with adhering macrophages at higher magnifications (B,D,F,H inset). The inset in G shows the pits at higher magnification, whereas the inset in H shows adhering macrophages at a higher magnification.

In vivo Behaviour of Gamma Irradiated (Co)polymer Films

The effect of crystallinity on the *in vivo* erosion of the TMC and CL (co)polymer networks was also investigated. Gamma irradiated films (50 kGy) prepared from (co)polymers containing 39 and 18 % TMC and (PCL) (0 % TMC), were implanted subcutaneously in rats for 5, 28 and 56 days.

Prior to implantation, *in vitro* cell viability assays using human skin fibroblasts were performed. Cell viability values of 39, 18, 0 % TMC containing networks were 100 ± 3 , 112 ± 3 , and 105 ± 7 %, respectively. The values obtained for the positive and the negative control were 13 ± 2 and 107 ± 7 %, respectively. Furthermore, the morphology of the cells was not adversely affected. This confirms that the gamma irradiated (co)polymer networks are non-cytotoxic.

After 5 days of subcutaneous implantation, the non-extracted networks containing 39 % and 18 % TMC, which are both amorphous at 37 °C, were found to have eroded to some extent. In this time period the erosion rate of networks containing 39 % TMC was 9.7 ± 5.6 $\mu\text{m/day}$, while that of networks containing 18 % TMC was 2.9 ± 1.4 $\mu\text{m/day}$. For PTMC gamma irradiated at 50 kGy, we previously reported subcutaneous erosion rates of 25.9 ± 5.2 $\mu\text{m/day}$ in rats ²².

The surfaces of the (co)polymer network films after 5 days of subcutaneous implantation are shown in Figure 5. The surface of these *in vivo* eroded networks resembled that of those eroded *in vitro* in macrophage cultures (Figure 4). After 5 days of implantation, the pits on the amorphous copolymer networks that were implanted in the current study (Figures 5B and 5C) seemed to be deeper than those seen on PTMC networks evaluated in an earlier study (Figure 5A, ²²).

After 28 days of implantation, the copolymer networks had already eroded almost completely. This indicates that the erosion rates of the copolymer networks increased after the first 5 days of implantation. An increase in erosion rate was also observed upon implantation of poly(ethylene carbonate) ³⁰. At 56 days, no material could be found at the implantation site.

The semi-crystalline PCL networks, however, did not show any sign of erosion (Figure 5D). During 56 days of implantation, their thickness remained essentially unchanged. Although we did not implant TMC-CL networks containing 13 % TMC, linear semi-crystalline TMC-CL copolymers containing 11 % TMC also eroded very slowly when subcutaneously implanted in rats ²¹, losing only 7 % of their mass in one year.

Figure 5. SEM micrographs showing the surfaces of the (co)polymer network films containing 100 % (A, from reference ²²) 39 % (B), 18 % (C), 0 % (D) TMC after 5 days of subcutaneous implantation in rats.

As was the case in the macrophage culturing studies, these *in vivo* results indicate a substantial effect of crystallinity on the erosion behaviour of TMC-CL copolymer networks.

The *in vivo* tissue response to the (co)polymer networks was assessed qualitatively by histological analysis and quantitatively by immunohistochemical analysis.

Representative micrographs of toluidine blue stained histological sections showing the tissue response to (co)polymer network films is given in Figure 6. After 5 days of implantation, the tissue responses to the 39 % and 18 % TMC containing (co)polymer networks were comparable (Figures 4A and 4C). Erosion of the films is evident from the presence of high numbers of macrophages and foreign body giant cells which had phagocytosed fragments of the films. In some cases a thin capsule with fibroblasts was observed. The tissue surrounding the copolymer networks was highly vascularised. Here, few polymorphonuclear cells, some lymphocytes and sporadic plasma cells were present. At this time point, a milder tissue response to the PCL networks (Figure 4F) than to the copolymer networks was observed. The interface between the tissue and the PCL networks contained less macrophages and giant cells. Also in the tissue surrounding the PCL implants, fewer macrophages and lymphocytes than that observed in the case of copolymer networks were

seen. This can be due to the much slower erosion of the PCL networks than the copolymer networks¹⁰⁻¹³.

Figure 6. Light micrographs showing the tissue response to non-extracted 39 % TMC (A,B), 18 % TMC (C,D,E), 0 % TMC (F,G,H) containing (co)polymer films gamma irradiated at 50 kGy on day 5 (A,C,F), on day 28 (B,D,E,G) and on day 56 (H) of implantation. Magnifications: 200X (A,C,D,F,G,H), 400X (B,E). (P) polymer, (fc) fibrous capsule, (g) giant cell, (→) macrophage, (L) lipid tissue, (V) blood vessel, (▲) phagocytosed intracellular fragments, (△) lymphocyte.

Immunohistochemical analysis revealed that the overall numbers of macrophages surrounding the 39 %, 18 % and 0 % TMC containing (co)polymer networks were similar, the values were 2724 ± 462 , 2449 ± 548 , and 2058 ± 671 cells/mm², respectively. The overall number of lymphocytes was found to be relatively low in the case of PCL networks (50 ± 23 cells/mm²) when compared to those of 39 % and 18 % TMC containing copolymer networks (148 ± 69 and 184 ± 56 , respectively). The extent of vascularisation did not differ significantly for all implanted materials, as the amount of tissue that stained positive for Collagen IV was 16.3 ± 5.4 , 10.8 ± 2.8 , and 9.7 ± 1.6 % for 39 %, 18 %, and 0 % TMC containing (co)polymer networks respectively.

After 28 days of implantation, most of the implanted copolymer specimens had eroded completely, and the tissue response to these materials could only be evaluated histologically. Macroscopically, no adverse effects were seen in the implantation site as the tissues looked normal. Only 6 out of the 12 implanted specimens of the copolymer network containing 39 % TMC could be retrieved. In these cases, minor amounts of material were found in the form of small fragments that had been surrounded or phagocytosed by macrophages and giant cells (Figure 4B). In the tissue surrounding these fragments more lymphocytes were seen than at day 5. In some cases, plasma cells were sporadically observed.

At day 28, only 7 out of 12 implanted network films containing 18 % TMC could be retrieved. Although the dimensions of three of these films were significantly smaller, the films had still not fragmented (Figure 4D). The rest of the films were found to have been fragmented (Figure 4E), as was the case for all 39 % TMC containing copolymer networks.

The tissue response to fragmented and to non-fragmented network films containing 18 % TMC was quite different. In the case of non-fragmented films, a thin fibrous capsule was visible and the tissue surrounding the copolymer network was relatively quiet with low numbers of inflammatory cells. In the case of fragmented films, however, higher numbers of macrophages, giant cells and lymphocytes could be observed. In this case, the tissue response was similar to that seen for the 39 % TMC containing copolymer networks and for the 79 % TMC containing copolymer network which was implanted in a previous study ²². As the surface area increases upon fragmentation, it can be expected that erosion will be faster. As discussed before, this might lead to a more pronounced tissue response ¹⁰⁻¹³. After 56 days of implantation, all copolymer networks had completely eroded and the implantation sites looked normal.

After 28 and 56 days of implantation, thicker fibrous capsules were seen around PCL network films than at day 5. Low numbers of macrophages and lymphocytes were present in the tissue surrounding the PCL implants at these time points.

Previously, we evaluated the tissue response to similar amorphous networks of TMC and CL copolymers that contained smaller amounts of CL (0 to 30 %) ²². In general, the tissue response to these networks was comparable to the response we observed for TMC and CL networks containing higher amounts of CL evaluated in this study.

Conclusions

Amorphous to semi-crystalline poly(trimethylene carbonate-co- ϵ -caprolactone) (co)polymers can be obtained by adjusting the monomer composition of the copolymer. Gamma irradiation can be used to crosslink these (co)polymers while sterilizing. The mechanical properties of the obtained networks vary between flexible and elastomeric to relatively rigid materials. Similarly, the erosion behaviour can be tuned from relatively rapid surface erosion to slow bulk erosion processes. The amorphous TMC and CL (co)polymer networks can be used in short term biomedical applications where elastomeric properties are desired such as controlled release or *in vitro* engineering of cardiovascular tissues, whereas the semi-crystalline (co)polymer networks would be more suited for applications requiring relatively stiff and slowly eroding materials.

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Appendix B

Thermoreversible Gelation Behaviour of Poly(trimethylene carbonate)-*block*-poly(ethylene glycol)-*block*-poly(trimethylene carbonate) Triblock Copolymers *

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Abstract

Triblock copolymers of trimethylene carbonate (TMC) and polyethylene glycol (PEG) were synthesized. Their gelation behaviour in water and the properties of the gels were investigated by particle size measurements, the vial tilting method, and dynamic rheology. As the temperature was decreased, the average particle size increased and sol-to-gel transition was observed. The storage modulus of these thermoreversible gels ranged from 220 Pa to 4700 Pa depending on the composition and concentration of the copolymer.

Introduction

Hydrogels have been extensively studied for drug delivery and tissue engineering applications. Amphiphilic block copolymers are used to form hydrogels, as intermolecular interactions of the hydrophobic blocks can form physical crosslinks. Biodegradable hydrogels can be prepared by selection of a biodegradable hydrophobic block formed from lactides and/or glycolides ¹. PTMC has been rarely used in preparing hydrogels despite its interesting

properties such as stability in buffers (pH=1-13) *in vitro* and relatively rapid enzymatic surface erosion *in vivo* ². Recently, Jeong et.al. reported reverse thermal gelling hydrogels from diblock copolymers of TMC and PEG ³. The gels did not degrade in water but did degrade when implanted subcutaneously in rats. However, these gels had low storage moduli. In this study, we aimed at developing thermoreversible hydrogels with improved mechanical properties from relatively high molecular weight PTMC-PEG-PTMC triblock copolymers. The effect of both hydrophilic and hydrophobic block lengths on particle formation, phase change behaviour, and rheological properties were investigated.

Materials and Methods

The synthesis of PTMC-PEG-PTMC triblock copolymers was carried out in the melt at 130 °C for three days using stannous octoate as catalyst. PEG diols ($M_{n, NMR} = 9,66 \times 10^3$ and $M_{n, NMR} = 33.3 \times 10^3$ g/mol) were used as initiator. The copolymers were purified by dissolution in chloroform and subsequent precipitation in cold hexane. Monomer conversion, copolymer composition, and molecular weight were determined by ¹H-NMR. The size and size distribution of particles in dilute polymer solutions (0.1 and 1 wt %) were determined using a Zetasizer (Malvern, Nano ZS). The measurements were performed over a range of temperatures (20-60 °C) one day after preparation of the solutions. Three measurements were taken at each temperature. Gel-to-sol transition temperatures of the hydrogels were determined by the vial tilting method. Hydrogel samples (≈1g) were prepared by mixing deionised water with appropriate amounts of polymer. The samples were heated from 4 °C to 60 °C at intervals of 2 °C and equilibrated for 10 minutes at each temperature. Rheological measurements were performed with a rheometer (Anton Paar, Physica MCR 301) using a parallel plate geometry (25 mm in diameter, gap size=0.5 mm), a frequency of 1 Hz, and a strain of 1 %. Thermoreversible gel-to-sol transition behaviour was investigated by increasing/decreasing the temperature at a rate of 1 °C/min.

Results and Discussion

Four PTMC-PEG-PTMC triblock copolymers were synthesized. The characteristics of the copolymers are given in Table 1. Two different PEG block lengths (EO₂₂₀ and EO₇₅₇) were used. For each PEG block, the length of the PTMC block was varied. Resulting copolymers had PEG contents ranging from 68 % to 82 %. The degree of polymerization (DP) values of PTMC were between 10.1 and 62.3. The TMC conversion for the lower molecular weight polymers was higher as compared to the higher molecular weight polymers.

This could be due to a lower viscosity of the PEG used (EO₂₂₀) which could ease miscibility with the TMC monomer during the synthesis. After purification, powder-like materials were obtained.

Table 1. Characteristics of the synthesized PTMC-PEG-PTMC block copolymers

Polymer	TMC conversion (%)	PEG content (wt %)	DP TMC	M _n (10 ³ g/mol)
EO ₂₂₀ (TMC ₁₀) ₂	99.3	82.4	10.1	11.7
EO ₂₂₀ (TMC ₂₂) ₂	99.2	68.3	22.0	14.1
EO ₇₅₇ (TMC ₃₄) ₂	94.6	82.6	34.3	40.3
EO ₇₅₇ (TMC ₆₂) ₂	96.8	72.4	62.3	46.0

Figure 1. Average particle size of the polymers as a function of temperature (A), (heating (filled symbols) and cooling (empty symbols) (0.1 wt/vol %). The heating and cooling curves show great overlap.), and as a function of temperature (heating) for different concentrations (B).

The block copolymers formed micelles in which PTMC is in the core and PEG is in the shell. The average particle sizes during heating and cooling were very close to each other at a given temperature (Figure 1A). The average size of the particles increased more than twice as the temperature was decreased from 60 °C to 20 °C. EO₂₂₀(TMC₁₀)₂ and EO₇₅₇(TMC₃₄)₂ formed micelles with a quite uniform size distribution (PDI=0.23-0.28). Micelles with a broader size distribution were obtained for EO₂₂₀(TMC₂₂)₂ (PDI=0.32-0.44) and for EO₇₅₇(TMC₆₂)₂ (PDI=0.48-0.70). The average size of micelles was also significantly higher for EO₇₅₇(TMC₆₂)₂ compared to the other copolymers. The broader size distribution for EO₂₂₀(TMC₂₂)₂ and EO₇₅₇(TMC₆₂)₂ suggests bridging between micelles which can lead to gel formation.

Increasing the copolymer concentration from 0.1 % to 1 % significantly increased the average particle size (Figure 1B). These results suggest that a possible mechanism for the gelation could be micellar expansion, bridging between micelles, and aggregation. This process is enhanced by an increase in concentration or a decrease in temperature. This mechanism was also seen in PLGA-PEG-PLGA triblock copolymers ⁴. Although no hydrogels were obtained with EO₂₂₀(TMC₁₀)₂, micelles with uniform size distribution (PDI=0.22-0.26) were formed in water. Such particles can be useful for the encapsulation of hydrophobic drugs.

Figure 2. Gel-to-sol transition diagram of PTMC-PEG-PTMC hydrogels

The hydrogels showed sol-to-gel transitions as the temperature was decreased (Figure 2). The concentration at which a gel was formed depended on the TMC block length at a given PEG length and the molecular weight of the copolymer. At a given PEG length, gelation occurred at lower concentrations as the length of PTMC block was increased. Similarly, increasing the molecular weight of the copolymer decreased the critical gelation concentration of copolymers having similar PEG contents. No gel could be obtained from EO₂₂₀(TMC₁₀)₂ up to 50 w/vol % concentration.

Figure 3A shows the thermoreversible gel formation behaviour of EO₂₂₀(TMC₂₂)₂. The gel-to-sol transition occurs at 54.1 °C while heating and sol-to-gel transition occurs at 51.1 °C while cooling. Storage modulus versus temperature curves during cooling and heating were largely overlapping. The plateau storage modulus of EO₇₅₇(TMC₆₂)₂ hydrogels increased as the concentration of polymer was increased (Figure 3B). The values ranged from 220 Pa to 4700 Pa. The plateau storage modulus values of EO₂₂₀(TMC₂₂)₂ (23 wt/vol %) and EO₇₅₇(TMC₃₄)₂ (33 wt/vol %) at 37 °C were 1150 Pa and 770 Pa, respectively. These gels are

much stronger than the reverse thermal gelling PEG-PTMC diblock copolymers where the storage modulus of aqueous solution (30 wt %) was 10 Pa at 37 °C³.

Figure 3. Storage and loss modulus of (A) $EO_{220}(TMC_{22})_2$ (23 w/vol %) as a function of time and temperature, the heating and cooling curves show significant overlap (B) plateau moduli of $EO_{757}(TMC_{62})_2$ as a function of concentration at 37 °C

Conclusions

Hydrogels could be obtained from triblock copolymers of PTMC and PEG. Gels are formed upon cooling and have improved mechanical properties compared to reverse thermal gelling, low molecular weight diblock copolymers of PTMC and PEG which were investigated previously³. Hydrogels or nanoparticles prepared from these PTMC-PEG-PTMC copolymers can be used for controlled release or tissue engineering applications.

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Summary

The number of applications for biomedical technologies is ever-increasing, and there is a need to develop new materials with properties that can conform to the requirements of a specific application. Synthetic polymers are of great importance in the biomedical field as they can be designed to exhibit a wide range of physical- and biological properties and a range of degradation profiles. Interest in biodegradable elastomers is increasing, particularly for the engineering of soft and elastic tissues. These materials are also being utilized more and more for controlled drug delivery purposes.

In this thesis, the development of biodegradable elastomers based on trimethylene carbonate polymers using different approaches is presented.

A general introduction to the thesis and the relevant scientific background information regarding this study is given in **Part I**. The aim of the study and the structure of the thesis are presented in **Chapter 1**. General information on degradation and erosion of synthetic biodegradable polymers and on tissue engineering is presented in **Chapter 2**. The need for biodegradable elastomers in preparing scaffolds in (soft-) tissue engineering is highlighted by outlining recent findings in the literature. In the final section of this chapter, a short literature review on biodegradable elastomers and trimethylene carbonate (TMC) based elastomers is provided. In **Chapter 3**, the mechanical- and biological properties of poly(trimethylene carbonate) (PTMC) crosslinked by gamma irradiation is evaluated. It was found that crosslinked PTMC films promote the adhesion and proliferation of neo-natal cardiomyocytes. The porous scaffolds had stiffness values close to those of human myocardium, making this material interesting as a scaffolding material for cardiac tissue engineering. It was also shown that these materials were angiogenic and eroded rapidly upon implantation on the heart of rats.

A first approach to obtain elastomeric networks with lower erosion rates was to prepare networks by crosslinking of TMC copolymers using different doses of gamma irradiation (**Part II**). Trimethylene carbonate was copolymerised with ϵ -caprolactone (CL) as poly(ϵ -caprolactone) can also be crosslinked by gamma irradiation and erodes at a much lower rate *in vivo* than PTMC. It is shown in **Chapter 4** that elastomeric networks could indeed be prepared by gamma irradiation of poly(trimethylene carbonate-*co*- ϵ -caprolactone)

films although their gel contents and network densities were relatively low. Nevertheless, even at low gel contents the tensile strength and elongation at break values were higher for gamma irradiated copolymers than for the non-irradiated ones. These materials degraded by surface erosion upon incubation in aqueous lipase solutions. The enzymatic erosion rates of these films could be tuned by adjusting the (co)polymer composition and the irradiation dose (for homopolymers). Contrary to our expectations, however, the erosion rates increased with increasing CL content. Although previous studies showed that aqueous lipase solutions could be used to predict the *in vivo* erosion rate of PTMC, apparently for CL containing (co)polymers this was not the case. Therefore, we then investigated the *in vivo* erosion of these networks by subcutaneous implantation in rats (**Chapter 5**). These studies showed that *in vivo* erosion of these networks was not affected much by copolymerisation of TMC with up to 30 mol % CL or by tuning the network density by adjusting the irradiation dose. The tissue response to these (co)polymer networks and their erosion rates were comparable to those of previously investigated non-irradiated PTMC films. These surface eroding (co)polymer networks are more suitable for short-term biomedical applications, and it was concluded that a different approach need to be taken to prepare more slowly eroding elastomers.

In the investigations described in **Chapters 4** and **5**, the CL content was limited to 30 mol %, as TMC copolymers having 30 to 70 mol % CL were previously reported to have poor mechanical strength. However, the results obtained in **Chapter 4** suggest that relatively strong and creep-resistant networks could be prepared by gamma irradiation of copolymers containing more than 30 % CL. The mechanical properties and biodegradation behaviour of these (co)polymer networks is presented in **Appendix A**.

In **Part III**, initially an *in vitro* macrophage culture model was first set-up to investigate the erosion behaviour of networks prepared from trimethylene carbonate polymers and to assess the effect of structural parameters on this behaviour. The development of this model is described in **Chapter 6**. Using this *in vitro* assay, it was clearly demonstrated that macrophages are able to erode gamma irradiated PTMC films effectively whereas no effect on gamma irradiated PCL films (which were used as controls) was observed. This study demonstrated that a physical contact between the macrophages and PTMC network films is a prerequisite for erosion to occur. It was shown that selected enzymes and reactive oxygen species that can be secreted by macrophages were likely to be involved in these erosion processes. Particularly, the erosion behaviour of PTMC- and PCL networks in aqueous solutions of cholesterol esterase was shown to be similar to that observed in *in vivo* experiments as well as in macrophage cultures.

As the elastomeric networks prepared from TMC and CL copolymers did not have significantly lower erosion rates than that of linear high molecular weight PTMC polymers and PTMC networks, we evaluated new strategies to prepare relatively slowly eroding, elastomeric trimethylene carbonate-based networks. It was hypothesized that further increasing the network densities and gel contents of PTMC networks would yield slow erosion rates. **Chapter 7** presents a systematic investigation of the effect of gamma irradiation dose and initial polymer molecular weight on the properties of PTMC networks. By gamma irradiation of PTMC films that incorporate pentaerythritol triacrylate (PETA) as a crosslinking aid, a remarkable increase in gel contents and network densities could be achieved. The gel contents and network densities could be increased by increasing (i) initial polymer molecular weight, (ii) PETA content, and (iii) irradiation dose. The densely crosslinked PETA containing PTMC networks were still flexible, elastomeric and non-toxic to cells. Moreover, upon incubation in aqueous cholesterol esterase solutions, PETA-containing PTMC networks prepared by gamma irradiation eroded at lower rates when compared to PTMC networks that did not contain PETA.

This promising approach was then extended to poly(trimethylene carbonate-*co*-D,L-lactide) (co)polymers, as presented in **Chapter 8**. PDLLA or (co)polymers of DLLA with TMC either degrade or crosslink very inefficiently upon sterilization by gamma irradiation. This adversely influences their strength and leads to a more rapid erosion (compared to non irradiated (co)polymers) of implants prepared from these materials. The work showed that by incorporating PETA into these copolymer films, amorphous TMC and DLLA (co)polymer networks with high gel contents and high network densities could be easily obtained. The networks had a wide range of elastic moduli and glass transition temperatures. Moreover, they exhibited large recoverability upon deformation, and were compatible with cells. The macrophage-mediated erosion of TMC and DLLA (co)polymers could be tuned by adjusting the (co)polymer composition and by crosslinking in the presence of PETA. The surface erosion behaviour of these materials was more pronounced with increasing TMC contents.

To be able to perform these crosslinking reactions under more moderate conditions in house, we investigated the possibility of crosslinking trimethylene carbonate polymers by ultraviolet light. This is reported in **Chapter 9**. Crosslinking in this manner would also permit stabilisation of the shape of any device prepared from these polymers immediately after processing at much lower costs. It was shown that by UV irradiation of PTMC films containing pentaerythritol triacrylate (PETA) and a photoinitiator, flexible, biodegradable networks with high toughness and excellent elastomeric behaviour could readily be prepared.

The network characteristics, mechanical properties, wettability, and *in vitro* enzymatic erosion behaviour of the networks could easily be tuned by selection of the initial molecular weight of PTMC or by crosslinking blends of PTMC with block copolymers containing PTMC, poly(ethylene glycol) and PCL blocks. We demonstrated the advantage of this crosslinking method by preparing scaffolds for tissue engineering by fused deposition modelling. PTMC and its blends with different block copolymers were thermally processed in the presence of a crystallisable solvent and subsequently crosslinked using ultraviolet light. These scaffolds had interconnected macropores. Due to leaching out of the solvent, micropores also formed on the fibres. Moreover, the scaffolds prepared from PTMC and blends with PCL containing block copolymers were shown to be suitable for the culturing of human mesenchymal stem cells making them attractive for tissue engineering applications. Scaffolds prepared from blends with PEG containing block copolymers reduced or prevented the adhesion of cells.

In **Chapter 10**, we report on the crosslinking of high molecular weight PTMC using only a (relatively) biocompatible photoinitiator or a photoinitiator together with methacrylate end-functionalised PTMC oligomers as crosslinking aids. By adjusting the methacrylate content or the irradiation time, the preparation of resorbable networks with high gel contents and tuneable network densities was possible. The formed flexible and elastomeric networks were compatible with (stem-) cells. Crosslinking with a macromer and/or a photoinitiator also allowed the preparation of TMC-based materials with lower erosion rates than non-crosslinked PTMC as assessed using macrophage cultures, aqueous solutions- of cholesterol esterase and potassium superoxide.

In **Part IV**, the work presented in **Chapters 4 and 5** is extended to copolymers of TMC and CL having higher CL contents (**Appendix A**). Although the mechanical properties of the amorphous (co)polymer could be improved by gamma irradiation, increasing the CL content in the copolymers also did not reduce the *in vivo* erosion rates of amorphous (co)polymer networks very significantly. Networks prepared from semi-crystalline (co)polymers of TMC and CL did erode at lower rates than amorphous (co)polymers. However, these networks deformed plastically upon application of stress, and were therefore not ideal for engineering of elastic (soft-) tissues. The suitability of macrophage cultures in assessing the effect of structural parameters of the materials on their erosion behaviour was confirmed in this study, as the results of macrophage-mediated erosion studies performed with these networks were similar with those obtained by *in vivo* studies.

In **Appendix B**, thermoresponsive hydrogels based on triblock copolymers synthesized from TMC and polyethylene glycol (PEG) are presented. The gelation behaviour

and the rheological behaviour of the gels depended on the composition and concentration of the copolymer in water.

The work described in this thesis shows that by using a crosslinking aid, elastomeric networks can be obtained in a very practical manner from linear trimethylene carbonate polymers. The erosion rates and the physical- and biological properties of these networks can be easily varied for instance by copolymerisation, blending, or by adjusting the crosslinking aid content, initial polymer molecular weight, etc. These materials exhibited interesting biological properties as was assessed by initial cell culturing assays performed using macrophages and mesenchymal stem cells. Nevertheless, these very interesting materials need to be further evaluated with regard to biological properties when a specific application in soft tissue engineering or controlled drug release is envisaged.

Samenvatting

Het aantal toepassingen voor biomedische technologieën stijgt sterk, en er is behoefte aan nieuwe materialen met eigenschappen die voldoen aan de eisen voor een specifieke toepassing. Synthetische polymeren zijn zeer belangrijk in het biomedische veld omdat ze kunnen worden ontworpen met een breed scala aan fysische en biologische eigenschappen en verscheidene degradatie profielen. De interesse in biodegradeerbare elastomeren neemt toe, met name in de weefseltechnologie, waar de materialen worden toegepast voor de vervanging van zachte en elastische weefsels. Biodegradeerbare elastomeren worden ook meer en meer gebruikt voor de gecontroleerde afgifte van geneesmiddelen.

In dit proefschrift worden verschillende benaderingen voor de ontwikkeling van biodegradeerbare elastomeren op basis van trimethyleen carbonaat polymeren gepresenteerd.

Een algemene inleiding op het proefschrift en de relevante wetenschappelijke achtergrond informatie zijn opgenomen in **Deel I**. Het doel van de studie en de structuur van het proefschrift worden gepresenteerd in **Hoofdstuk 1**. Algemene informatie over degradatie en erosie van synthetische biodegradeerbare polymeren en over weefseltechnologie worden gepresenteerd in **Hoofdstuk 2**. Een overzicht van recente bevindingen in de literatuur laat zien dat in de weefseltechnologie biodegradeerbare elastomeren nodig zijn voor de bereiding van scaffolds. In het laatste gedeelte van dit hoofdstuk wordt een kort literatuuroverzicht gegeven van biodegradeerbare elastomeren en op trimethyleen carbonaat (TMC) gebaseerde elastomeren. In **Hoofdstuk 3** worden de mechanische en biologische eigenschappen van poly(trimethyleen carbonaat) (PTMC) gecrosslinkt door gamma bestraling geëvalueerd. Gecrosslinkte PTMC films blijken hechting en proliferatie van neo-natale hartspiercellen te stimuleren. De poreuze scaffolds hadden moduli dichtbij die van de menselijke hartspier, waardoor dit materiaal interessant is als drager materiaal voor vervanging van hartspierweefsel. Er werd ook aangetoond dat deze materialen angiogeen zijn en snel degraderen na implantatie op het hart van ratten.

Een eerste aanzet tot het verkrijgen van elastomere netwerken met een tragere erosiesnelheid was om netwerken te bereiden door TMC copolymeren te crosslinken met verschillende doses gammastraling (**Deel II**). Trimethyleen carbonaat werd gecopolymeriseerd met ϵ -caprolacton (CL), omdat poly (ϵ -caprolacton) (PCL) ook kan

worden gecrosslinkt door gamma bestraling en *in vivo* veel trager degradeert dan PTMC. **Hoofdstuk 4** laat zien dat elastomere netwerken inderdaad kunnen worden verkregen door gamma bestraling van poly(trimethyleen carbonaat-co- ϵ -caprolacton) films hoewel de gel percentages en de dichtheden van de netwerken relatief laag waren. Niettemin, zelfs bij lage gel percentages ware de treksterkte en rek bij breuk hoger voor gamma bestraalde copolymeren dan voor de niet bestraalde polymeren. Deze materialen degradeerden door oppervlakte erosie bij incubatie in waterige lipase oplossingen. De enzymatische erosiesnelheid van deze films kan worden afgestemd door aanpassing van de copolymeer samenstelling en de stralingsdosis (voor homopolymeren). In tegenstelling tot onze verwachtingen, nam de erosiesnelheid toe met toenemend CL gehalte. Hoewel uit eerdere studies is gebleken dat waterige lipase oplossingen kunnen worden gebruikt om de *in vivo* erosiesnelheid van PTMC te voorspellen, is dit kennelijk niet het geval voor copolymeren met CL. Daarom hebben we vervolgens de *in vivo* erosie van deze netwerken onderzocht door middel van subcutane implantatie bij ratten (**Hoofdstuk 5**). Deze studies toonden aan dat *in vivo* erosie van deze netwerken niet sterk wordt beïnvloed door copolymerisatie van TMC met tot 30 mol % CL of door het variëren van de netwerk dichtheid door aanpassing van de stralingsdosis. De reactie van weefsel op deze (co)polymeer netwerken en hun erosiesnelheid waren vergelijkbaar met die van eerder onderzochte niet bestraalde PTMC films. Deze oppervlakte eroderende (co)polymeer netwerken zijn voornamelijk geschikt voor korte termijn biomedische toepassingen, en er moet een andere benadering worden gekozen om langzamer eroderende elastomeren te ontwikkelen.

In het onderzoek beschreven in de **Hoofdstukken 4 en 5** werd de hoeveelheid CL beperkt tot 30 mol %, omdat bekend is dat TMC-copolymeren met 30 tot 70 mol % CL lage mechanische sterktes hebben. Echter, uit de resultaten in **Hoofdstuk 4** blijkt dat relatief sterke en kruip vrije netwerken kunnen worden bereid door gamma bestraling van copolymeren die meer dan 30 % CL bevatten. De mechanische eigenschappen en het degradatie gedrag van deze copolymeer netwerken worden beschreven in **Bijlage A**.

In **Deel III**, werd in eerste instantie een *in vitro* macrofaag kweek model opgesteld om het erosie gedrag van trimethyleen carbonaat polymeer netwerken te onderzoeken en om het effect van structurele parameters op dit gedrag te bepalen. De ontwikkeling van dit model wordt beschreven in **Hoofdstuk 6**. Met behulp van deze *in vitro* test, werd duidelijk aangetoond dat macrofagen in staat zijn om gamma bestraalde PTMC films te eroderen, terwijl er geen effect op gamma bestraalde PCL-films (die werden gebruikt als controle) werd waargenomen. Deze studie toonde verder aan dat direct contact tussen macrofagen en de

PTMC netwerk films een noodzakelijke voorwaarde is voor erosie. Er werd daarnaast aangetoond dat de geselecteerde enzymen en reactieve zuurstofsoorten die kunnen worden uitgescheiden door macrofagen waarschijnlijk betrokken zijn bij deze erosie processen. Met name het erosie gedrag van PTMC en PCL netwerken in waterige cholesterol esterase oplossingen was vergelijkbaar met het erosie gedrag *in vivo* en in macrofaag culturen.

Omdat de elastomere netwerken bereid uit TMC en CL copolymeren geen significant langzamere erosiesnelheid lieten zien dan lineair hoog moleculair gewicht PTMC en PTMC netwerken, evalueerden we nieuwe strategieën om relatief langzaam eroderende, trimethyleen carbonaat gebaseerde elastomeer netwerken te bereiden. De hypothese was dat het verhogen van de netwerk dichtheden en gel percentages van PTMC netwerken langzame erosie zouden opleveren. **Hoofdstuk 7** presenteert een systematisch onderzoek naar het effect van de dosis gammastraling en het initiële moleculaire gewicht van het polymeer op de eigenschappen van de PTMC netwerken. Door gamma bestraling van PTMC films die pentaerytritol triacrylate (PETA) bevatten om crosslinking te bevorderen, kon een opmerkelijke stijging van gel percentages en netwerk dichtheden worden bereikt. De gel percentages en netwerk dichtheden konden worden verhoogd door verhoging van (i) het initiële moleculair gewicht van het polymeer, (ii) hoeveelheid PETA, en (iii) de stralingsdosis. De dicht gecrosslinkte PETA bevattende PTMC netwerken waren nog steeds flexibel, elastisch en niet toxisch voor cellen. Bovendien degradeerden PETA bevattende PTMC netwerken, bereid door gamma bestraling, langzamer bij incubatie in waterige cholesterol esterase oplossingen dan PTMC netwerken die geen PETA bevatten.

Deze veelbelovende aanpak werd vervolgens uitgebreid tot poly(trimethyleen carbonaat-co-D,L-lactide) copolymeren, zoals gepresenteerd in **Hoofdstuk 8**. Poly(D,L-lactide) (PDLLA) en copolymeren van D,L-lactide (DLLA) met TMC, degraderen of crosslinken zeer inefficiënt bij sterilisatie door middel van gamma bestraling. Dit heeft nadelige gevolgen voor de sterkte en leidt tot snellere erosie (ten opzichte van niet bestraalde (co)polymeren) van implantaten bereid uit deze materialen. Uit het beschreven werk is gebleken dat door het opnemen van PETA in deze copolymeren, gemakkelijk amorfe TMC en DLLA (co)polymeer netwerken met hoge gel percentages en netwerk dichtheden konden worden verkregen. De netwerken lieten een breed scala aan elasticiteitsmoduli en glasovergangstemperaturen zien. Bovendien herstelden ze goed na vervorming, en waren ze compatibel met cellen. De macrofaag gemedieerde erosie van TMC en DLLA (co)polymeren kon worden afgestemd door de aanpassing van de copolymeer samenstelling en door

crosslinken in de aanwezigheid van PETA. Het oppervlakte erosie gedrag van deze materialen was uitgesprokener bij toenemend TMC gehalte.

Om deze crosslink reacties onder mildere omstandigheden in het laboratorium kunnen uitvoeren, zijn de mogelijkheden van het crosslinken van trimethyleen carbonaat polymeren door middel van ultraviolet licht onderzocht. Dit wordt beschreven in **Hoofdstuk 9**. Crosslinken op deze manier zou ook stabilisatie van de vorm van een medisch implantaat, bereid uit deze polymeren, mogelijk maken direct na de verwerking tegen veel lagere kosten. Er werd aangetoond dat door UV bestraling van PTMC films met PETA en een foto-initiator, gemakkelijk flexibele, biodegradeerbare netwerken met een hoge taaiheid en uitstekend elastisch gedrag konden worden bereid. De kenmerken van het netwerk, mechanische eigenschappen, bevochtigbaarheid, en het enzymatisch erosie gedrag *in vitro* van de netwerken konden eenvoudig worden aangepast door de keuze van het moleculair gewicht van PTMC of door mengsels van PTMC met blokcopolymeren van PTMC, poly(ethyleen glycol) (PEG) en PCL te crosslinken. We toonden de voordelen van deze methode aan bij de bereiding van gecrosslinkte scaffolds voor weefselengineering door middel van *fused deposition modelling* (een gecontroleerde polymeerextrusie techniek). PTMC en mengsels van PTMC met verschillende blokcopolymeren zijn thermisch verwerkt in de aanwezigheid van een kristalliseerbaar oplosmiddel en vervolgens met ultraviolet licht gecrosslinkt. Deze scaffolds bevatten onderling verbonden macroporiën. Door het uitwassen van het oplosmiddel, vormden zich ook microporiën op de vezels. De scaffolds bereid uit PTMC en mengsels van PTMC met PCL bevattende blok copolymeren bleken geschikt voor het kweken van menselijke mesenchymale stamcellen waardoor deze scaffolds aantrekkelijk zijn voor toepassingen in de weefseltechnologie. Scaffolds bereid uit mengsels van PTMC met PEG bevattende blok copolymeren verminderden of voorkwamen de hechting van cellen.

In **Hoofdstuk 10**, beschrijven we het crosslinken van hoog moleculair gewicht PTMC met behulp van slechts een (relatief) biocompatibele foto-initiator of een foto-initiator samen met methacrylaat gefunctionaliseerde PTMC oligomeren. Door aanpassing van het methacrylaat gehalte of de belichtingstijd, konden resorbeerbare netwerken met hoge gel percentages en verschillende netwerk dichtheden worden bereid. De gevormde flexibele en elastische netwerken waren compatibel met de (stam-)cellen. Macrofaag culturen en incubatie in waterige cholesterol-esterase oplossingen en kalium superoxide lieten zien dat crosslinken met een macromeer en/of een foto-initiator het mogelijk maakt om TMC gebaseerde materialen met een lagere erosiesnelheid dan niet gecrosslinkt PTMC te bereiden.

In **Deel IV** wordt het werk gepresenteerd in **Hoofdstuk 4** en **5** uitgebreid tot copolymeren van TMC en CL met een hoger CL gehalte (**Bijlage A**). Hoewel de mechanische eigenschappen van de amorfe copolymeren konden worden verbeterd door gamma bestraling, leidde het verhogen van de hoeveelheid CL in de copolymeren niet tot significante verlaging van de *in vivo* erosiesnelheid. Netwerken bereid uit semi-kristallijne copolymeren van TMC en CL erodeerden langzamer dan amorfe (co)polymeren. Echter, deze netwerken vervormden plastisch wanneer spanning werd aangebracht, en zijn daardoor niet ideaal voor de vervanging van elastische (zachte) weefsels. In dit onderzoek werd de geschiktheid van macrofaag culturen voor het beoordelen van het effect van structurele parameters van de materialen op het erosie gedrag bevestigd; de resultaten van macrofaag gemedieerde erosie studies waren vergelijkbaar met die verkregen uit *in vivo* studies uitgevoerd met deze netwerken.

In **Bijlage B** worden thermoresponsieve hydrogelen op basis van triblok copolymeren gesynthetiseerd uit TMC en PEG gepresenteerd. Het gelerings gedrag en het rheologische gedrag van de gels hing af van de samenstelling en de concentratie van het copolymeer in water.

Het werk beschreven in dit proefschrift laat zien dat als het crosslink proces wordt ondersteund, op een zeer praktische manier elastische netwerken kunnen worden verkregen uit lineaire trimethyleen carbonaat polymeren. De erosiesnelheid en de fysische en biologische eigenschappen van deze netwerken kunnen gemakkelijk worden gevarieerd bijvoorbeeld door copolymerisatie, mengen, of door aanpassing van de hoeveelheid crosslinker, moleculair gewicht van het polymeer, enzovoort. In initiële celkweek tests uitgevoerd met macrofagen en mesenchymale stamcellen lieten de materialen interessante biologische eigenschappen zien. Niettemin moeten de biologische eigenschappen van deze zeer interessante materialen nog verder worden geëvalueerd als een specifieke toepassing in de weefseltechnologie of gecontroleerde geneesmiddel afgifte wordt overwogen.

Curriculum Vitae

Erhan Bat was born on the 16th of January 1981 in Nusaybin (Mardin), Turkey. He was raised in İzmir and lived in this lovely city by the Aegean Sea till 1999. That year, he graduated from İzmir Private Turkish Anatolian High School where he was awarded an education scholarship for seven years. Following his graduation, he studied Chemical Engineering at Middle East Technical University (METU), Ankara. In 2003, he received a Bachelor of Science degree in honours. He continued studying in the same department under the supervision of Prof. Dr. Gngr Gndz. For his master project, he worked on hyperbranched and air drying fatty acid based resins for surface coatings. With this work, he received the best thesis award of Graduate School of Natural and Applied Sciences of METU. After finishing his studies in 2005, he joined the Polymer Chemistry and Biomaterials Group of University of Twente, Enschede, The Netherlands as a Ph.D. student. During his Ph.D. study, he worked under supervision of Prof. Dr. Jan Feijen and Prof. Dr. Dirk W. Grijpma. The results of his research are presented in this thesis. Since November 2009, he has been working as a “post”-doctoral researcher for the Molecular NanoFabrication Group of Prof. Dr. Jurriaan Huskens at University of Twente.