Development of Biodegradable Adhesives for Meniscus Repair

Agnieszka Bochyńska

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The research described in this thesis was conducted between 2012 and 2016 in the research group Biomaterials Science and Technology at the Institute for Biomedical Technology and Technical Medicine (MIRA), University of Twente, Enschede, The Netherlands, and in the Orthopaedic Research Laboratory, Radboud Institute for Molecular Life Sciences (RIMLS), Radboudumc, Nijmegen, The Netherlands. The research was financially supported by Reumafonds, the Dutch arthritis foundation.

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DEVELOPMENT OF BIODEGRADABLE ADHESIVES FOR MENISCUS REPAIR

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Table of Contents

Chapter 1	General Introduction	1
Chapter 2	Tissue Adhesives for Meniscus Tear Repair – An Overview of Current Advances and Prospects for Future Clinical Solutions	9
Chapter 3	Development of Tissue Adhesives Based on Amphiphilic Isocyanate-Terminated Trimethylene Carbonate Block Copolymers	47
Chapter 4	Adhesion of Tissue Glues to Different Biological Substrates	63
Chapter 5	Development of Biodegradable Hyper-Branched Tissue Adhesives for the Repair of Meniscus Tears	77
Chapter 6	Evaluation of Novel Biodegradable Three-Armed- and Hyper-Branched Tissue Adhesives in a Meniscus Explant Model	105
Chapter 7	The Effect of Tissue Surface Modification with Collagenase and Addition of TGF- β 3 on the Healing Potential of Meniscal Tears Repaired with Tissue Glues <i>In Vitro</i>	121
Chapter 8	<i>In Vitro</i> and <i>In Vivo</i> Characterization of Biodegradable Reactive Isocyanate-Terminated Three-Armed- and Hyper-Branched Block Copolymeric Tissue Adhesives	135
Chapter 9	Development of a Fast Curing Tissue Adhesive for Meniscus Tear Repair	165
Summary		185
Samenvatting		191
Acknowledgements		197
List of publications		202

Chapter 1

General Introduction

General Introduction

Menisci are fibrocartilaginous tissues present in the knee joint. They are crucial structural elements of the knee as they play a key role in shock absorption and load distribution, protecting the articular cartilage [1].

Meniscus tears are among the most common knee injuries. Approximately one third of middle-aged and elderly people has a damaged meniscus [2]. Such tears lead to pain, swelling and locking of the knee joint [3]. Moreover, it has been shown that in the long-term the risk of the knee osteoarthritis (OA) dramatically increases if menisci are damaged by injury or degenerative processes.

Treatment for meniscus injuries usually consists of (partial) meniscectomy. This results in instant pain relief and restoration of the knee function, but in the long-term leads to accelerated cartilage degradation due to increased peak stresses acting on the articular cartilage [4, 5]. These increased intra-articular contact stresses inevitably lead to mechanical damage and biochemical changes of the cartilage, leading to development of degenerative changes and OA in the knee [6].

The cascade of events leading to OA can be prevented by repairing the meniscus tear directly after the trauma [7]. The anatomical location of the tear in the meniscus influences the healing capacity of the tear. Tears located in the peripheral, vascularized part of the meniscus have a higher chance of successful repair [8, 9]. However, most tears are located in the inner, highly loaded area of the meniscus with limited vascular supply. These lesions have shown only limited capacity for repair and healing [8]. The reported healing rates of meniscal tears repaired using mechanical devices, such as sutures, staples, arrows, and screws are highly variable [10]. Despite recent advances, a large proportion of meniscal tears observed during arthroscopy remains irreparable [11].

It has been recognized that tissue adhesives could provide optimal meniscal repair and facilitate tissue healing by minimizing gap formation, providing contact of the bonded surfaces and by that restore the integrity of the damaged meniscus [12, 13]. Once brought into a meniscal tear, such an adhesive would fill the tear, bind to the meniscal tissue and hold the torn region together during the period of healing.

Additionally, it would permit cell migration and extra-cellular matrix formation in the torn zone. After tear repair, there would be no further enlargement of the tear and no deterioration of the adjacent cartilage.

Tissue adhesives have already been used in clinical practice for other purposes, for example cyanoacrylates as topical skin adhesives, fibrin glue for pulmonary leaks, and a recently FDA approved isocyanate/polyurethane-based adhesive for abdominoplasty [14-17]. However, they are not suitable for meniscus repair as they either do not have adequate mechanical properties or are cytotoxic.

Aim and outline of this thesis

The aim of the work described in this thesis was to develop novel biodegradable polymeric materials that can be used as adhesives for meniscus tear repair. More specifically, isocyanate-terminated hyper-branched amphiphilic block copolymers based on trimethylene carbonate (TMC), polyethylene glycol (PEG) and citric acid (CA), and three-armed star block copolymers based on trimethylolpropane ethoxylate (TMPE) and TMC, were synthesized. The obtained materials were characterized in terms of their adhesion to meniscus tissue, the mechanical properties of the cured networks, and their biocompatibility *in vitro* and *in vivo*.

Chapter 2 introduces the background of the anatomy, physiology and biomechanics of the meniscus, and currently used surgical techniques to repair meniscus tears. It gives an overview of tissue adhesive materials that are currently used in clinical practice for other applications as well as new developments in the research field of tissue adhesives. The requirements for tissue adhesives suitable for meniscus repair are identified and compared with the properties of existing materials [18].

In **Chapter 3** the development of isocyanate-terminated linear and three-armed star-shaped amphiphilic copolymers is described. Their physical and mechanical properties are determined. Their adhesion to chamois leather (dermal sheep collagen), which is used as a tissue model, is determined and compared with that of two clinically used adhesives: fibrin glue and Dermabond® [19].

In **Chapter 4** gluing of different biological substrates, which serve as tissue models, is evaluated. Three different adhesive materials: Dermabond®, fibrin glue and an

isocyanate-terminated three-armed adhesive are used to glue Achilles tendon tissue, muscle tissue, meniscus tissue, chamois leather and collagen films. The adhesive strength of the glues is compared and discussed in relation to the working mechanisms of the glues, the contact angle between the glues and the substrates, as well as in relation to the properties of the substrates such as their biochemical content and surface morphology.

Chapter 5 describes the development and characterization of a series of isocyanate-terminated hyper-branched amphiphilic copolymers based on citric acid (CA), polyethylene glycol (PEG) and trimethylene carbonate (TMC). The physical properties of the newly developed cured adhesive materials are determined. Moreover, the degradation profiles of the cured network films and their tensile properties are determined. Finally, the adhesion of the copolymers to meniscus tissue is tested and their potential cytotoxicity is evaluated in a cell-based *in vitro* assay [20].

In **Chapter 6** the application of the two recently developed three-armed star-shaped and hyper-branched adhesives in a full thickness meniscus explant model is evaluated. Cylindrical meniscal explants are glued with the two new materials, Dermabond[®] and fibrin glue, after which they are cultured *in vitro*. The adhesive strength of the glues and the tissue response are determined by a mechanical push out test and by histological screening, respectively. Moreover, the use of collagenase, which partially digests the surface of the explants to potentially enhance tissue healing and improve adhesion of the glues, is evaluated [21].

In **Chapter 7** further evaluation of the newly developed three-armed and hyper-branched adhesives in a cylindrical explant model is performed. The glues are applied in such a way that the glue only partially covers the simulated tear, allowing parts of the two sides of the gap to stay in direct contact with each other. Such design allows investigating if cells are able to migrate into the gap and facilitate repair. Additionally, the influence of the TGF- β 3 and the collagenase treatment on the number and location of cells is investigated [22].

In **Chapter 8** the characterization of the three-armed and hyper-branched adhesive copolymers is further assessed. The mechanical properties of the networks degrading both *in vitro* and *in vivo* and the changes of their structure are monitored by tensile

testing and scanning electron microscopy (SEM) imaging of the surfaces of the network films. The two adhesive copolymers are injected subcutaneously in rats and their potential cytotoxicity is assessed by histological evaluation at predetermined time points.

Chapter 9 describes the design and development of a fast curing adhesive composition. In order to accelerate curing times of the isocyanate-terminated glues two strategies are employed. Firstly, a double component curing adhesive composition is introduced using a double chamber syringe equipped with a static mixer. In one chamber the isocyanate-terminated copolymer is contained and in the second chamber an amine-terminated cross-linker. During extrusion of the two components from the syringe, the components are mixed and curing is initiated immediately. The second strategy employs the use of a catalyst, which is added to the isocyanate-terminated copolymers before application. The application and the adhesive properties of these newly developed adhesive compositions are evaluated in bucket-handle tears in a bovine meniscus model [23].

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Chapter 2

Tissue Adhesives for Meniscus Tear Repair – An Overview of Current Advances and Prospects for Future Clinical Solutions

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Abstract

Menisci are crucial structures in the knee joint as they play important functions in load transfer, maintaining joint stability and in homeostasis of articular cartilage. Unfortunately, ones of the most frequently occurring knee injuries are meniscal tears. Particularly tears in the avascular zone of the meniscus usually do not heal spontaneously and lead to pain, swelling and locking of the knee joint. Eventually, after a (partial) meniscectomy, they will lead to osteoarthritis. Current treatment modalities to repair tears and by that restore the integrity of the native meniscus still carry their drawbacks and a new robust solution is desired. A strong tissue adhesive could provide such a solution and could potentially improve on sutures, which are the current gold standard. Moreover, a glue could serve as a carrier for biological compounds known to enhance tissue healing. Only few tissue adhesives, e.g., Dermabond[®] and fibrin glue, are already successfully used in clinical practice for other applications, but are not considered suitable for gluing meniscus tissue due to their sub-optimal mechanical properties or toxicity. There is a growing interest and research field focusing on the development of novel polymer-based tissue adhesives, but up to now, there is no material specially designed for the repair of meniscal tears. In this review, we discuss the current clinical gold standard treatment of meniscal tears and present an overview of new developments in this field. Moreover, we discuss the properties of different tissue adhesives for their potential use in meniscal tear repair. Finally, we formulate recommendations regarding the design criteria of material properties and adhesive strength for clinically applicable glues for meniscal tears

Introduction

Menisci are crescent shaped discs of fibrocartilage with a triangular cross-section, present in duplicate in each knee joint. The menisci have been recognized as crucial structures in maintaining knee joint stability and articular cartilage homeostasis [1-3]. During normal functioning, they are exposed to shear, tension and compression forces and serve a variety of (bio)mechanical functions, such as load bearing, constituting contact area, guiding rotation and stabilizing translation [4-7]. They also

play a role in lubrication and nutrition of the underlying articular cartilage surfaces and by that might help to prevent the development of osteoarthritis [1].

The most commonly occurring injuries of the knee joint are meniscus tears. They originate either from acute injuries of the knee joint (e.g. sport, trauma) or are caused by degenerative changes (mostly in case of elderly patients) associated with early osteoarthritis [8]. Irrespective to their origin, they might cause pain, swelling and locking of the joint, and they may ultimately lead to osteoarthritis [9]. Therefore, it is of utmost importance to provide an effective treatment modality and to prevent this negative scenario.

In the past, it was believed that menisci could be removed without any immediate or long-term consequences for the function of the knee joint and consequently a (partial) meniscectomy was performed as a gold standard treatment. In fact, short-term results were very satisfactory. It resulted in instant pain relief and restoration of knee function, but as osteoarthritis develops very slowly, it took decades to find out that even a partial meniscectomy inevitably leads to joint degeneration [10, 11]. In 1975 Krause et al. showed that menisci have a function in load transmission and stress absorption in the knee and that peak stresses acting on articular cartilage increase after meniscectomy [6]. Removal of only 15-34% of the meniscus, a partial meniscectomy, will produce a 350% increase in contact stress and lead to development of degenerative changes and eventually osteoarthritis in a majority of the patients [12]. Ever since, the preferred strategies aim at restoration of the integrity of the meniscus rather than at its removal by (partial) meniscectomy [13]. These treatment methods can only be effective if the torn parts of the tissue are kept in close proximity to each other enabling a healing process to take place. Many devices have been developed and tested in clinical practice for this purpose, such as sutures, stingers, staples, arrows and darts [14, 15]. However, the success rate of these devices highly depend on the location of the meniscal lesion [16]. They are mainly effective in treating lesions located in the peripheral vascularized region of the meniscus [17, 18]. Moreover, the techniques are challenging, surgical procedure is time-consuming and devices are expensive. Therefore, it would be ideal to find an alternative treatment method, which should be easier and faster to perform, and

would allow a more universal successful treatment of tears, also those located in the avascular region of the meniscus.

Recently, much interest has been paid to tissue adhesives as an alternative to sutures. Tissue adhesives are already used in clinical practice for other purposes, such as Dermabond® (2-octyl cyanoacrylate) as topical skin adhesive, fibrin glue for pulmonary leaks, and recently TissuGlu® (an FDA approved urethane-based adhesive) for abdominoplasty surgery [19, 20]. However, up to now, there is no clinically available glue suitable for the treatment of meniscal tears.

The purpose of this paper was to summarize current advances in the development of tissue adhesives, compare them with the current clinical gold standard treatments and critically evaluate their potential application in the treatment of meniscal tears. Mechanical and biological properties of the meniscus are discussed and requirements for a suitable material are formulated. Subsequently, characteristics of the main groups of tissue adhesives found in literature are discussed and conclusions and recommendations for future clinical directions are made.

Biology of the meniscus

The healthy meniscus contains 72 % water, the remaining 28% are organic compounds: 22 % collagen, 0.8 % glycosaminoglycans (GAGs, of which the major part (40%) is chondroitin 6-sulphate), DNA (2%), adhesion glycoproteins (<1%) and elastin (<1%) [21]. These proportions may vary depending on age or degenerative status. For instance, with increasing degeneration the water content can increase up to 85% [21] and the cellularity of the meniscus decreases [22]. The blood supply of the meniscus is also age dependent: the meniscus of an infant is fully vascularized, while an adult meniscus is only partly vascularized, i.e. the outer part. An adult meniscus can be divided into three zones: the outer (red-red), middle (red-white) and inner (white-white) zone, see Figure 1, [23-25]. The outer two-thirds of the meniscus (red-red and the red-white zone) are vascularized, the cells are fibroblast-like and the collagen fibers present there are mainly type I. The inner one-third of the meniscus (white-white zone) is avascular, contains both collagen type I and II and the cells in this region are more (fibro)chondrocyte-like [26, 27]. Moreover, the inner two thirds of the meniscus contain more GAGs, whose main function is to enable

the meniscus to take up water in order to improve their visco elastic behaviour and counter compressive loads on the tissue [23]. Due to differences in the structure of the meniscus, particularly related to the vascularization and differences in cell phenotype, tears that occur in the outer red-red part of the meniscus have the ability for self-repair. Tears in the middle part are less likely to heal spontaneously, while tears in the inner white-white part are unable to repair themselves [28, 29].

Figure 1. Regional variations in vascularization and cell populations of the meniscus, showing on the left the red-red, white-red and white-white regions, and on the right the differences in cell populations and the location of the blood vessels. Reprinted from [23].

Biomechanics of the meniscus

The two menisci cover an area of approximately 2/3 of the tibial plateau [30]. They stabilize the knee, provide congruity and lubrication of the articular cartilage, and constitute a large contact area between tibial plateau and femoral condyle. The main attachment sites of the medial and lateral menisci in the joint are the menisco-tibial ligamental attachments [31]. Additional ligaments are the transverse ligament and the posterior and anterior meniscus-femoral ligaments (Wrisberg and Humphry) [31]. The medial meniscus is more firmly attached to the joint capsule than the lateral. The lateral meniscus is also more flexible. Throughout the whole range of flexion and extension of the knee joint menisci play a crucial role in transferring

loads [32]. They are subjected to tensile, compressive and shear forces and they are displaced radially from the center of femoral condyles due to compressive forces in combination with different flexion angles of the knee [33, 34]. As a result of the compressive loading on the meniscus, the stress is transferred to a vertical force, whose radial component causes a radial displacement of the meniscus. As the meniscus is firmly connected with the tibial plateau through its anterior and posterior attachments, its displacement is constrained by the circumferentially orientated collagen bundles resulting in a circumferentially directed component of force and tensile stress (see Figure 2a), [34]. The collagen fibers in the meniscus structure are organized in a manner that optimally resists these tensile stresses [35]. The surface layer of the meniscus contains sheets of radially oriented collagen fibres, to cope with shear forces, while the deep zone has circumferentially organized collagen fibres, to withstand the circumferential forces (see Figure 2b).

Figure 2. a) A schematic representation of the forces acting on the meniscus. F_f is a joint load on top of the meniscus, F_t is the reaction force at tibial plateau, radially oriented force F_{fh} and vertically oriented F_{fv} are balanced by forces generated by anterior and posterior horns F_a and F_p . A combination of these forces results in tensile hoop stress F and axial and radial components of stress in the meniscus (F_r and F_c) while loading, [34], b) Schematic representation of collagen fibers alignment in meniscal tissue, reprinted from [36].

Biomechanical properties of meniscal tissue (see Figure 3) have been extensively described in literature. Fithian *et al.* reported that the tensile modulus measured in circumferential direction was in the range 100-300 MPa and in radial direction between 10-30 MPa. The shear modulus of the meniscus was measured to be approximately 120 kPa [35]. Lechner *et al.* and Tissakht *et al.* reported a tensile modulus in circumferential direction ranging from 40 up to 140 MPa [37, 38].

Figure 3. Overview of the tensile properties of anterior, central and posterior part of the human medial and lateral meniscus, based on [35, 37, 38]. View on the right knee. E – Elastic modulus, ϵ_{max} – elongation at maximum applied force, UTS – ultimate tensile strength (only in circumferential direction).

Meniscal tears and their healing potential

There are two main causes of meniscus tears: trauma (e.g. sports injuries) mostly occurring in young and active patients, and degeneration, which are mainly associated with early stages of osteoarthritis and are more frequently observed in elderly patients [39]. An overview of the classification of meniscal tears is shown in Figure 4. Longitudinal tears, such as commonly occurring bucket-handle lesions, are classified as traumatic, whereas flap, horizontal and tears in menisci with deteriorating changes are classified as degenerative. Radial tears may be classified both as traumatic and degenerative [39]. Unfortunately, the tears are usually located

in the highly loaded, avascular, inner region of the meniscus and do not heal spontaneously. Moreover, it has been reported that there are significantly better results after treating traumatic than degenerative lesions [10]. This is mainly due to the fact that degenerative tears are often associated with early stages of osteoarthritis and disturbed homeostasis of the knee joint, thus in this condition it is more difficult to successfully repair them [40]. The orientation of the tear also influences its ability to heal. Tears that disrupt the circumpherentially oriented collagen fibers may be more difficult to heal or do not heal at all since they interfere with the circumpherential mechanical properties of the meniscus tissue [41-43]. Thus, longitudinal tears are relatively the easiest to heal and the native mechanical function will be restored after healing [29, 44-46]. Therefore, as traumatic lesions are usually oriented in the circumpherential direction, only in these cases in young patients, restoration of the native tissue is the preferred treatment. Elderly patients with degenerative changes are offered alternative treatment methods, such as partial meniscectomy [29], allograft transplantation or implantation of a permanent implant, however these methods are not covered by this review, and are widely described elsewhere [47].

Figure 4. Classification of meniscal tears. Reprinted from [39].

Current clinical treatment methods of meniscal tears

There are numerous techniques used in clinical practice to restore the integrity and function of torn menisci, most used are sutures, staples, stingers and screws [48].

These devices are employed to keep the torn meniscus together enabling tissue healing. Among these techniques, suturing is superior, since it provides optimal mechanical stability and has the highest strength at failure [8, 11, 48, 49]. Different suturing techniques are used: all-inside, outside-in, inside-out, in order to provide the most suitable treatment for particular tears [50]. The use of vertical sutures provides the best results and is therefore the current gold standard [51]. An alternative to sutures, which are usually difficult to apply during minimally invasive surgery as the procedure is time-consuming and may induce scarring and cause problems with healing, might be using Meniscus Arrows® or Smart Nails®, which are easier to apply [52]. However, they carry the risk of chondral abrasion, due to the fact that a part of these devices remain exposed at the surface of the meniscus and harm articular cartilage [53]. In addition, these implants might fail, and as they are usually prepared from non-degradable materials, articular cartilage might be damaged due to the long-term presence of material debris in the knee joint.

In clinical practice, procedures aimed at increasing the rate of healing in the avascular zone are also proposed. Rasping the tissue to expose collagen fibers and induce vascularization, trephination and creation of access channels to facilitate new tissue ingrowth have been employed [54-58]. However, the success rate is found to mainly depend on the type and location of the tear.

Advances in research of meniscal tears repair

Recent advances in meniscus research suggest that the low cellularity, dense ECM and poor vascularity together with the inflammatory condition of the knee joint in general might also be responsible for the lack of healing potential. Reprogramming of the wound environment is one of the potential targets to increase the healing potential of the meniscus, but this requires extra steps in treating the tissue adjacent the tear [59]. Amongst the procedures which were proposed are the delivery of therapeutics by biomaterial-based technologies and the partial digestion of the matrix in the torn region by collagenase which might enable the migration of cells to the wound margin and stimulate healing [60, 61]. On the other hand an approach in which the level of proteolytic enzymes is controlled may also improve meniscus capacity to repair, as the present inflammation is associated with increased synovial inflammation and increased level of the proteolytic enzymes, especially

metalloproteinases (MMP) [62, 63]. In an *in vitro* repair and animal models it has been shown that by introducing inflammatory cytokines, such as interleukin-1, the integration of the meniscus can be reduced, while inhibiting MMPs and catabolic cytokines can restore its integration capability [64-66]. Another approach which was already proven to have a beneficial influence on meniscal repair is the injection of progenitor cells into the joint, a procedure which enhances tissue regeneration. Also growth factors, especially TGF- β , have been shown to increase proteoglycans synthesis by fibrochondrocytes from different zones of the meniscus in an *in vitro* cultures [67-69]. Further development of these techniques to clinical use could improve the healing capacity of the meniscus, potentially making the whole healing process shorter and more efficient.

In the light of the complexity of the biological modifications developed to improve the meniscus repair capacity, there might also be a great potential in the use of tissue adhesives. They could serve as an alternative or support for sutures, and would keep the wound edges of the tear close to each other. The use of slowly resorbable tissue adhesives would have numerous advantages, such as easy and fast application, no need of removal after the tissue is healed, and at the same time they could act as a carrier to deliver cells, growth factors, enzymes and other biological factors to the meniscus. Thus, they could be used in combination with the treatment modalities described in the previous section. There already have been attempts in the clinic to use fibrin gels in meniscus repair treatments, either as a glue or as a support to suturing [50, 51, 70-73]. More details of these procedures are given later. However, until now they have not been adopted in clinical practice. It remains a challenge to develop a tissue adhesive which has all the required properties for adequate meniscal tear repair. In this review we focus on a discussion on the desired properties of glues for meniscal tear repair and how current developments in the field comply with them.

Requirements of tissue adhesives for use in meniscal repair

Tissue adhesives are defined as materials capable to attach and remain on surfaces of biological substrates with ability to interact with biological factors [74]. Any tissue adhesive, regardless its intended application, should fulfil a large number of requirements to be suitable for biomedical use [75]. Most importantly, good

attachment to (wet) tissue surfaces is a prerequisite. The material must be non-cytotoxic and not induce any adverse tissue reaction. An adhesive should initially be in a liquid state with an adequate viscosity to be able to apply it only locally on the tissue in adequate amounts. There should be no need for elaborate application techniques or devices, and the adhesive should preferably be injectable. It should wet and spread over the entire surface of the tissue to be glued, resulting in effective and close contact between adhesive molecules and the surface of the tissue. Thereafter, it should solidify shortly after its application [76]. The adhesive should achieve a stable union between the tissue surfaces until proper healing has occurred. To allow this, it must withstand forces present on the site of application. Finally, it should degrade and potentially be resorbed by the body so that the two edges of the wound can reunite to accomplish complete wound healing. Biodegradation products of the cured tissue adhesives must be not cytotoxic and must have sizes allowing their clearance by the kidney (maximally 50 kDa [77]). Moreover, the tissue adhesive should be shelf-stable and preferably coloured for easy visualization [75]. Abovementioned requirements are critical for every tissue adhesive material, regardless of the site of application. For materials intended for specific purposes, additional criteria should be formulated [78].

To formulate specific design criteria for tissue adhesives for meniscus tear repair, the particularity of the tissue in terms of its structure, vascularisation, healing potential and its performance in loaded conditions must be considered. The adhesive has to spread well on the wet surfaces of the meniscus in a synovial environment. Subsequently, it should cure forming a solid network strong enough to withstand the forces present in the knee joint. Therefore, it should be sufficiently hydrophilic to facilitate spreading on the tissue. Secondly, in order to obtain a strong network after curing, the adhesive should preferably contain hydrophobic components as well. These components will contribute to good mechanical properties of the network after curing. The tensile modulus of the cured network should be in the same range as that of meniscus tissue in the circumferential direction, as the majority of injuries fitting criteria for repair occur in the circumferential direction [79]. This means that it should be in the range of values between 40 and 150 MPa (see Figure 3). The ultimate tensile strength (UTS) of the adhesive should be either higher or comparable

to that of meniscus tissue, which is approximately between 12 and 19 MPa in circumferential direction (see Figure 3).

When designing the degradation profile of the adhesive, the healing rate of the meniscus must be taken into account. In general, patients are advised to use crutches for 8-10 weeks post-surgery after repair of a meniscal tear using sutures to avoid overloading the meniscus tissue during its healing process [29]. At the same time it has been reported that visual evidence of healing of a meniscus requires a 4 month time interval [80]. Therefore, it would be beneficial if the tissue adhesive remains functional at the site of application for at least 4 months before it starts to degrade without rapid loss of its mechanical properties. Finally, the adhesive strength of the adhesive material should be sufficient to keep the torn meniscus together during this period of healing. However, it is hard to determine what exactly should be the adhesive strength due to variability of lesions, intended technique to be used and specificity of each patient's case. If referred to the mechanical strength of the currently used devices for meniscus repair (sutures, stingers, screws), those depend not only on the device itself but also on the fixation method.

Rimmer et al. compared the failure strengths of 3 arthroscopic meniscal suturing methods [81]. Depending on the technique used (a single horizontal loop, a double vertical loop, and a single vertical loop), the failure strength was between 29.3 N and 67.3 N. Barber et al. compared the performance of sutures with other surgical devices [15]. The load to failure was measured of e.g. double vertical stitch (113 N), single vertical stitch (80 N), BioStinger (57 N), a horizontal mattress stitch (56 N), a meniscus arrow (33 N) and the Biomet staple (27 N). It is evident that the variation in mechanical failure strength is broad, and the use of a particular method strongly depends on the individual case, type of lesion and the number of sutures used. Moreover, it is impossible to directly relate tensile strength of sutures with the required adhesive strength of the tissue glue. In studies analysing forces on sutured menisci (both lateral and medial) in human cadaver knee models, it was reported that the distraction forces did not exceed 5 N on average when the applied load was 300 N [82, 83]. In another study, where the distraction forces were assessed indirectly on bucket-handle lesion, the occurring forces were determined to be less than 10 N [84]. These forces would correlate to a stress of 50-100 kPa for a tear

measuring 1 cm^2 , and in the non-weight bearing recovery period these forces would probably be even lower. Therefore, tissue adhesives should be designed to resist physiological stresses and by that stabilize the meniscus and prevent gap formation [85]. An adhesive material able to hold a torn meniscus together under a stress of 50-100 kPa should therefore be already sufficient for this application.

Another important aspect that should be taken into consideration in the design of a tissue adhesive for meniscus repair is the incorporation of cells or growth factors. These could enhance healing of the meniscus, especially in the inner a-vascular and a-cellular zone. The tissue adhesive can serve as a carrier for the molecules, which can be released on the site of application e.g. via diffusion process or, if the factors are bound to the glue, they could be released during degradation of the adhesive. The beneficial influence of incorporating both cells [86] and other biological compounds, such as bone marrow or platelet rich plasma (PRP) [85, 87], on meniscus healing have been already reported in literature.

A summary of the most important requirements of tissue adhesives to be used in the repair of meniscus tears is depicted in the Table 1.

Table 1. Summary of the key requirements of tissue adhesives for meniscus repair.

Tissue adhesive		Cured adhesive network		
Application	- Application via syringe/needle (adequate viscosity) - Can be applied via minimally invasive arthroscopical procedures - Easy spreading on the tissue surface (sufficiently hydrophilic), but does not leak to surrounding tissues	Mechanical properties	Elastic modulus	40-150 MPa
	UTS		12-19 MPa	
	Curing rate	-Cures within few minutes after application allowing surgeon to precisely place it in the tear	Biodegradation	-Starts to gradually degrade after 4 months of application -Degrades to non-toxic, low molecular weight products (max 30-50 kDa) , which can be renally excreted
Adhesive shear strength		Biocompatibility	-Does not induce chronic inflammation -Non-toxic degradation products	
	At least 50 kPa			
Biocompatibility	-Not cytotoxic, does not induce acute inflammatory response			

Current developments in the field of tissue adhesives

Recently, there has been much interest in the research field of tissue adhesives. Besides a few products which are already approved for clinical use (e.g. Dermabond[®], Fibrin glue, TissuGlu[®]), most research is directed towards the development of new adhesive biomaterials and improvement of the properties of existing ones.

In general, the biomaterials used for the synthesis of glues might be composed of natural, synthetic or combination of both types of polymeric materials [88]. Synthetic biomaterials are usually cheap and their ground substances are widely

available, the mechanical and physico-chemical properties might be easy to control, but they can be toxic and may have a sub-optimal biocompatibility. Natural materials, on the other hand, offer better biological properties such as biocompatibility and good cell adhesion, but the production is more difficult to standardize due to variability of the ground substances, they are expensive and carry a risk of disease transmission [89]. In terms of mechanisms of curing and adhesion to the tissue, the materials can be divided into several subcategories. Since numerous reviews were published recently on the chemistry and mechanisms of tissue adhesion [90-92], here only a brief description of the basic mechanisms and examples from literature are outlined.

Natural protein-based tissue adhesives

Fibrin adhesives

The most widely used natural tissue adhesive is fibrin glue (commercially available Raplixa, Artiss, Evarrest, Tisseel). It is a mixture of fibrinogen and thrombin and its curing mechanism mimics the final stages of blood coagulation [78], (Figure 5a). Fibrin binds to the tissue by three modes: covalent bonding, hydrogen- and electrostatic bonds and mechanical interlocking [93]. Other similarly working adhesives are FloSeal and Proceed (a combination of bovine thrombin and bovine collagen that form the matrix for the clot) and CoStasis, which is a combination of autologous human plasma obtained from patients' blood and a mixture of bovine collagen and thrombin. The use of fibrin gels in meniscal tear repair has been already described in literature. Ishimura et al. reported results of a study where meniscal tears of 40 patients using a purified fibrin glue were repaired [70]. Within the follow up period of up to 11.4 years post intervention, the rate of recurrence of tears in the red-red zone or red-white zone was below 10%, whereas that of tears in the white-white zone was 17%. In another study, Henning et al. repaired a series of meniscal tears using an exogenous fibrin clot [48]. It was reported that in 64% of cases meniscus was healed. Van Trommel et al. reported on outside-in repair of a radial tear of the lateral meniscus using an exogenous fibrin clot in 5 patients. All 5 menisci were found to have healed on follow-up arthroscopy [73]. In a more recent study, Ra et al. described the use of a fibrin clot together with 2 sutures in the repair of

radial meniscal tears in 12 patients [51]. Follow-up MRI performed after 11 ± 3 months postoperatively, revealed complete healing in 11 patients (and incomplete healing in 1 patient). Also recently, Kamimura *et al.* reported the use of fibrin glue together with sutures in the treatment of degenerative horizontal tears in 18 patients [94]. The follow-up results of 10 patients showed that complete healing was achieved in 70% of cases. Nevertheless, despite successful repair results of meniscal tears reported in literature, the use of fibrin has not been widely adopted in clinical practice.

Gelatine adhesives

Gel-forming mechanisms similar to blood coagulation have been further investigated. Liu *et al.* [95] proposed that gelatine (as a structural protein) and calcium independent transglutaminase (mTG) (as a cross-linking enzyme) can be used (Figure 5b). They reported that gelatine-mTG adhesives gelate *in situ* within few minutes (<5), adhere to tissue in the presence of blood *via* covalent bonds (Figure 5c) and provide sufficient strength to be used as a surgical sealant. Also Chen *et al.* investigated the use of gelatine-mTG adhesives [96]. The materials did not induce structural damage of the retina after injection into the vitreous cavity in rats and their adhesive strength to bovine retinal tissue *in vitro* was between 15-45 kPa. Moreover, peptide-conjugated polymer hydrogels formed by tissue transglutaminase cross-linking were evaluated as an adhesive on guinea pig skin and collagen membrane [97]. The results show that the adhesive strength to the tissue was comparable to that of fibrin glue.

Other adhesives of this class are gelatine-resorcinol-formaldehyde (GRF) and gelatine-resorcinol-formaldehyde-glutaraldehyde (GRFG) glues. The gelatine is cross-linked by aldehyde *via* reaction with its amine groups. At the same time, aldehyde groups form covalent bonds with amines from the tissue. These materials have been utilized in aortic dissections, liver surgeries, gastrointestinal and urinary track operations [88]. However, safety issues have arisen regarding their toxic degradation product – formaldehyde.

Figure 5. a) Schematic for fibrin glue cross-linking mechanism [95], b) Cross-linking mechanism of gelatine with Calcium-independent microbial transglutaminase (mTG) [95], c) Adhesion mechanism of transglutaminase-cross-linked gelatine to the tissue [98].

Albumin adhesives

In addition, albumin has been investigated as a structural protein for tissue adhesive compositions. Currently BioGlue, one such adhesive that comprises bovine serum albumin (45%) and glutaraldehyde (10%) (Figure 6), is approved by the FDA for the repair of aortic dissections [99]. It closes the cavity of the aorta and provides a stronger arterial wall for its repair, it binds within 30 seconds and reaches its maximum bonding strength after 3 minutes [100].

Although natural protein-based cross-linked adhesives have excellent biocompatibility, their bonding strength to the tissue is relatively low, usually in the range of 10-40 kPa. After curing they form networks with the relatively low mechanical properties of soft hydrogels, which limit their application in load bearing sites in the body. Moreover, they are rapidly degraded by enzymes from the body. Normally they degrade within 2 weeks [101], which is too fast for meniscus repair. These glues are however applicable for pulmonary leaks, for haemostasis in vascular surgery and for treatment of cerebrospinal leaks.

Figure 6. Cross-linking of a commercially available albumin-based adhesive (BioGlue®).

Nature-inspired (biomimetic) tissue adhesives

Nature-inspired adhesives, for instance based on glues secreted by marine sessile organisms (mussels, barnacles, tube worms), perform very well in hydrated conditions. The working mechanism is depicted in Figure 7. Mussels enzymatically oxidize the phenolic residues of their adhesive proteins. Then, the oxidized residue undergoes a crosslinking reaction that results in the formation of a 3-dimensional polymeric network. The attachment to the tissue takes place by bonding to amine and thiol groups present on the tissue surface [75]. Due to their high adhesion strength and ability to adhere to wet surfaces, these glues have been proposed for use in biomedical applications. These properties are mainly due to the presence of a redox functional group: 3,4-dihydroxyphenyl-L-alanine (DOPA). The key element of DOPA is ortho-dihydroxylphenyl (catechol), which exhibits strong affinity towards organic surfaces [102]. Therefore, development of biomimetic adhesives have received much attention these last years and artificial materials that mimic natural forms, such as co-polypeptides containing DOPA [103], poly (ethylene glycol) hydrogels and Pluronics functionalized with DOPA, have been synthesized [104, 105].

Figure 7. Working principle of a mussel adhesive. [106].

Another similar mechanism is present in red and brown algae, which also, like mussels, excrete phenolic compounds that can bind to both hydrophobic and hydrophilic surfaces in aqueous conditions. The produced polyphenols are activated by a vanadate-peroxidase type of enzyme that allows the cross-linking of the polyphenol with the extracellular carbohydrate fibers, which will finally lead to adhesion to a surface [107]. Natural algal-borne polyphenol can be successfully replaced by phloroglucinol. Formulations composed of phloroglucinol, alginate and calcium ions are capable of adhering to a variety of surfaces including porcine tissue [108].

One of the strategies to induce a better and stronger interaction with a surface is the increase of the contact area. Geckos have used this in an interesting adhesive strategy that relies on foot pads composed of keratinous foot-hairs that are split into terminal spatula of a size of 200 nm [109]. The adhesion mechanism is based on capillary forces and van der Waals interactions. Inspired by this natural phenomena, Geim *et al.* developed an adhesive composed of dense arrays of flexible pillars to ensure their collective adhesion. However, problems related to their durability were encountered [110]. To resolve these problems Lee *et al.* developed an adhesive inspired by mussels and geckos, which combine excellent properties of both mechanisms, and can bind to the surfaces both in dry and wet environment in multiple cycles. The adhesive force in a wet environment was calculated to be equivalent to 90 kPa [111]. Szomor and Murrell tested a naturally-sourced glue secreted by Australian frogs (genus *Notaden*) on sheep meniscus tissue and reported that the adhesive strength of

this glue (~90 N) is superior both to fibrin glue (5 times stronger) and gelatine (2.5 times stronger) [112].

Based on recent literature, it can be concluded that biomimetic tissue adhesives have the potential to be used in numerous applications. In general, the adhesive strength of this class of materials is reported to be 20-90 kPa [105, 111, 113]. The cytocompatibility is dependent on the amount of material added to cells, but *in vivo* tests showed no adverse tissue reactions [105]. However, there is almost no information about degradation profiles of the reported adhesives and of the mechanical properties of the cured networks. Therefore, it cannot be indisputably concluded that these materials could be suitable for treating meniscal tears.

Synthetic and hybrid tissue adhesives

Another group of adhesives are polymers, natural, synthetic or combination of both, whose end-groups have been modified in a way that allows simultaneous curing and attachment to the tissue. Examples of this class are cyanoacrylates, materials containing N-hydroxysuccinimide (NHS)- or isocyanate-terminated polymers, as well as polymers that can be photo cross-linked or those forming networks due to physical interactions e.g. due to thermal gelation.

Cyanoacrylate adhesives

Cyanoacrylate adhesives are very attractive materials for tissue fixation due to their high bonding strength and ability to bond in a wet environment. The cyanoacrylates monomers polymerize through contact with water (hydroxide ions) or a weak base, such as cell membranes and tissue [114], (see Figure 8). They are applied to the wound site as cyanoacrylate monomer that undergoes immediate polymerization to form a polymer film, [115]. However, cyanoacrylates still have some serious drawbacks for biomedical application. They degrade reactively in aqueous media with toxic formaldehyde as their degradation product [116, 117]. They are brittle and rigid with the values of elastic modulus in the range of 500-1500 MPa [118]. Additionally, cytotoxic effects may occur due to oxidative conversion of membranous lipids [119]. Nevertheless, clinical use of cyanoacrylate adhesives has

been reported as a good replacement for sutures, because of its better cosmetic effect, reduced pain and recovery period [120].

Commercially available Dermabond® and Histoacryl® are widely used as topical skin adhesives, but they are not bio-absorbable and therefore in clinical practice they are used only for skin wounds [99]. Still, the use of Histoacryl® has been compared with sutures in delaying the formation of a 2 mm meniscal gap [121]. The bovine menisci were placed in a tensile loading machine and the force needed for gap formation was measured. The tear was repaired with either vertical sutures, Histoacryl® or both. It was found that Histoacryl® gluing is superior to vertical sutures regarding gap delaying. However, the best results were obtained when glue and sutures were used together. Reckers et al. reported an *in vivo* study of cyanoacrylate surgical adhesives used for the fixation of transplanted menisci in rabbits [122]. It was concluded that the glue induced necrosis from the cortex to the bone marrow of transplanted bone surface. In order to improve the biocompatibility and mechanical properties of cyanoacrylates, Lim et al. mixed cyanoacrylate with poly-L-DOPA [123] in order to improve the physiochemical properties of cyanoacrylate for use as tissue glue. Lim and Kim also proposed to mix the cyanoacrylate component with poly(L-lactide-co-caprolactone) to obtain a biodegradable elastomer [124]. They reported that, although the adhesive strength was decreased compared to a pure cyanoacrylate compound, the biocompatibility and flexibility were improved. This indicates that these materials are more suitable for specific biomedical applications.

In conclusion, of all known adhesives, cyanoacrylates are the class of adhesives that form the strongest bond with tissue. Their adhesive strength values, depending on the tissue type and type of test performed, are reported to be in the range of up to a few MPa (30kPa adhesive strength to skin up to 2 MPa to bone) [87, 125, 126]. So far, their toxicity limits their application within the body and they have been FDA-approved only for topical skin wound closure. Moreover, their high stiffness (high E modulus), limits their application in soft tissues, such as meniscus.

Figure 8. Schematic illustrating the polymerization principle of cyanoacrylates.

Chemically cross-linkable adhesives

In the first class of these materials, the presence of N-hydroxysuccinimide activates carboxylic groups and allows them to react with amine groups present on the surface of the tissues to form amide bonds. In contrast, an unactivated carboxylic acid group would just lead to salt formation with an amine. Strehin et al. characterized chondroitin sulphate-PEG adhesive hydrogels (CS-PEG), which covalently adhere to tissue through an amide bond. CS-NHS was mixed with PEG-(NH₂)₆ to tune hydrophilicity and the molecules react to form a hydrogel, but at the same time, CS-NHS also reacts with tissue [127], see Figure 9a. Simson et al. described an adhesive hydrogel composed of a mixture of bone marrow aspirate and chondroitin sulphate end-functionalized with NHS to bind and stabilize the interface of bovine meniscus tissue [85]. The results showed that the adhesive strength to meniscus was in the range of 60-335 kPa, which should be enough to keep a torn meniscus together. Moreover, the viability and proliferation of fibrochondrocytes was positively influenced by the glue. Therefore, it was concluded that this system could be used to mechanically stabilize a tear and stimulate regeneration of the tissue across the injury site.

Taguchi et al. [128] investigated biodegradable adhesives composed of human serum albumin (HSA) and organic acid-based cross-linkers (trisuccinimidyl citrate, disuccinimidyl tartarate (DST) and disuccinimidyl malate) with activated NHS groups. The best results were obtained using DST. The adhesive strength of these biocompatible and bioresorbable materials was better than that of fibrin glue. The active ester groups present in DST react with the amine groups from HAS and collagen molecules from the tissue. The adhesive composed of DST/HSA was evaluated in the gluing of meniscal tears [129]. Glued and sutured porcine menisci

were implanted subcutaneously in rabbits, and after 3 months, tensile tests and histology were done. Comparisons were made between tears treated with sutures, tears treated with sutures soaked with adhesive and tears treated with only adhesive. The results showed best performance when the sutures were soaked with adhesive (~70N), followed by sutures (~61N) and adhesive (~60N) only. This indicates the potential of this class of materials in meniscal repair.

Adhesives comprising isocyanate-terminated molecules have a similar bonding mechanism. They form a covalent bond with the tissue and cure to form polyurethane networks upon contact with the surrounding fluids [130], see Figure 9b. Compared with other biomaterials, polyurethanes possess many advantages, such as high tenacity, toughness, water and chemical resistance and mechanical flexibility [131]. Biodegradable polyurethanes are designed to undergo (enzymatic) hydrolytic degradation to non-cytotoxic products [132]. Mostly aliphatic diisocyanates are used in the design of biomaterials intended for biomedical use, since it has been shown that they exhibit lower toxicity comparing to aromatic ones [133].

Hadba et al. described the development of two compositions of branched isocyanate-functional adhesives based on poly(ethylene glycol) and adipic acid functionalized with 2,4-toluene diisocyanate (TDI) or 4,40-methylene-bis(phenyl isocyanate) (MDI) [134]. The adhesive strength of the formulated materials to porcine stomach tissue depended on the specific formulation, the highest value determined was approximately 100 kPa (1093gf/cm²).

Rohm et al. and Sternberg et al. [119, 135] reported the use of mixtures of 1,2-ethylene glycol bis-(dilactic acid) functionalized with hexamethylene diisocyanate (HDI) with different biodegradable polymers such as hyaluronic acid, gelatin, chitosan acetate and chitosan chloride. The obtained products had promising properties in terms of biodegradability, biocompatibility and adhesive strength (which was similar or better than fibrin glue). The solidification speed could be adjusted by using aqueous solution of chitosan chloride as a second component of the adhesive system.

Nayeb-Habib et al. developed a isocyanate pre-polymer and chitosan gel based tissue adhesive in order to combine good adhesion of isocyanates and biocompatibility and healing properties of chitosan [136]. Degradation products of this polymer did not induce any toxicity. Field et al. evaluated two isocyanate-based adhesives to repair full thickness meniscal wounds in an ovine model *in vivo* over a period of 1 month [137]. A 10 mm defect in the medial meniscus was repaired with these adhesives, and after 1 month signs of repair and tissue regeneration were observed and no adverse inflammatory reaction was present. Nevertheless, despite the progress in the development of less toxic materials with suitable mechanical properties and satisfactory curing times, until today there is only one isocyanate-based tissue adhesive commercially available: TissuGlu[®] (Cohera Medicals Inc[®]). It is FDA-approved for use in abdominoplasty surgery.

Figure 9. a) Scheme of the adhesion and network formation mechanism of CS-PEG hydrogels. These hydrogels attach via covalent bonding of the NHS-activated carboxyl groups to the amine groups of the tissue (reprinted from [127]). b) Scheme of the attachment of isocyanate-terminated polymer molecules to the surface of the tissue by covalent bonding and the formation of a polymer network by cross-linking.

Photo cross-linkable adhesives

Another interesting class of tissue adhesives are those that are photo cross-linkable, an example is commercially available FocalSeal. It is a copolymer composed of hydrophilic polyethylene glycol (PEG), and glycolide or lactide, trimethylene carbonate and acrylic acid moieties. These materials are crosslinked by light, which also initiates their adhesion to tissue [138]. The acrylate groups covalently bind to the amine groups of the tissue and form an interpenetrating network upon curing. The material is biodegradable; resorption takes approximately 3 months [99]. The

application process, however, is complicated because delivery of a source of curing light to the application site can be technically difficult.

In another study Lang *et al.* developed acrylate-functionalized copolymers based on sebacic acid and glycerol that could be cross-linked with light. [139]. The materials were used *in vivo* to attach a polymeric patch to the beating heart of rats and pigs. The patch remained in place even under higher than normal heart beat rates.

Ishihara et al. [140] worked on chitosan derivatives that could be crosslinked with UV light. The chitosan hydrogel obtained could stop bleeding from a cut mouse tail and keep two pieces of sliced mouse skin together. Moreover, application of the chitosan hydrogel induced significant wound contraction and accelerated wound closure and healing.

In a recent study by Jeon et al. a light-activated adhesive based on mussel proteins and insect dityrosine crosslinking chemistry is reported [141]. The adhesive could be easily photo-cross-linked using visible light, the adhesion and cross-linking mechanism is depicted in Figure 10. The adhesive strength to porcine skin was around 50 kPa, and no adverse tissue response after closing incisions on the back of rats was observed.

Figure 10. Network formation of a tissue adhesive with visible light using dityrosine groups (reprinted from [141]).

Thermo-responsive adhesives

A different group of materials are those, which can be cross-linked physically. Lando et al and Cohn et al. developed an interesting approach, in which thermo-responsive biodegradable copolymers based on lactide - ϵ -caprolactone and three-armed trimethylolpropane-lactide- ϵ -caprolactone were used as tissue adhesives [142, 143]. These low molecular weight materials were in a flow state with low viscosity at the temperature of application and could attain high viscosity at the body temperature. The adhesive failure strength to polyamide (6,6-nylon) films, which was used as a model for a tissue, was measured to be up to 2N/cm width of the film. Although chemically modified tissue adhesives hold great potential for use in a variety of medical applications, further studies are required to develop materials for specific applications such as the treatment of meniscus tears.

Formulation of tissue adhesives with biological factors

It has been recognized that incorporation of biological molecules, growth factors, platelet rich plasma (PRP), bone marrow (BM) and living cells to the adhesive can

enhance healing of the tissue [87]. Ishimura et al. reported an experimental study in which they incorporated marrow cells into fibrin gel and compared the healing rate of the torn meniscus with fibrin gel that did not contain cells [86]. The results showed faster and more mature healing of defects in the group, which contained marrow cells. A similar approach was used by Scotti et al.: To improve the bonding between two swine meniscal slices, articular chondrocytes were added to a fibrin glue gel. The bonding capacity was evaluated in a nude mouse model after implantation for 4 weeks [144]. The results showed firm gross bonding in the experimental samples, while no bonding was observed in the control samples that did not contain cells. Although the success of this approach has only been described in a limited number of studies, the very promising results obtained justify the further investigation of cell-containing adhesives for meniscus repair.

How do tissue adhesives compare with the requirements for repair of the damaged meniscus and the current gold standard treatment?

The most important prerequisites for a tissue adhesive to be suitable for meniscus tear repair include: adequate mechanical properties; suitable degradation profile, sufficient adhesive strength, short curing times and good biocompatibility (see Table 1). Comparing these requirements with the properties of the tissue adhesives described above, it can be concluded that up to now no tissue adhesive material has yet been developed that fully complies with all requirements. However, this not only due to sub-optimal properties of the adhesives, such as insufficient bonding strength in the case of enzymatically cross-linked adhesives or poor biocompatibility in the case of cyanoacrylates. It is also due to lack of the data that is required to be able to conclude if a certain material is suitable for the intended application. Simson *et al.*, for example, described a promising bone marrow-chondroitin sulphate tissue adhesive for meniscus repair, however no information regarding its biodegradability was presented.

Moreover, although lap shear adhesion tests are one of the most often employed methods to assess the adhesive strength of newly developed glues, the conditions under which these tests are conducted are not standardized. There have been many substrates and tissue models used to evaluate the adhesion strength of tissue

adhesives, e.g. polyamide films [142, 143], gelatin pieces [145-147], collagen membranes [97], and even pig skin and wood [74]. Additionally, very different environments (wet or dry), gluing temperature and the curing times have been used. These large variations make direct comparison of the outcomes of described experiments difficult.

Up to now, there have been only few clinical studies reporting the use of tissue adhesives in the repair of meniscal tears, all studies involved fibrin glue. As this particular adhesive has poor mechanical- and adhesive properties, it cannot be used as to replace the use of sutures as gold standard. There is much interest in the development of new adhesive materials. However, more systematic (clinical) research is needed before the suitability of a newly developed tissue adhesive for the intended application can be proven.

Conclusions

Among the methods currently used to repair meniscus, the ideal solution has not yet been found. Tissue adhesives hold great potential to replace or support sutures and staples. Many new adhesive materials with a good prognosis for use in a variety of applications have been developed. However, most of them have not been sufficiently characterized to be able to qualify them as being suitable for meniscus repair. Nevertheless, chemically cross-linked adhesives seem the most versatile as these are based on existing natural or synthetic polymers and can be readily modified. Their properties can be adjusted by careful molecular design and chemical functionalization to make them suitable for the intended application.

Standardized relevant biomechanical and biological models need to be defined to compare different available and developed tissue adhesives, and to be able to address their suitability for the repair of meniscal tears. In clinical practice, the most successful treatment modality remains the use of sutures. Nevertheless, there is great need for a suitable meniscus tissue adhesive. Such adhesive should be easily applied in a meniscal tear, bind strongly to meniscal tissue, hold the torn region together, facilitate its healing, and then gradually degrade into non-toxic products.

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Chapter 3

Development of Tissue Adhesives Based on Amphiphilic Isocyanate-Terminated Trimethylene Carbonate Block Copolymers

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Abstract

In this study, novel three-armed star-shaped isocyanate-terminated block copolymers based on trimethylolpropane ethoxylate (TMPE) and trimethylene carbonate (TMC) varying in composition were designed and synthesized. By reaction with water, networks were formed. The characteristics of these networks were compared with those prepared from analogous linear structures based on polyethylene glycol (PEG) and TMC.

Tensile testing of the networks highlighted good mechanical properties. In lap shear adhesion tests to chamois leather, very good bonding strengths of up to 0.6 MPa were obtained. These values compare favorably with those of Dermabond[®], which is currently one of the strongest clinically used tissue adhesives.

Introduction

Biodegradable tissue adhesives have great potential to be used in the biomedical field as replacement for sutures and staples. They allow easier and faster application and the procedure is less painful for the patient [1, 2].

In practice, only two types of adhesives are mainly applied in the clinic: fibrin glue and cyanoacrylates. Fibrin glues are natural materials that crosslink to form hydrogels via a mechanism that resembles the final stages of blood coagulation [3]. Fibrin is biocompatible and biodegradable, it rapidly degrades within 2 weeks. However, its bonding strength to tissue is too low to be used for load bearing applications and it can be a source of disease transmission [4, 5].

Secondly, cyanoacrylates are often applied. They have very good adhesive properties and cure quickly [6], however they are hard, rigid and brittle. These compounds are degradable, but since formaldehyde is a degradation product, their toxicity is an issue [7, 8]. For this reason their application is limited to topical skin wound closure. Commercial products are Dermabond[®] and Histoacryl[®].

There is a clear need for a new tissue adhesive, with good mechanical- and adhesive properties that at the same time is biodegradable and non-toxic. It has been shown that isocyanate-functionalized polymers potentially can be used as tissue adhesives [9]. Our group reported that linear copolymers (oligomers) based on poly(ethylene glycol) (PEG) and trimethylene carbonate (TMC), which were end-functionalized

with isocyanate groups have adhesive properties to intervertebral disc tissue [10]. PEG is a biocompatible hydrophilic polymer, while TMC is hydrophobic and flexible and provides enzymatic degradability [11]. Variation of the copolymer composition allows adjusting the hydrophilicity of the system and optimizing the wetting of the tissue surface [12]. The isocyanate groups allow chemical reactions with the amine, thiol and hydroxyl groups present in the tissue forming covalent bonds [13]. At the same time, reaction with water leads to the formation of amine end-groups which can react with isocyanate end-groups to form biuret bonds and network [14]. As carbon dioxide is formed during this reaction as well, these amphiphilic networks can be foamed.

In this study, we designed novel three-armed star oligomeric copolymers based on trimethylolpropane ethoxylate (TMPE), TMC and hexamethylene diisocyanate (HDI). Low molecular weight reactive copolymers with different contents of TMC were prepared, to assess the influence of the hydrophilic to hydrophobic ratio on the properties of the cured materials. The properties of the star-shaped copolymers and resultant networks were compared with those of analogous linear structures.

Experimental Part

Materials

Trimethylene carbonate (TMC) was purchased from Boehringer Ingelheim (Germany). Polyethylene glycol (PEG) ($M_n=400$ g/mol), trimethylolpropane ethoxylate (TMPE) ($M_n=450$ g/mol), acetic anhydride, stannous octoate (tin 2-ethylhexanoate, SnOct_2), chloroform-d (CDCl_3), fibrinogen and thrombin (both isolated from bovine plasma) were purchased from Sigma Aldrich (Netherlands). Hexamethylene diisocyanate (HDI) was purchased from Merck Schuchardt (Germany). Dermabond[®] (2-octyl cyanoacrylate) was purchased from Ethicon, Johnson & Johnson (Netherlands). Phosphate buffered saline (PBS) was purchased from B Braun Melsungen AG (Germany). Diethyl ether was purchased from Biosolve (Netherlands) and dried over molecular sieves A4 prior to use. PEG and TMPE were dried at 140 °C under vacuum before use. All other products were used

as received.

Synthesis and characterization of oligomers and isocyanate-functionalized oligomers

Oligomers were synthesized by ring opening polymerization of TMC using PEG or TMPE as the initiator. In a typical procedure, TMC was reacted with PEG or TMPE using $\text{Sn}(\text{Oct})_2$ (0.02 mmol/mol of monomer) as a catalyst. The polymerization was conducted at 130 °C for 3 days under nitrogen atmosphere. Oligomers with TMC and PEG molar ratios of 2:1 and 4:1, and oligomers with TMC and TMPE with molar ratios of 3:1 and 6:1 were prepared. The used nomenclature for the synthesized oligomers is: $\text{PEG}-(\text{TMC}_m)_2$ or $\text{TMPE}-(\text{TMC}_m)_3$, where m is the number of TMC units per oligomer arm.

The oligomers were then reacted with HDI to obtain reactive molecules. In a typical procedure, a metered volume of HDI was placed in a flask under the flow of nitrogen. The oligomer was then added drop-wise to maintain an excess of the isocyanate compound. The final molar OH/NCO ratios for the linear- and three-armed compounds were 2.08 and 3.12, respectively. The reaction was allowed to proceed at 75 °C for 8 hours. The excess of unreacted HDI was removed by precipitation in dry diethyl ether, the product was dried under vacuum overnight. The resulting products were labeled: $\text{PEG}-(\text{TMC}_m\text{-HDI})_2$ or $\text{TMPE}-(\text{TMC}_m\text{-HDI})_3$. Figure 1 shows the synthesis of the oligomers and their subsequent functionalization with HDI.

The chemical structure of the oligomers and functionalized oligomers was assessed using ^1H NMR spectroscopy (Bruker 400MHz NMR spectrometer) with CDCl_3 as the solvent and FTIR spectroscopy using Perkin-Elmer Spectrum 1000 FTIR spectrometer. The accuracy of this method is $\pm 5\%$.

Figure 1. Synthesis of PEG-(TMC_m-HDI)₂ (a) and TMPE-(TMC_m-HDI)₃ (b) isocyanate-functionalized oligomeric copolymers. The value of *m* is 1 or 2.

Network formation and network characterization

Network films were prepared by casting the isocyanate-functionalized oligomers on glass with a 0.3 mm thick casting knife and immersing in excess of water. The cast films were left overnight at room temperature (RT) to allow reaction with water and curing.

To evaluate the network characteristics, their gel content, degree of swelling in chloroform and water uptake were determined. The prepared networks were dried in a vacuum oven at 40 °C until constant weight, then their initial mass was determined (*m_i*). The specimens were then placed in chloroform or water for 24 hours and their wet mass (*m_{wc}* and *m_{ww}*) was determined. Finally, the samples were re-dried in a vacuum oven at 40 °C until constant weight and their dry mass was determined (*m_d*). The experiments were performed in triplicate.

The gel content (GC) in chloroform was calculated using:

$$GC = \left(\frac{m_d}{m_i} \right) \cdot 100\%$$

The degree of swelling (DS) in chloroform was calculated using:

$$DS = \left(\frac{m_{wc}}{m_d} \right) \cdot 100\%$$

The water uptake (WU) was calculated using:

$$WU = \left(\frac{m_{ww} - m_d}{m_d} \right) \cdot 100\%$$

The thermal properties of the water-cured networks were determined by differential scanning calorimetry (DSC) using Perkin Elmer Pyris 1 before and after drying. The precision of this method for the temperature performance is ± 0.1 °C. Samples of 5-10 mg were heated from -100 °C to 100 °C at 10 °C/min.

Dumbbell-shaped test specimens of the water-cured network films were prepared according to ISO 37:1994 (E), Type 3 and subjected to tensile testing using a Zwick Z020 universal tensile tester. The grip separation was 35 mm and the crosshead speed was 50 mm/min. The mechanical properties of the films were evaluated both in the wet and in the dry state after drying in a vacuum oven at 40 °C for 24 hours. All measurements were performed in triplicate.

Application as tissue adhesive

Chamois leather (dermal sheep collagen) was chosen as a model for tissue. Strips of approximately 0.5x10x30 mm were cut, immersed in water and blotted with tissue to remove excess water. The different isocyanate-functionalized compounds were applied onto the chamois leather to evaluate their adhesive properties. On each strip, the adhesive covered an area of approximately 100 mm². Two of these strips were pressed together, immersed in water and left overnight at RT to allow curing and network formation.

To determine the adhesion strength, the wet specimens were subjected to a lap shear tensile test analogous to ASTM F2255-05 using a Zwick Z020 universal tensile tester. This is shown schematically in Figure 2. The grip to grip separation was 25 mm and the crosshead speed 50 mm/min. The shear adhesive bond strength expressed in MPa is the maximum shear force divided by the glued area. Each

measurement was performed at least in triplicate.

Two currently applied tissue adhesives Dermabond[®] and fibrin glue were used as controls. Dermabond[®] was used as received, fibrin glue was prepared by dissolving fibrinogen and thrombin in PBS at concentrations of 100 mg/mL and 100 U/mL, respectively. Subsequently, these two solutions were mixed in double syringe system with a static mixer and directly applied onto the wet chamois leather.

Figure 2. Testing of the lap shear adhesion strength after curing of the different tissue adhesives.

Preliminary cytotoxicity evaluation

The cytotoxicity of PEG-(TMC₁-HDI)₂ was evaluated using primary human annulus fibrosus cells (ScienCell, USA) and corresponding media (ScienCell). The cells were incubated at 37 °C and 5% CO₂ for 3 d to reach 90% confluence. The PEG-(TMC₁-HDI)₂ was added to the cell culture medium (0.3 g/ml) and incubated overnight at 37 °C. The extract was then filter-sterilized and added to the annulus fibrosus cells in culture at different concentrations varying from 1 to 20 % (ml extract per ml culture medium), 5 wells not containing the extract were used as control. The cells were then incubated for a further 24 h at 37 °C and 5% CO₂. At this time point, cell viability was assessed by basic light microscopical morphological investigation.

Results and Discussion

Synthesis and characterization of oligomers and functionalized oligomers

Several oligomers varying in composition were synthesized by ring opening polymerization of TMC using PEG or TMPE as initiator and subsequently reacted with HDI giving isocyanate-terminated reactive oligomers. NMR analysis showed that TMC conversion was higher than 98 % in all cases.

The molecular weight of PEG was determined by NMR after functionalization with

acetic anhydride, the molecular weight of TPME was confirmed by comparing the integral values of the methylene group at 0.9 ppm with the repeating ethylene oxide units ($-\text{O}-\text{CH}_2-\text{CH}_2-$) at 3.5-3.75 ppm. The TMC content and the number average molecular weights (M_n) of the non-functionalized oligomers were then determined by comparing the integral values of the TMC peak ($-\text{COO}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{O}-$) at 4.1-4.3 ppm or at 2.1 ppm and the integral values of the PEG peak ($-\text{O}-\text{CH}_2-\text{CH}_2-$) at 3.5-3.7 ppm in the ^1H NMR spectra. In case of TMPE, the integral values of TMC were compared with those of the TMPE methylene group at 0.9 ppm.

A typical spectrum of a PEG-(TMC₁)₂ oligomer is shown in Figure 3a.

Figure 3. Characteristic ^1H NMR spectra of (a) a PEG-(TMC₁)₂ oligomer and (b) a corresponding isocyanate-functionalized PEG-(TMC₁-HDI)₂ oligomer.

Table 1 shows that the TMC contents and the determined number average molecular weights of PEG, TMPE and the synthesized block copolymeric oligomers are close to the expected values.

The extent of functionalization of the oligomers was also assessed by ^1H NMR spectroscopy, see Figure 3b for a characteristic spectrum. The degree of functionalization was calculated by comparing the integral values of the TMC peaks at 2.1 ppm with those of the methylene peaks of HDI ($-\text{OOCHN}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NCO}$) at 3.3 ppm or at 1.44-1.6 ppm. As shown in Table 1, the degree of functionalization was high with values above 91% in all cases. In addition, FTIR spectroscopy showed the presence of both the unreacted free isocyanate end-groups

at 2235-2300 cm^{-1} and the urethane bonds at 3250-3500 cm^{-1} .

Table 1. Characteristics of the prepared oligomers. The TMC content, number average molecular weight (M_n) as well as the degree of functionalization (DF) after subsequent reaction with HDI were determined by ^1H NMR.

Oligomer	TMC content in oligomer [mol%]	$M_n^{\text{a)}}$ [g/mol]	M_n [g/mol]	DF after reaction with HDI [%]
PEG	-	400	398	-
TMPE	-	450	439	-
PEG-(TMC ₁) ₂	66.5	604	601	94
TMPE-(TMC ₁) ₃	74.5	756	739	100
PEG-(TMC ₂) ₃	80.0	809	806	99
TMPE-(TMC ₂) ₃	85.7	1062	1037	91

^{a)} expected value

Formation and characterization of networks

To evaluate the physical properties of the polyurethane networks, networks in the form of films were formed by reaction of cast isocyanate-functionalized oligomers with water. In a series of reactions, the formation of biuret linkages leads to covalent crosslinking of the block copolymeric chains. Also carbon dioxide, which can lead to foaming, is formed. In our experiments, foaming was limited and films with thicknesses of 0.3-0.4 mm were obtained.

The network properties of these crosslinked amphiphilic films are presented in the Table 2. It can be noted that the gel content of the networks is high for all the compositions, confirming successful functionalization of the oligomers with HDI and crosslinking with water at room temperature. The degree of swelling of the networks in chloroform varied with architecture of the functionalized oligomers. Networks prepared from the linear functionalized oligomers based on PEG could be swollen 800 to 1000 %, while networks prepared from TMPE based functionalized oligomers could only be swollen up to approximately 300%. This indicates that the latter networks are more densely crosslinked. The effect of composition seems to be minimal. (Note that the PEG-(TMC₂-HDI)₂ network with the highest degree of swelling had the lowest gel content).

Table 2 also shows that the water uptake of the networks depends on composition as well as on architecture of the functionalized oligomers used. For more or less comparable compositions, water uptake is significantly higher for networks based on PEG than for those based on TMPE as was the case for swelling in chloroform. The effect of TMC content is clear as well, especially for the networks prepared using linear structures. The highest water uptake is observed for PEG-(TMC₁-HDI)₂, in which the weight content of the hydrophilic PEG component is 43.6%. It was found that after uptake of water, the PEG-containing networks were opaque, while those based on TMPE remained transparent.

Table 2. Characteristics of the prepared networks. The gel content (GC), degree of swelling in chloroform (DS) and water uptake (WU) of the networks were determined in triplicate.

Network	GC [%]	DS [%]	WU [%]	TMC content [wt%]	PEG content [wt%]
PEG-(TMC ₁ -HDI) ₂	100±0	813±73	65±2	21.8	43.6
TMPE-(TMC ₁ -HDI) ₃	99±1	340±38	11±1	24.1	35.3
PEG-(TMC ₂ -HDI) ₂	86±5	1019±59	29±4	35.4	35.2
TMPE-(TMC ₂ -HDI) ₃	99±1	341±18	9±1	40.0	29.3

The thermal properties of the networks were evaluated, results are shown in Table 3. In the wet state, only single glass transition temperatures (T_g) can be observed. The networks prepared using PEG-containing functionalized oligomers had significantly lower glass transition temperatures than those prepared using TMPE. For both architectures an increase in TMC content leads to an increase in T_g . After drying the water-cured networks, melting points corresponding to the PEG or TMPE component in the soft segment ranging from approximately 35 to 48 °C were measured. Melting points at higher temperatures of approximately 80 °C that correspond to the urethane bonds in the hard segments could also be observed.

Table 3. Thermal properties of the prepared networks in the water swollen state and in the dry state.

Network	Wet	Dry		
	T _g ^{a)} [°C]	T _g ^{a)} [°C]	T _{m(soft)} ^{b)} [°C]	T _{m(hard)} ^{c)} [°C]
PEG-(TMC ₁ -HDI) ₂	-52.5	-40.2	38.4	80.0
TMPE-(TMC ₁ -HDI) ₃	-35.9	-13.6	48.7	80.0
PEG-(TMC ₂ -HDI) ₂	-36.1	-33.8	35.1	81.0
TMPE-(TMC ₂ -HDI) ₃	-24.2	-15.6	39.9	80.0

a) glass transition temperature

b) melting temperature of PEG or TMPE in the soft segment

c) melting temperature of the urethane bond in the hard segment

In the dry state, T_g of the networks is higher than in the hydrated state and varies from -40 to -34 °C for networks prepared from linear PEG-TMC structures and from -16 to -14 °C for three-armed TMPE-TMC structures. In dry PEG-TMC networks, the glass transition temperature increases with increasing TMC contents, this effect is not evident in TMPE-TMC networks.

The tensile properties of the prepared networks were assessed in the wet and in the dry state, the data is presented in Table 4. Especially for the networks based on linear isocyanate-functionalized PEG-TMC oligomers, composition and water content (see Table 2) has a significant influence on the mechanical properties. The tensile strength (σ), elasticity modulus (E) and elongation at break (ε) of PEG-(TMC₁-HDI)₂ networks that contain the lowest amounts of TMC are very much increased upon drying. For networks prepared from PEG-(TMC₂-HDI)₂ this effect is not as pronounced. As networks based on TMPE-TMC do not take up as much water (Table 2), the difference in tensile properties in the dry state of these networks do not differ very much from those in the wet state. In all cases the tensile strength and the elasticity modulus of the networks increase with the TMC content.

Table 4. Tensile properties of the prepared networks in the water-swollen state and in the dry state.

Network	Wet			Dry		
	σ [MPa]	E [MPa]	ϵ [%]	σ [MPa]	E [MPa]	ϵ [%]
PEG-(TMC ₁ -HDI) ₂	0.1±0.01	1.2±0.2	14.0±1.1	2.6±0.3	14.4±0.2	52.6±14.3
TMPE-(TMC ₁ -HDI) ₃	3.3±1.4	34.5±4.8	21.6±10.5	3.8±0.2	33.9±4.9	26.4±4.7
PEG-(TMC ₂ -HDI) ₂	3.9±0.5	21.5±5.0	108.6±27.4	3.5±0.1	35.3±4.1	48.0±0.7
TMPE-(TMC ₂ -HDI) ₃	5.0±2.8	45.1±11.6	22.7±7.6	4.4±0.5	48.9±10.2	21.7±1.3

Application as tissue adhesives

PEG-TMC and TMPE-TMC isocyanate-functionalized oligomers were applied as tissue adhesives using chamois leather (dermal sheep collagen) as a model for tissue. The reactive oligomers were spread onto the leather and allowed to cure overnight in a wet environment. Under these conditions the terminal isocyanate groups can react with amine- thiol- and hydroxyl groups present on the surface of the leather as well as with water to form the network.

The adhesive strength of the cured networks (the strength of the adhesive bond) was assessed in a lap shear test. Their strength in gluing chamois leather was compared with that of fibrin glue and Dermabond[®]. The results are shown in Figure 4. The figure shows that not only the composition (and concomitant water-uptake) has an important effect on the adhesive bond strength, but that also the architecture of the used isocyanate-functionalized oligomers is of great importance. With increasing TMC content of networks prepared from linear isocyanate-functionalized PEG-TMC oligomers the bond strength increased from 0.03 to 0.35 MPa. The bond strength of networks based on three-armed isocyanate-functionalized TMPE-TMC was even higher and increased from 0.56 to 0.68 MPa. In all cases, we observed cohesive failure of the glue, the interface between the cured adhesive and the leather remained intact. It is noteworthy that the TMPE-(TMC₁-HDI)₃ and TMPE-(TMC₂-HDI)₃ networks with the lowest water uptake had the highest adhesive bonding strengths to chamois leather. Apparently, in this system, it seems that further increasing hydrophilicity in order to enhance wetting of the leather surface is not necessary.

Figure 4. The adhesive bond strength in the wet state of glued chamois leather upon curing isocyanate-functionalized PEG-TMC and TMPE-TMC oligomers with water. Fibrin glue and Dermabond® were used as controls. Values are expressed as mean $\pm$ standard deviation.

The adhesive bonding strength of fibrin glue was found to be very low, as is known from literature [15]. Dermabond® in contrast, is a much stronger adhesive [16], and in glueing chamois leather we found high adhesive strength values of approximately 0.4 MPa. The adhesive bond strength of the TMPE-TMC based networks was found to be even better than that of Dermabond®.

Cytotoxicity of isocyanate-functionalized oligomers

As isocyanates can be toxic to cells, we conducted a first experiment to assess the cytotoxicity of PEG-(TMC₁-HDI)₂ using human annulus fibrosus cells. The adhesive was placed in cell culture medium (0.3 g/ml), the extract of this large quantity was then added to proliferating annulus fibrosus cells. It was found that the extract was not toxic to the cells at concentrations lower than 2 % (ml extract per ml cell culture medium) as cell viabilities remained equal to cell viabilities in cell culture medium only. With increasing concentration of extract in the culture

medium, cell viabilities decreased: at 3, 5, 8, 10 and 20 %, the values of the cell viabilities were respectively 80, 40, 0, 0, 0 % compared to the control. This indicates that cytotoxicity is strongly dependent on the amount of extract administered to the cells. At the lowest concentrations, cell viability is not much affected. Nevertheless, it will be important to conduct more extensive investigations of possible cytotoxicity.

Conclusions

We have designed and synthesized PEG-TMC and novel three-armed star-shaped copolymers based on TMPE and TMC, which were both end-functionalized with isocyanate groups for use as water-curing tissue adhesives. The mechanical properties and the bond strength to chamois leather of the three-armed star-shaped adhesives were significantly better than those of the linear structures and those of fibrin glue and Dermabond®, which were used as controls. Although more extensive experiments are needed to confirm the compatibility of these adhesives with cells and tissues, the developed materials are promising for use as degradable and resorbable tissue adhesives.

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Chapter 4

Adhesion of Tissue Glues to Different Biological Substrates

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Abstract

Tissue adhesives are attractive materials with potential to replace the use of sutures and staples in the repair of the injured tissues. The research field of tissue adhesives is dynamically growing, and different methods and tissue models are employed to evaluate the adhesive properties of newly developed materials. It is thus difficult to directly compare the properties of materials developed by researchers from different groups. Moreover, the extrapolation of results obtained using different tissue models to the targeted human tissues is often not possible.

Therefore, the purpose of the current study was to evaluate the adhesive properties of the three different tissues adhesives: the clinically used Dermabond® and fibrin glue, and the recently developed isocyanate-terminated three-armed adhesive block copolymer. Different biological substrates were used to assess their adhesion strengths: bovine Achilles tendon, meniscus tissue and skeletal muscle, chamois leather and collagen films. Comparisons of the adhesive properties of the glues to these substrates were made. The obtained results were analyzed in terms of the chemistry and the adhesion mechanisms of the glues, and the composition and properties of the substrates like their hydrophilicity and surface morphology.

We conclude that standardized procedures and models should be developed to allow direct comparison between the different (developed) tissue adhesives and to enable extrapolation of the results towards the targeted human tissues.

Introduction

Tissue adhesives are an attractive class of materials with the potential to be used as a replacement of other surgical techniques, such as sutures or staples. Tissue adhesives cause minimal tissue trauma, they are easy to apply and there is no need to remove them after the tissue has healed [1]. Therefore, much interest has recently been paid to the development of new adhesive materials which are aimed at the repair of different types of tissues. Adhesives are already used in clinical applications such as the repair of skin lacerations, pulmonary leaks, meniscus tears, abdominoplasty and cardiovascular vessels [2-4].

However, the availability of the human tissues for evaluating adhesive properties of newly developed adhesives is limited. Therefore, different materials have been employed in laboratory practice to serve as tissue models. Examples are polyamide films [5, 6], gelatin pieces [7-9], collagen membranes [10], animal tissues and even models using pieces of wood and pig skin [11]. However, the adhesion strength of the glues may significantly differ when tested using these tissue models instead of the targeted living tissues. Moreover, even though norms for testing adhesive materials exist, *e.g.* ASTM F2255-05, still the variability in the preparation of the substrates, the gluing procedures and the curing times can introduce large variations in the obtained results and direct comparison of the novel materials developed in different research groups is often not possible.

Therefore, the aim of this study was to evaluate and compare the adhesive strength of three different tissue glues to different biological substrates, using the clinically applied Dermabond[®] and fibrin glue, and a recently developed novel three-armed adhesive block copolymer. The differences in the observed adhesive strengths are discussed in terms of the hydrophilicity of the substrates, their surface morphology and the contact angle between applied glues and the surfaces. Recommendations towards choosing adequate substrates for evaluating adhesion strengths of newly developed glues are formulated.

Materials and Methods

Tissue adhesives

Three tissue adhesives were used in this study: Dermabond[®] (Ethicon, Johnson and Johnson, the Netherlands), fibrin glue (mixture of fibrinogen (100 mg/mL) and thrombin (100 U/mL) in PBS, both isolated from bovine plasma, Sigma Aldrich, the Netherlands) and a three-armed block copolymeric isocyanate-terminated adhesive, TMPE-(TMC₂-HDI)₃, which was synthesized as described in detail previously [12]. The building blocks of the copolymer were trimethylolpropane ethoxylate (TMPE, $M_n=450\text{g/mol}$), trimethylene carbonate (TMC) and hexamethylene diisocyanate (HDI).

Preparation of the substrates for gluing experiments

Bovine meniscus, bovine Achilles tendon and bovine skeletal muscle tissue were obtained from a local slaughterhouse. Meniscus tissue was cut with a cryotome (Microm GmbH, Germany) in the direction along the alignment of the collagen fibers to obtain strips measuring approximately 0.5x10x25 mm. Achilles tendons were cut lengthwise in the same manner. Skeletal muscle tissue was frozen and then cut with a scalpel to strips of approximately 2x10x25 mm. Chamois leather (dermal sheep collagen) was cut in strips of 1x10x25 mm. Collagen films were prepared by swelling 1 w/v % of type I insoluble collagen (from bovine Achilles tendon, Sigma Aldrich, Germany) in acetic acid (0.05 M) overnight at 4 °C. Subsequently, the mixture was shredded for 10 min at 9500 rpm at 0 °C using a Braun MR 500 HC blender (Braun, Germany). The resulting slurry was filtered through a nylon filter (average pore size 100 µm) and it was cast on a glass plate using a 3 mm thick casting knife and then left to dry under fume hood at room temperature (RT). After drying, collagen films of a thickness of approximately 100 µm were obtained. Due to their weak mechanical properties, they were mounted on steel plates of a size 0.5x10x30 mm with a cyanoacrylate glue prior to adhesion testing. All substrates were kept in water before use.

Application of the tissue adhesives and evaluation of their bonding strength

All tissues were thawed and together with chamois leather and collagen films were kept in demi water to prevent drying. The surfaces of the substrates were blotted dry and the different tissue adhesives were applied onto the strips. Dermabond® was used as received. Fibrin glue was prepared by mixing two solutions (fibrinogen and thrombin) in a double syringe equipped with a static mixer (Sulzer, Switzerland) and directly applied onto the substrates. The three-armed copolymeric adhesive was applied using a spatula. In all cases, the adhesives covered an area of approximately 100 mm². Two of these strips were then pressed together, immersed in water and left overnight at RT to allow attachment of the adhesives to the substrates and curing.

To determine the strength of the adhesive bonding of the glues to the substrates after curing, the wet specimens were subjected to lap shear adhesion testing using a Zwick

Z020 universal tensile machine according to ASTM F2255-05 standard (n=5). The grip to grip separation was 22 mm and the crosshead speed was 50 mm/min. The shear adhesive bond strength (S) was calculated as the maximum shear force divided by the glued area.

Contact Angle Measurements

Static Contact Angle (SCA) measurement between different tissue adhesives and substrates were measured using optical contact angle device (OCA15, Dataphysics, Germany). The drop of adhesive was deposited onto the surface of the wet substrates, after which the drop contour was fitted by the Young-Laplace method. Each measurement was performed in triplicate.

Determination of the water content of the substrates

All tissues were thawed and together with chamois leather and collagen films were kept in demi water to prevent drying. Subsequently, the substrates (n=3) were blotted dry and their initial mass was determined (m_i). The samples were frozen in liquid nitrogen, freeze-dried and their mass in the dry state was again determined (m_d).

The water content of the substrates, expressed as a weight %, was calculated as:

$$WC = \frac{m_i - m_d}{m_i} \cdot 100\%$$

Surface morphology of the substrates

The surfaces of the substrates and the surfaces to which the glues were applied were visualized using scanning electron microscopy (SEM, n=1). The adhesives were applied on strips of the different substrates, then kept in water overnight to let the adhesives attach and cure. Subsequently the samples were mounted on the SEM holders and were dried in an oven with convective air flow at 40 °C. The surfaces of the samples were gold-sputtered for 60 seconds. The morphology was studied using Zeiss Gemini Sigma 300 SEM operating at 10 kV electron beam. Cross-sections of chamois leather and meniscus tissue were also imaged after application of the three-armed adhesive. These samples were prepared as described above, but they were

fractured in liquid nitrogen before drying. An FEI ESEM XL30 apparatus operating at 5 kV electron beam was used in this case.

Results

The adhesive strength of the glues to the different substrates was determined in the lap shear adhesion tests. The observed failure mechanism was in all cases adhesive failure: the glues detached from the substrates. The values of the adhesive strengths of particular glues were highly dependent on the type of the glued substrate (Figure 1). The adhesion strength of Dermabond® varied from 19.9 ± 5.7 kPa up to 868.3 ± 372.6 kPa for muscle tissue and collagen films, respectively. Fibrin glue had adhesive strengths ranging from 1.9 ± 0.8 kPa to meniscus up to 53.8 ± 35.0 kPa to collagen films. The three-armed block copolymeric glue had the highest adhesive strength to chamois leather (719.4 ± 225.0 kPa) and collagen films (883.0 ± 118.8 kPa) and the lowest to muscle tissue (5.3 ± 4.3 kPa).

Figure 1. Adhesive strength of the different glues to different biological substrates determined in lap-shear testing. Data are presented as mean $\pm$ standard deviations.

The static contact angles between drops of the glues and particular substrates are presented in Figure 2. Contact angles between Dermabond® and different substrates

varied from $17.5 \pm 5.8^\circ$ to collagen films up to $35.8 \pm 2.1^\circ$ to muscle tissue. The contact angle between Dermabond[®] and chamois leather could not be determined, as drops of the glue were directly adsorbed into the surface of the substrate. The contact angle between fibrin glue and muscle tissue and chamois leather was relatively low; $11.1 \pm 2.1^\circ$ and $16.7 \pm 13.5^\circ$, respectively. For the other substrates it was slightly higher, ranging from $29.8 \pm 11.1^\circ$ for Achilles tendons up to $43.7 \pm 16.2^\circ$ for collagen films. The three-armed glue had similar contact angles to all the substrates, ranging from $43 \pm 3.3^\circ$ for collagen films to $54.3 \pm 2.6^\circ$ for chamois leather.

Figure 2. Static contact angles between glues and different hydrated substrates. Data are presented as mean $\pm$ standard deviations. *Note that the contact angle between Dermabond[®] and chamois leather could not be determined due to immediate adsorption of the glue drop.

The water content of the substrates was between 63.3 ± 1.8 wt % for Achilles tendon up to 77.5 ± 1.5 wt % for muscle tissue (Figure 3). The collagen content of the human Achilles tendon is reported to be approximately 90% of the dry mass (mostly type I collagen). If we assume similar compositions of animal and human tissues, then taking the experimentally determined water content of 63.3 wt %, the collagen content would constitute for approximately 24% of the wet mass. The

experimentally measured meniscus water content of 68.9 ± 0.8 wt% is very close to values reported in literature, which are found to be approximately 72 wt% for healthy human meniscus [13]. The collagen content of meniscus tissue is approximately 22 wt%, mostly type I and II. In muscle tissue, on the other hand, collagen constitutes only between 2 and 6% of the wet mass [14]. Assuming that both chamois leather and collagen films consist of only collagen, their collagen contents are approximately 26% of their wet mass.

Figure 3. Water content of the investigated substrates. Data are presented as mean $\pm$ standard deviations.

The SEM images of the surfaces of the substrates showed clear differences in their morphology (Figure 4). Chamois leather had numerous exposed fibers at the surface. The surface of the collagen film on the other hand, was smooth. The structure of the surfaces of the living tissue was relatively smooth with visible fibers aligned parallel to the surface. Dermabond[®] and the three-armed block copolymeric glue formed a smooth film on the surfaces of the substrates. Fibrin glue, on the other hand seemed to be partially absorbed by the substrates, which is especially pronounced on the chamois leather surface. This, however, might be the effect of the drying procedure of the samples.

Figure 4. SEM images of the top surfaces of the investigated biological substrates (top row), and the surfaces partially covered with the glues. The scale bar indicates 200 μm .

Additionally, images of cross-sections of the chamois leather and meniscus specimens after application of the three-armed adhesive, clearly showed that the glue had penetrated in-between the fibers of the chamois leather (Figure 5a). This was not the case with meniscus tissue, where the glue remained on the surface of the tissue (Figure 5b).

Figure 5. SEM images of the cross-sections of chamois leather (a) and meniscus tissue (b) after application of the three-armed adhesive on their surface.

Discussion

In order to understand the differences in adhesive strength of particular glues to various substrates, the gluing process must be analyzed. The gluing process consist of three main steps: (1) spreading of the liquid glue on the tissue surface (wetting), (2) attachment of the glue to the tissue and (3) curing of the glue (solidification). Steps (2) and (3) usually take place simultaneously. The attachment to the tissue can happen by means of chemical bonding, physical interactions (ionic interaction, van der Waals forces) or mechanical interlocking after solidification onto the surface of the substrate [15]. Therefore, both the chemistry and adhesive mechanisms of the glues and factors such as chemical composition and hydrophilicity of the substrates and their surface morphology are critical in achieving adhesive bond.

Dermabond[®] is based on 2-octyl cyanoacrylate, it polymerizes by reaction with trace amounts of water or upon contact with cell membranes and tissues [16]. The working mechanism of fibrin glue resembles the final stages of the blood coagulation process. It binds to the tissue by covalent bonding, and the formation of hydrogen- and electrostatic bonds [17]. The newly developed three-armed glue cures in the aqueous environment and attaches to the tissue via the formation of covalent bonds [12]. All

glues additionally attach by mechanical interlocking onto the surface of the substrate.

Contact angle measurement gives information of how well the glue can spread and wet the surface of the substrate. The lower the contact angle between the glue and the substrate, the better the wetting of the surface is and the higher the adhesive strength is to be expected.

Dermabond[®] had similar contact angles to Achilles tendons, meniscus and collagen films, and its adhesive strength to these substrates was high. The somewhat higher contact angle between Dermabond[®] and muscle tissue and the fact that the glue was absorbed immediately by chamois leather are probably the reasons for the lower adhesive strength to these substrates. Fibrin glue had various contact angles with the different substrates. Its (poor) adhesive properties did not show clear dependence on these values. Also for the three-armed block copolymeric adhesive, no clear trend between adhesion strength and contact angle was established.

The collagen content and the hydrophilicity of the substrates are other factors determining the adhesive bond that can be achieved between glues and their surfaces. Muscle tissue had the lowest collagen content and the highest water percentage of all the substrates, and the adhesion strength of all three tested glues was relatively poor to muscle tissue when compared to other substrates. Moreover, both fibrin glue and the three-armed block copolymeric glue had higher adhesion strengths to chamois leather and collagen than to living tissues. It seems therefore, that the collagen content, which is higher for these two substrates than for the other substrates, is another critical factor determining the adhesion strength of these glues. Moreover, although the use of such synthetic substrates does not have the same clinical relevance as tests performed on living tissues, their production can be easily controlled thereby allowing to directly compare results obtained by different researchers. Furthermore, as their composition is well-defined, their use may increase the accuracy of the obtained adhesive results.

SEM images showed that chamois leather had numerous fibers exposed at the surface, possibly also allowing good mechanical interlocking of the glues. Indeed, the images of cross-sections of chamois leather and meniscus tissue after application

of the three-armed adhesive clearly showed that the glue had penetrated in-between the fibers of the chamois leather. This explains the much higher adhesive strength of the three-armed glue to chamois leather (719 ± 225 kPa) than to meniscus tissue (88.2 ± 50.8 kPa). In addition, except for Dermabond[®] which due to its low viscosity was immediately absorbed, also the fibrin glue had better adhesion to chamois leather than to the other substrates. Furthermore, collagen films were a substrate that could readily be glued too by all three glues.

Based on the obtained results, it seems that collagen content, hydrophilicity and surface topography are critical factors for achieving good adhesive bonds. Employment of substrates that mimic the natural living tissues of interest, are the most promising materials with which to perform preliminary screening experiments in the development of novel adhesive materials. Nevertheless, in further evaluation of new materials, examination on the targeted animal- or human tissues should be performed in a well-controlled and standardized manner.

Conclusions

The results of adhesion strength determinations showed that not only the glues, but also the biological substrate had a significant influence on the adhesive strength of the bond. Therefore, there is a need for the development of unified tissue models and procedures to evaluate adhesive strengths in the development of novel tissue adhesives. Employing such a universal and standardized strategy would allow directly comparing the adhesive properties of different glues and extrapolating the results to the targeted human tissues.

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Chapter 5

Development of Biodegradable Hyper-Branched Tissue Adhesives for the Repair of Meniscus Tears

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Abstract

Meniscus tears are one of the most commonly occurring injuries of the knee joint. Current meniscus repair techniques are challenging and do not bring fully satisfactory results. Tissue adhesives are a promising alternative, since they are easy to apply and cause minimal tissue trauma.

In this study, a series of amphiphilic copolymers based on polyethylene glycol, trimethylene carbonate and citric acid were synthesized and subsequently end-functionalized with hexamethylene diisocyanate to form reactive adhesive materials. The shear adhesive strength of the networks to bovine meniscus tissue measured in a lap-shear adhesion test ranged between 20 - 80 kPa, which was better than for fibrin glue (10kPa). The elastic modulus of the networks depended on composition and was in the same range as that of human meniscus. Cell compatibility was assessed using Alamar Blue staining after incubation of the bovine meniscus cells with different concentrations of the glues for 7 days. Cell viability was not affected after adding up to 3 mg of the adhesive/mL of medium.

The proposed materials are suitable candidates to be used as resorbable tissue adhesives for meniscus repair. They have excellent mechanical and adhesive properties that can be adjusted by varying the composition of the copolymers.

Introduction

Menisci are fibro-cartilaginous tissues present in duplicate in the knee joint. They play a crucial role in shock absorption and distribution of forces in the joint, as well as in lubrication and nutrition of articular cartilage [1]. Most injuries of the knee are associated with meniscus trauma; the meniscus tears which lead to pain, swelling and locking of the joint. Since menisci are only well-vascularized in the outer, peripheral zone, they have very limited capacity to self-repair [2, 3].

The preferred treatment strategy is to repair the meniscus using sutures or staples to restore the integrity of the tissue [4], or in case of tears in the avascular outer part, a partial meniscectomy is frequently performed. However, both solutions carry their drawbacks. Meniscectomy brings instant pain relief, but removal of only 15-34% of the meniscus will lead to a 350% increase in the contact stress in the joint and thus

eventually will lead to development of degenerative changes and osteoarthritis [5]. Using sutures on the other hand, is mostly successful in the vascularized, peripheral zone of the meniscus, where cells have access to nutrients and growth factors [6, 7]. The arthroscopic procedure using sutures is technically challenging, time-consuming, the sutures are expensive and the success rate very much depends on the location and type of tear [8].

An attractive alternative for suturing would be use of biodegradable tissue adhesives [9]. Ideally, these materials provide stability by holding the torn tissue together during the period of healing, and gradually degrade to biocompatible products [10]. The application procedure could be faster, easier and cheaper than in the case of sutures. Tissue adhesives are already successfully used in other surgical applications, such as Dermabond[®] (2-octyl cyanoacrylate) for skin lacerations [11] or fibrin glue in hemostasis or pulmonary air leaks [12, 13]. However, none of them is suitable for gluing the damaged meniscus. Cyanoacrylates are rigid and brittle materials, their elastic modulus (E_{mod}) is approximately 500 MPa [14], while the E_{mod} of meniscus tissue (in circumferential direction) is only between 40-160 MPa [1, 15]. Moreover, formaldehyde is a degradation product, which limits their application within the body [16]. Fibrin glue is a natural biocompatible compound, but has poor mechanical and adhesive properties and a rapid degradation profile [17, 18]. Furthermore, it can be a source of disease transmission [19].

With these limitations in mind, we have been developing new polymeric tissue adhesives that are flexible and strong, and at the same time biocompatible and biodegradable. The adhesion process to the tissue consists of two steps: 1) wetting the surface of the tissue and 2) attachment to the tissue and curing of the material (network formation) [20]. Therefore, in order to provide optimal gluing of the meniscus, the glue must first spread well on the wet tissue surface. The material should therefore be sufficiently hydrophilic. At the same time, the polymeric network that is formed upon curing of the adhesive should be able to withstand the mechanical forces present at the site of application. For this, we envisaged the use of amphiphilic block-copolymers with hydrophilic blocks that ensure good spreading on the tissue surface and hydrophobic blocks that lead to networks with adequate mechanical properties.

Previously, we reported on the synthesis and characterization of three-armed star-shaped isocyanate-terminated block copolymers based on trimethylene carbonate (TMC) and trimethylolpropane ethoxylate (TMPE) [21]. These materials are capable to react with primary amines present in the proteins at the surface of the tissue and form stable covalent urea bonds with them [22]. At the same time they react with surrounding water forming a solid polyester-polyurethane network.

We showed that these three-armed copolymers had good adhesive potential and demonstrated better properties than analogous linear copolymers. Based on these results, we expect that by increasing degree of branching of the copolymers, and then the number of reactive groups which attach to the surface, it will be possible to enhance their adhesive properties.

In this paper, we report on the synthesis and characterization of novel hyper-branched amphiphilic, isocyanate-terminated copolymeric oligomers that vary in composition and in architecture and their possible application as a tissue adhesive for meniscus repair.

Materials and Methods

Materials

Poly(ethylene glycol) with number average molar mass (M_n) of 200 g/mol (PEG₂₀₀) and 600 g/mol (PEG₆₀₀), citric acid (CA), chloroform-d (CDCl₃), fibrinogen and thrombin (both isolated from bovine plasma), phosphate buffered saline (PBS), cholesterol esterase (from porcine pancreas, 35 U/mL) (CE) were purchased from Sigma Aldrich (the Netherlands). Trimethylene carbonate (TMC) was obtained from ForYou (China). Hexamethylene diisocyanate (HDI) was purchased from Merck Schuchardt (Germany). Diethyl ether was purchased from Biosolve (the Netherlands). PEG₂₀₀ and PEG₆₀₀ were dried at 120 °C under vacuum, diethyl ether was dried over molecular sieves prior to use. All other chemicals were used as received. Dermabond[®] was obtained from Ethicon (Johnson & Johnson, The Netherlands). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and Antibiotic-Antimycotic (PSF, contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone[®]) were purchased from

Gibco® (UK). Collagenase type II was purchased from Worthington (USA). Bovine meniscus tissue was obtained from a local abattoir.

Synthesis of oligomers and isocyanate-terminated hyper-branched oligomers

A series of reactive isocyanate-terminated hyper-branched oligomers was prepared in a three step synthesis process, which is schematically depicted in the Figure 1. Ring opening polymerization of TMC using PEG as initiator was performed as previously described [21] (Figure 1a). TMC was reacted with PEG₂₀₀ or PEG₆₀₀ using Sn(Oct)₂ (0.02 mmol/mol of monomer) as a catalyst. The polymerization was conducted at 130 °C for 3 days under argon atmosphere. Linear oligomers with TMC to PEG molar ratios of 2:1 and 4:1 were prepared. The used nomenclature for these blockcopolymeric precursors is: PEG_m-(TMC_n)₂, where m is molar mass of PEG, n is the number of TMC units (in PTMC chain) per PEG chain end.

Then, the linear oligomers were reacted with CA in a polycondensation reaction at 120 °C for 1 day under vacuum (Figure 1b). In this manner hyper-branched oligomers were obtained with PEG_m-(TMC_n)₂ to CA molar ratios of 4:1, 7:1 and 10:1, respectively. The used nomenclature of these hyper-branched oligomers is: CA-xPEG_m-(TMC_n)₂, where x is 4, 7 or 10. To obtain reactive isocyanate-terminated molecules, these hyper-branched oligomers were subsequently reacted with excess of HDI at 75 °C for 1 day under argon atmosphere (Figure 1c). Drop-wise addition of the hyper-branched oligomer to HDI ensured that the molar excess of HDI was at least 1:3. Unreacted HDI was removed by washing in dry diethyl ether. The product was then dried under vacuum overnight. The resulting products were labelled: [CA-xPEG_m-(TMC_n)₂]-HDI.

An overview of prepared reactive isocyanate-terminated hyper-branched oligomers is given in Table 1.

Figure 1. Scheme of the three-step synthesis of reactive isocyanate-terminated hyper-branched oligomers, a) Step 1: ring opening polymerization of TMC using PEG results in the formation of linear amphiphilic oligomers, b) Step 2: polycondensation of the linear amphiphilic oligomers and CA resulting in hyper-branched structures, c) Step 3: reaction of the end groups of these hyper-branched structures with excess HDI resulting in the formation of reactive isocyanate-terminated hyper-branched oligomers.

Table 1. Overview of the prepared reactive isocyanate-terminated hyper-branched oligomers.

	1 TMC unit per PEG chain end	2 TMC units per PEG chain end
PEG ₂₀₀	[CA-4PEG ₂₀₀ -(TMC ₁) ₂]-HDI	[CA-4PEG ₂₀₀ -(TMC ₂) ₂]-HDI
	[CA-7PEG ₂₀₀ -(TMC ₁) ₂]-HDI	[CA-7PEG ₂₀₀ -(TMC ₂) ₂]-HDI
	[CA-10PEG ₂₀₀ -(TMC ₁) ₂]-HDI	[CA-10PEG ₂₀₀ -(TMC ₂) ₂]-HDI
PEG ₆₀₀	[CA-4PEG ₆₀₀ -(TMC ₁) ₂]-HDI	[CA-4PEG ₆₀₀ -(TMC ₂) ₂]-HDI
	[CA-7PEG ₆₀₀ -(TMC ₁) ₂]-HDI	[CA-7PEG ₆₀₀ -(TMC ₂) ₂]-HDI
	[CA-10PEG ₆₀₀ -(TMC ₁) ₂]-HDI	[CA-10PEG ₆₀₀ -(TMC ₂) ₂]-HDI

The composition and chemical structure of the intermediate linear- and hyper-branched oligomers, and of the isocyanate functionalized end-products was evaluated using ¹H NMR spectroscopy (Bruker 400MHz NMR spectrometer) using CDCl₃ as the solvent. The –NCO content of the reactive hyper-branched oligomers was determined by back-titration of the isocyanate groups according to the ASTM D2572 standard.

Characterization of the networks obtained after curing the isocyanate-terminated hyper-branched oligomers

Cross-linked polyurethane networks were obtained by casting 0.3 mm thick films of the isocyanate-terminated hyper-branched oligomers on glass plates, subsequent overnight immersion in water at room temperature (RT) allowed the curing reaction to take place.

In order to determine the physical characteristics of the obtained water-cured network films, they were dried in vacuum at 40 °C until constant weight and their initial mass was determined (m_i). To assess the process of network formation, the gel content of the networks was determined after vacuum-drying. The specimens were incubated in chloroform, which was determined to be a good swelling agent and a suitable solvent to extract the sol fraction of the network, for 24 hours and dried in vacuum until constant weight (m_{dc}).

The gel content (GC) was calculated according to:

$$GC = \left(\frac{m_{dc}}{m_i} \right) \cdot 100\%$$

To assess their swelling behavior, the obtained water-cured network films were also placed in water for 24 hours, and their wet mass (m_w) was determined. The samples were then re-dried (in vacuum at 40 °C) until constant weight, and their dry mass was determined (m_{dw}).

The water uptake (WU) was calculated using:

$$WU = \left(\frac{m_w - m_{dw}}{m_{dw}} \right) \cdot 100\%$$

Of each network, three different specimens were evaluated.

The thermal properties of the water-cured networks in their wet as-prepared state were determined by differential scanning calorimetry (DSC) using a Perkin Elmer Pyris 1. Samples weighing 5-10 mg were heated from -100 °C to 100 °C at 10 °C/min.

Dumbbell-shaped test specimens of the water-cured network films were prepared as described above. The wet as-prepared samples were subjected to tensile testing at room temperature in triplicate according to ISO 37:1994 (E), Type 3 using a Zwick Z020 universal tensile tester. The grip to grip separation was 35 mm and the crosshead speed was 50 mm/min.

***In vitro* degradation of water-cured polyurethane networks**

The *in vitro* degradation of the water-cured networks was assessed in PBS solutions (pH=7.4, containing 0.02 wt% of sodium azide as bactericide) at 37 °C. Disc-shaped specimens (10 mm in diameter and thicknesses varying between 100 and 500 µm) were punched out from the network films and placed in 48 well-plates containing 1 mL of PBS and let to degrade. The medium was changed twice a week. At predetermined time points, the specimens were recovered and their mass and thickness were determined in the wet state and after drying in vacuum until constant weight. Of each network triplicate specimens were evaluated.

Additionally, enzymatic degradation experiments were conducted in the same manner using CE solution in PBS (20 $\mu\text{g/mL}$, 0.02 wt% of sodium azide). The results of these experiments are presented in the Supplementary Material.

Application of the reactive isocyanate-terminated hyper-branched oligomers as tissue adhesives

The reactive isocyanate-terminated hyper-branched oligomers were used as an adhesive to glue bovine meniscus tissue. The bovine menisci were cut in circumferential direction to obtain strips measuring approximately 2x10x25 mm. The strips of tissue were kept hydrated until the gluing experiment. The surfaces of the tissue strips were blotted dry and the different isocyanate-functionalized oligomers, which are viscous liquids, were applied to the strips of tissue using a spatula. They were then pressed together and immersed in water overnight. The adhesive covered an area of approximately 100 mm². Two of these strips were then pressed together and immersed in water and left overnight at RT to allow attachment of the adhesive to the substrate, curing and network formation. This environment is a simplification of the wet synovial fluid-containing knee joint, but it allows us to investigate the curing characteristics of the different networks and their adhesive strengths to the tissue. Figure 2a and 2b illustrate this process.

To determine the strength of the adhesive bond after curing, the wet specimens were subjected to lap shear adhesion testing (Figure 2c) using a Zwick Z020 universal tensile tester in analogy to ASTM F2255-05. The grip to grip separation was 25 mm and the crosshead speed was 50 mm/min. The shear adhesive bond strength (S) expressed in kPa is the maximum shear force divided by the glued area. For each adhesive, the experiments were conducted in triplicate.

Additionally, the performance of two commercially available tissue adhesives, fibrin glue and Dermabond[®], was evaluated. Fibrin glue was prepared by dissolving fibrinogen and thrombin in PBS at concentrations of 100 mg/mL and 100 U/mL, respectively. These two solutions were mixed in a double barrel syringe equipped with a static mixer and directly applied onto the strips of bovine meniscus tissue. Dermabond[®] was used as received and also directly applied onto the meniscus strips.

In these cases the lap shear adhesion tests were conducted directly after applying the adhesives, as these adhesives cure within a few seconds.

Cell culturing in the presence of reactive isocyanate-terminated hyper-branched oligomers

Meniscus cells were isolated from bovine meniscus tissue (cut to pieces of approximately 1 mm³) after digestion in collagenase solution for 2 days at 37 °C and 5% CO₂. The solution contained collagenase type II (3mg/mL of medium), DMEM, 10% FBS and 1% PSF. The cells were isolated from remaining extracellular matrix using a 70 µm cell bag.

The bovine meniscus cells were cultured for 5 days in DMEM medium containing 10% FBS and 1% of PSF using 24 well-plates in standard culture conditions (37 °C, 5% CO₂, 95% humidity). The cell concentration was 25,000 cells/well, the wells contained 1 mL of medium. On day 5 the cells were stained using the Alamar Blue[®] (Bio-Rad, AbD Serotec[®], UK) cell metabolic activity assay and counted. The cells were then further cultured in the presence of varying amounts of isocyanate-terminated hyper-branched oligomeric adhesives. Amounts of 0.5 to 30 mg of these reactive hyper-branched oligomers were placed on permeable Transwell[®] membranes, which were subsequently immersed in the medium. Cell viability was again assessed after 1, 3 and 7 days upon contacting the medium with the isocyanate-terminated hyper-branched oligomeric adhesives (this corresponds to day 5, 8 and 12 of overall culture time). The experiments were conducted at least in triplicate.

In addition, the Live/Dead[®] assay (Molecular Probes, the Netherlands) in which calcein AM (2µM solution) stains living cells green and ethidium homodimer-1 (4µM solution) stains dead cells red, was used to visualize the viable and non-viable cells.

Figure 2. a) Application of the reactive isocyanate-terminated hyper-branched oligomer (adhesive) on strips of meniscus tissue (the covered area is approximately 1 cm^2 , b) Two strips of tissue are glued, pressed together and kept immersed in water overnight, c) The glued specimens are then subjected to lap shear testing.

Statistical analysis

Descriptive statistics were used to summarize the data. Data were expressed as mean values $\pm$ standard deviations. For the adhesion tests, all compositions of the tissue adhesives were compared with fibrin glue using one-way ANOVA followed by Fisher's LSD post-hoc test. For the degradation study, the main effects of mass loss in time between networks containing PEG₂₀₀ and PEG₆₀₀ were determined using two-way ANOVA. P-values <0.05 were considered statistically significant. Statistical analyses were performed using SPSS (IBM, version 21).

Results and Discussion

Synthesis and characterization of reactive isocyanate-terminated hyper-branched oligomers

A series of hyper-branched isocyanate-terminated reactive oligomers was synthesized in a three-step procedure. First linear amphiphilic copolymers containing PEG₂₀₀ or PEG₆₀₀ and TMC (PEG_m-(TMC_n)₂) were prepared. Then the linear copolymers were reacted with CA in a polycondensation reaction. As the functionality of CA is higher than 2, this would lead to the formation of branched structures [23]. To obtain oligomers with different degrees of branching, the ratio of PEG_m-(TMC_n) to CA was varied. Oligomers with PEG_m-(TMC_n) to CA ratios of 4:1, 7:1 or 10:1 were prepared. In these molecules the ratio of the hydroxyl-to carboxyl groups was 3:1, 5:1 and 7:1, respectively. As the linear PEG_m-(TMC_n)₂ component was always used in excess, gelation during the polycondensation reaction was avoided and hyper-branched polyesters with unreacted terminal hydroxyl groups were obtained. The hyper-branched polyesters were then end-functionalized by reaction with excess with HDI and removal of the unreacted HDI by washing.

In the different steps of the reaction, NMR was used to follow the synthesis process. The successful synthesis of linear PEG_m-(TMC_n)₂ oligomers was confirmed by the presence of peaks corresponding to PEG repeating units at 3.5 ppm and to peaks corresponding to TMC at 4.2 and 2.1 ppm. After reacting the linear PEG_m-(TMC_n)₂ copolymers with CA, additional peaks corresponding to CA at 2.75 ppm could be detected. After end-functionalization of the hyper-branched oligomers with the diisocyanate, peaks in the NMR spectra at 3.3 and 1.3-1.7 ppm that correspond to HDI could be discerned. All synthesized oligomers were viscous liquids at RT and at 37 °C.

Comparison of the areas of the different peaks in the NMR spectra allowed the determination of the composition of the synthesized reactive isocyanate-terminated hyper-branched oligomers. Table 2 gives an overview of the composition of the adhesives.

Table 2. Content of PEG, TMC and HDI (in wt %), and of free isocyanate groups (-NCO) in the reactive hyper-branched oligomers.

Hyper-branched reactive oligomer	PEG content in the isocyanate-terminated oligomer [wt%] ^{a)}	TMC content in the isocyanate-terminated oligomer [wt%] ^{a)}	HDI content in the isocyanate-terminated oligomer [wt%] ^{a)}	Free - NCO groups [%] ^{b)}
[CA-4PEG ₂₀₀ -(TMC ₁) ₂]-HDI	27.1	26.5	32.7	11.8 (1.5)
[CA-7PEG ₂₀₀ -(TMC ₁) ₂]-HDI	21.5	24.7	47.7	11.7 (0.2)
[CA-10PEG ₂₀₀ -(TMC ₁) ₂]-HDI	19.1	22.8	38.1	14.7 (2.0)
[CA-4PEG ₂₀₀ -(TMC ₂) ₂]-HDI	15.1	35.1	41.5	13.1 (0.7)
[CA-7PEG ₂₀₀ -(TMC ₂) ₂]-HDI	13.4	31.5	37.9	9.5 (0.9)
[CA-10PEG ₂₀₀ -(TMC ₂) ₂]-HDI	15.1	32.8	43.7	12.1 (0.7)
[CA-4PEG ₆₀₀ -(TMC ₁) ₂]-HDI	41.8	14.7	37.6	10.9 (0.7)
[CA-7PEG ₆₀₀ -(TMC ₁) ₂]-HDI	48.3	14.4	27.7	13.5 (1.6)
[CA-10PEG ₆₀₀ -(TMC ₁) ₂]-HDI	51.4	12.6	33.6	6.9 (1.2)
[CA-4PEG ₆₀₀ -(TMC ₂) ₂]-HDI	36.5	25.6	30.5	10.8 (0.2)
[CA-7PEG ₆₀₀ -(TMC ₂) ₂]-HDI	37.1	26.7	31.4	7.1 (1.9)
[CA-10PEG ₆₀₀ -(TMC ₂) ₂]-HDI	33.0	21.6	40.0	5.8 (0.3)

^{a)} determined by ¹H-NMR in single experiments

^{b)} determined by titration according to ASTM D2572, data is presented as a mean value, the standard deviation is given in brackets.

The table shows that no clear influence of the extent of branching, indicated by the different CA to PEG_m-(TMC_n)₂ ratios, can be seen. This holds for compositions based on PEG₂₀₀ as well as for compositions based on PEG₆₀₀.

The number of free isocyanate groups of the reactive oligomers based on PEG₂₀₀, which according to the testing standard is expressed as a mass percentage, is in the

range of 10-15%. There does not seem to be a clear effect of composition or degree of branching on the number of free isocyanate groups. For PEG₆₀₀-based oligomers, the number of free reactive isocyanate groups seems to decrease from approximately 11 to approximately 7% with increasing CA/PEG₆₀₀-(TMC_n)₂ ratio. This implies that with an increasing extent of branching the number of available reactive isocyanate groups does not increase. It seems therefore not beneficial to further increase the CA/PEG₆₀₀-(TMC_n)₂ ratio to increase the number of reactive end-groups per molecule.

In these reactive hyper-branched oligomers, no apparent relationship between the HDI content and the number of free isocyanate groups can be seen either. It is thus likely that during the synthesis, purification or drying process, the (highly) reactive terminal isocyanate groups have reacted prematurely for example with traces of moisture present in the air or in the used solvents. The result of this is that these materials are not as well-defined as would be desired. Nevertheless, as shown later, these materials do have good adhesive properties.

Physical properties of the water-cured polyurethane networks

When an isocyanate-terminated tissue adhesive is applied to tissue, it not only forms covalent bonds with functional groups present on the tissue surface (-NH₂, -COOH, -SH, -OH), it simultaneously also cures to form a network by reaction with water in the surrounding fluids [22]. It is therefore of great importance to evaluate the physical and mechanical properties of such networks and how these depend on the chemical composition of their precursors.

Polyurethane network films with a thickness of 100-500 μm were prepared by casting the reactive hyper-branched oligomers onto a glass plate, and cross-linking by reaction with water. An overview of the physical properties of the obtained networks is presented in Table 3. It can be seen in the table that in general networks with very high gel contents are obtained, especially for networks based on PEG₂₀₀, where gel contents range from 93 to 100 %. For networks based on PEG₆₀₀ gel contents are also high, although somewhat lower (61 to 99 %). This indicates that the cross-linking process is more efficient for hyper-branched oligomers based on PEG₂₀₀.

The water uptake of the networks is mainly related to the content of the hydrophilic PEG component in the network and was significantly higher for networks containing PEG₆₀₀. In these networks the weight percentage of the PEG component was higher, rendering the networks more hydrophilic. In addition, longer PEG chains increase the mesh size of the networks, allowing them also to take up more water. Water uptake is influenced by the content of hydrophobic TMC as well: with increasing TMC content, water uptake decreases. This is especially pronounced for the networks based PEG₆₀₀.

An overview of the mechanical properties of the networks in the wet state as determined in tensile testing experiments is also given in Table 3. Both their tensile strength (σ) and elastic modulus (E) were found to be strongly dependent on the composition of the hyper-branched reactive oligomers used in network formation.

In the wet state, the tensile strength of networks based on PEG₂₀₀ (6-9 MPa) was higher than the tensile strength of the ones based on PEG₆₀₀ (approximately 1 MPa). The elastic modulus of the PEG₂₀₀ networks was also higher than that of the PEG₆₀₀ networks, with elastic modulus values of respectively 50-150 MPa and 1.5-6 MPa (see Table 3). These differences were the result of the differences in hydrophilicity of the networks; networks containing PEG₂₀₀ were less hydrophilic and took up less water. Importantly, the elastic moduli of hydrated networks based on PEG₂₀₀ were in the same range as the elastic modulus of human menisci measured in the circumferential direction (40-160 MPa) [1, 15, 24]. The elongation at break of the networks ranged from 30-100%, no clear influence of composition could be observed.

Thermal analysis of the polyurethane networks after their preparation was done by DSC. It was shown that in the wet state the networks were essentially amorphous with only a minor melting endotherm corresponding to the urethane bonds at approximately 80 °C. For all networks a clear glass transition lower than 37 °C was observed, their flexible rubber-like mechanical characteristics at physiological conditions. For networks containing PEG₂₀₀ the glass transition temperature (T_g) was significantly higher than for those containing PEG₆₀₀. The T_g of the former networks ranged between -10 and -17°C, while T_g of the latter networks varied between -38 and -52 °C. In both cases T_g decreased with increasing TMC content.

Table 3. Overview of the physical and mechanical properties of the prepared networks in the wet state. Values are expressed as mean values with standard deviations in brackets.

Hyper-branched reactive oligomer	PEG content in the reactive oligomer ^{a)} [wt%]	Gel Content [%]	Water uptake [%]	Tensile strength [MPa]	E _{mod} [MPa]	Elongation at break [%]
[CA-4PEG ₂₀₀ -(TMC ₁) ₂]-HDI	27.1	97.6 (0.7)	35.5 (4.2)	3.4 (1.2)	52.8 (16.7)	14.2 (10.4)
[CA-7PEG ₂₀₀ -(TMC ₁) ₂]-HDI	21.5	92.3 (0.6)	19.6 (0.7)	6.2 (1.6)	74.4 (7.6)	41.4 (36.2)
[CA-10PEG ₂₀₀ -(TMC ₁) ₂]-HDI	19.1	100.0 (1.8)	10.9 (1.5)	7.6 (1.3)	67.8 (5.4)	57.8 (21.2)
[CA-4PEG ₂₀₀ -(TMC ₂) ₂]-HDI	15.1	100.0 (2.1)	42.8 (2.8)	14.4 (1.5)	151.0 (7.1)	48.2 (16.0)
[CA-7PEG ₂₀₀ -(TMC ₂) ₂]-HDI	13.4	96.7 (0.4)	14.6 (0.9)	4.9 (3.1)	62.5 (11.4)	28.6 (28.1)
[CA-10PEG ₂₀₀ -(TMC ₂) ₂]-HDI	15.1	97.4 (1.8)	18.1 (2.8)	7.8 (2.1)	85.4 (15.8)	56.6 (33.1)
[CA-4PEG ₆₀₀ -(TMC ₁) ₂]-HDI	41.8	97.3 (4.6)	80.3 (5.6)	1.2 (0.2)	6.5 (2.2)	35.0 (12.2)
[CA-7PEG ₆₀₀ -(TMC ₁) ₂]-HDI	48.3	87.9 (5.9)	149.4 (8.3)	1.0 (0.3)	2.05 (0.6)	83.7 (26.0)
[CA-10PEG ₆₀₀ -(TMC ₁) ₂]-HDI	51.4	79.8 (40)	164.8 (19.6)	0.6 (0.1)	1.5 (0.4)	75.5 (29.4)
[CA-4PEG ₆₀₀ -(TMC ₂) ₂]-HDI	36.5	98.9 (2.2)	86.7 (0.6)	0.4 (0.1)	0.85 (0.2)	43.8 (19.3)
[CA-7PEG ₆₀₀ -(TMC ₂) ₂]-HDI	37.1	77.4 (16.3)	92.1 (12.3)	1.0 (0.2)	4.3 (0.9)	30.8 (5.9)
[CA-10PEG ₆₀₀ -(TMC ₂) ₂]-HDI	33.0	60.7 (10.4)	115.5 (3.8)	0.3 (0.0)	3.0 (0.6)	9.9 (3.6)

^{a)}determined by ¹H-NMR

***In vitro* degradation of the water-cured polyurethane networks**

The *in vitro* degradation of the polyurethane networks formed upon curing of the adhesives by reaction with water, was evaluated during incubation in PBS over a period of 26 weeks. At predetermined time points changes in mass and thickness of the specimens were determined after drying. The remaining mass of the networks is depicted in the top part of Figure 3. The figure shows that the mass loss of networks containing PEG₆₀₀ was significantly faster than that of networks containing PEG₂₀₀ ($p < 0.05$). After a meniscus tear repair procedure with sutures, it can take 2-4 months before evidence of healing can be seen [25]. It would therefore be desired that the adhesive, which can be applied as a series of drops on the surfaces of the tear, holds the meniscus together during this period enabling its healing before fully degrading. The degradation profile of networks containing PEG₂₀₀ and of some networks containing PEG₆₀₀ seem suitable for this application, since they gradually degrade and start losing mass after 4-8 weeks. After 26 weeks networks containing PEG₆₀₀ had lost 30% to almost 100% of their mass (they were fragmented), while networks containing PEG₂₀₀ had lost 5-40%). Networks containing PEG₆₀₀ were more hydrophilic than networks containing PEG₂₀₀ (see Table 3) and therefore more prone to hydrolytic degradation. The networks with the highest water uptake: [CA-7PEG₆₀₀-(TMC₁)₂]-HDI and [CA-10PEG₆₀₀-(TMC₁)₂]-HDI showed the most rapid loss of mass during degradation. While the degree of branching did not seem to have an effect on the rate of mass loss, it is apparent the networks with higher contents of hydrophobic TMC degraded at a lower rate. This is especially obvious for networks containing PEG₆₀₀.

Figure 3. Remaining mass (top) and thickness (bottom) of networks in the dry state after in vitro incubation in PBS. a) networks based on PEG₂₀₀ and b) networks based on PEG₆₀₀. Mass loss of networks containing PEG₆₀₀ was significantly faster than that of networks containing PEG₂₀₀ ($p < 0.05$). *Note that after 4 weeks the thickness of CA 4PEG₂₀₀-(TMC)₁)₂-HDI network specimens was very irregular and could not be determined accurately; after 4 weeks the thickness and the mass of CA-7PEG₆₀₀-(TMC)₁)₂-HDI network specimens could not be determined due to loss of integrity of these samples; after 8 weeks the thickness of CA-10PEG₆₀₀-(TMC)₁)₂-HDI network specimen could not be determined due to loss of integrity of these samples. After 12 weeks their mass could not be determined either.

Changes in the thickness of the network specimens was also monitored during incubation in PBS (see bottom part of the Figure 3). It is shown that the networks based on PEG₂₀₀ exhibited only minor changes in specimen thickness of discs height during degradation. In contrast, the thickness of all specimens based on PEG₆₀₀ gradually decreased in time. These materials also became fragile during the degradation experiment. Plotting the thickness of the specimens as a function of their mass loss (not shown), indicated that these materials degraded by a bulk erosion process. As linear high molecular weight PTMC degrades due to activity of enzymes [24], degradation experiments were also conducted in CE solutions (see Supplementary Material). Similar degradation profiles were observed and changes in mass loss and thickness incubated in CE and PBS were not significant. This means that the major mechanism of degradation *in vitro* is non-enzymatic hydrolysis. Upon hydrolysis of the network, the network will ultimately degrade into PEG, CA, 1,3-propane diol, CO₂, water and 1,6-hexanediamine. These water soluble compounds can be metabolized or excreted by the kidneys.

Adhesion strength

The adhesive properties of the reactive isocyanate-terminated hyper-branched oligomers were determined in lap shear adhesion tests with bovine meniscus tissue as the substrate. The adhesives were applied to strips of tissue, which were pressed together and immersed in water overnight. Figure 4 shows that the adhesion strength varied between approximately 20 to 80 kPa, this is the case for PEG₂₀₀-based compositions as well as for PEG₆₀₀-based compositions. These adhesive strengths should be sufficient to hold a torn meniscus together; using a human cadaver knee model it was determined that on average the distraction forces on sutured menisci did not exceed 5 N when the applied load was 300 N [26, 27]. In another study, where the forces were assessed indirectly, the distraction forces in a bucket-handle lesion were determined to be less than 10 N [28]. For a 1 cm² tear, this corresponds to a required adhesive strength in the order of 50 to 100 kPa. During the non-weight bearing recovery period of the patient, the required adhesive strengths would be even lower. Although the differences in adhesion strength of the prepared adhesives were low and the standard deviations of the means of the measurements high, it seemed that the adhesive strength of reactive oligomers is somewhat higher for the

compositions with the higher TMC contents. No clear effect of the content of free isocyanate end-groups can be discerned (see the results presented in Table 2).

In all cases the observed mode of failure of the adhesive bond was adhesive failure: the network detached from the surface of the tissue, while the cured network itself remained undamaged. The smooth surface of the meniscus tissue used in the experiment did not result in deep penetration of the adhesive into the tissue and therefore in adequate mechanical interlocking of the cured network of the tissue. Roughening the meniscus surface before gluing by rasping could improve the adhesive strength of the oligomers due to increased area of contact, exposure of collagen fibers and the chemical groups capable of reacting with the isocyanate glues. This technique is already used in clinical practice to improve the meniscus healing potential [2, 29].

The adhesive strengths of the reactive hyper-branched oligomers were compared with those of fibrin glue and Dermabond[®], which are currently used in the clinic. Dermabond[®] is only approved for topical use as a skin adhesive, but as it has a high bonding strength to tissue it was used as a positive control in our study. The adhesive strengths to bovine meniscus tissue were respectively 10 kPa and 650 kPa for fibrin glue and Dermabond[®]. Although the adhesive strength of our hyper-branched adhesives was lower than that of Dermabond[®], they were significantly higher (seven times stronger) than the adhesive strength of fibrin glue ($p < 0.05$). Even though the performed statistical tests showed significant differences between particular compositions of our adhesives and fibrin glue, it should be kept in mind that the sample size was relatively low. Therefore, the main trends are more important than the differences between the different tissue adhesives. The properties of the adhesives will need to be assessed in more detail under physiological conditions using synovial fluid, biomechanical- and cadaveric models and animal experiments. It should also be noted that, as mentioned before, Dermabond[®] is a very rigid, non-degradable material and therefore not suited for our intended application.

Figure 4. Lap shear adhesion strength of the different reactive hyper-branched adhesives and fibrin glue and Dermabond® controls to bovine meniscus tissue. Values are expressed as mean values $\pm$ standard deviation. Asterisks indicate statistically significant differences between a particular adhesive and fibrin glue ($p < 0.05$). Adhesion strength values of Dermabond® were not included in the statistical comparisons.

Compatibility of the reactive hyper-branched oligomers with meniscus cells

To assess the potential cytotoxicity of the reactive hyper-branched isocyanate-terminated oligomers, different amounts of the adhesives were brought in indirect contact with bovine meniscus cells in culture. As could be concluded from the experimental results described above, the main factor that determines the physical properties of the reactive oligomers and cured networks is the molar mass of the PEG component in the reactive hyper-branched precursor. To assess the compatibility of the adhesives with meniscus cells, two compositions of the reactive hyper-branched oligomers were chosen: CA-7PEG₂₀₀-(TMC₂)₂-HDI and CA-4PEG₆₀₀-(TMC₂)₂-HDI. Both these materials spreads well on the surface of the tissue and had satisfactory adhesion strengths to meniscus tissue.

After culturing the meniscus cells for 5 days, amounts of 0.5 to 30 mg of CA-7PEG₂₀₀-(TMC₂)₂-HDI and CA-4PEG₆₀₀-(TMC₂)₂-HDI were brought into contact

with the cell culture medium using permeable Transwell® membranes. The volume of cell culture medium in the wells containing the cells was 1 mL.

Figure 5. a) Viability of bovine meniscus cells after contacting 1 mL of medium with different amounts of CA-4PEG₆₀₀-(TMC₂)₂-HDI adhesive during culture expressed as the relative number of living cells prior to the addition of the adhesives to the medium, data is presented as mean $\pm$ standard deviation b) Microscopic images of the cells in culture after Live/Dead staining at day 7 after the indicated amounts of the adhesive were brought into contact with the medium. The scale bar indicates 200 μ m.

The viability of the bovine meniscus cells in time after contacting the medium with the reactive hyper-branched CA-4PEG₆₀₀ (TMC₂)₂-HDI adhesive is presented in Figure 5a. When no adhesive was added, the cells continued to proliferate until confluency was reached and number of cells was reduced. When the hyper-branched adhesive (which contains reactive isocyanate endgroups) was added to the medium, cell viability initially decreased. This *in vitro* decrease in viability at day 1 after adding the adhesives strongly depended on the amount of adhesive placed in the culture medium. In all cases, nevertheless, cell viability then increased and the cells continued to proliferate until confluency was reached. This was even the case for high amounts of 18 and 30 mg adhesive per ml of cell culture medium. However in these cases the viability of cells initially decreased dramatically and after 7 days of culture only approximately 50% of the initial number of cells was reached. When the lower amounts of hyper-branched adhesive were added, the viability of the cells

was not significantly affected when compared to the control experiment and no toxic effects were observed when the amounts were 3 mg/mL medium or less. The results of the Live-Dead assays presented in Figure 5b confirm that 7 days after adding the reactive adhesives up to concentrations of 8 mg per ml of medium, a confluent layer of living cells is present.

Similar results were obtained for the CA-7PEG₂₀₀-(TMC₂)₂-HDI adhesive (Supplementary Material).

These results imply that, although the non-cured adhesives did affect cell viability, once the hyper-branched adhesive cured in the medium, a toxic effect was not observed anymore. We assume that for repair of the meniscus lesion six drops of adhesive would be needed. Given that the mass of such drop is 3 mg, a total of 18 mg would be required. As the volume of synovial fluid present in the knee joint is 6.7 +/- 2.3 ml (and even higher in an arthritic joint [30]), the amount of adhesive per mL of fluid would be even lower than the safe limit of 3 mg/mL, which we determined in this experiment. Therefore, no severe tissue reactions would be observed. Currently, subcutaneous implantations of these adhesive materials in small animals are being conducted to confirm their biocompatibility *in vivo*.

Conclusions

We have shown that hyper-branched isocyanate-terminated oligomers based on citric acid, poly (ethylene glycol) and trimethylene carbonate are favourable candidates for use as biocompatible resorbable tissue adhesives for gluing meniscus tears. These reactive oligomers are viscous liquids that can easily be applied in an arthroscopic procedure using a syringe. The mechanical properties are suitable after curing, they can be varied by changing the composition and architectural design of the oligomeric precursors. Particularly of interest are networks based on PEG₂₀₀, which had mechanical properties close to those of human meniscus tissue. For the different compositions, the lap shear adhesion strengths to bovine meniscus tissue did not significantly differ. *In vitro* degradation studies showed that networks based on PEG₆₀₀ degraded much faster than networks based on PEG₂₀₀. Cell culturing experiments using meniscus cells demonstrated that an adverse tissue response is not to be expected in the presence of suitable amounts of the reactive adhesives.

For application in the clinic, the adhesive should not only have good adhesion to the tissue, suitable degradation characteristics and minimal toxicity, it should also be rapidly curing. Ongoing work is aimed at optimizing the composition and architecture of these reactive oligomers.

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Supplementary Material

Figure SI 1. Remaining mass (top) and thickness (bottom) of networks in the dry state after in vitro incubation in CE solution. a) networks based on PEG₂₀₀ and b) networks based on PEG₆₀₀. Mass loss of networks containing PEG₆₀₀ was significantly faster than that of networks containing PEG₂₀₀ ($p < 0.05$). *Note that after 12 weeks the thickness and mass of CA-7PEG₆₀₀-(TMC₁)₂-HDI network specimens and after 18 weeks the thickness of CA-10PEG₆₀₀-(TMC₁)₂-HDI network specimen could not be determined due to loss of mechanical integrity of these samples.

Figure SI 2. Viability of bovine meniscus cells after contacting 1 mL of medium with different amounts of CA-7PEG₂₀₀ (TMC₂)₂-HDI adhesive during culture in vitro (expressed as a relative number to the amount of living cells before adding the reactive hyper-branched adhesive to the medium), data is presented as mean $\pm$ standard deviation.

Chapter 6

Evaluation of Novel Biodegradable Three-Armed- and Hyper-Branched Tissue Adhesives in a Meniscus Explant Model

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Abstract

Current treatment methods to repair meniscal tears do not bring fully satisfactory results. Tissue adhesives are considered promising alternatives, since they are easy to apply and cause minimal tissue trauma.

The first aim of this study was to analyze the adhesive properties of and tissue response to two recently developed biodegradable block copolymeric three-armed- and hyper-branched tissue adhesives. The second aim was to investigate if tissue surface modification with collagenase improves the attachment of the adhesives and increases the healing potential of the tissue.

Cylindrical explants were harvested from bovine menisci. The central core of the explants was removed and glued back into the defect, with or without incubation in collagenase solution prior to gluing, using one of the novel glues, Dermabond® or fibrin glue. The repair constructs were cultured in vitro for 1 and 28 days. Adhesion tests and histology were performed to analyze the effects of the glue in combination with the additional treatment.

The adhesive strength of the novel glues was 40-50 kPa, which was significantly higher than that of fibrin glue (15 kPa). Cells were present in direct contact with the glues, and the tissue remained vital during the whole culture period. Increased cellularity around the tear in the collagenase treated explants was observed after 1 day.

The two newly developed tissue adhesives are attractive materials to be used for repair of meniscal tears. The beneficial influence of collagenase treatment in treating meniscal tears with glues still needs to be confirmed in more clinical relevant studies.

Introduction

Meniscus tears are one of the most commonly occurring injuries of the knee joint. Unfortunately, most tears do not heal spontaneously, since meniscus tissue has a very limited capacity for self-repair [1]. This is mainly due to its limited vascular supply, which is only present in the outer part of the tissue [2]. Moreover, the high density of the extracellular matrix (ECM) of the meniscus limits the mobility of the

relatively low number of cells populating the tissue [3, 4]. In addition, the meniscus is constantly loaded and prone to being displaced continuously. This is a critical factor, as wound sites need to be kept in close proximity to each other to allow healing.

Current treatment methods consist of either suturing of the torn meniscus or partial meniscectomy [5-7]. However, neither of them brings fully satisfactory results. Partial meniscectomy leads to increased contact stresses acting on articular cartilage ultimately leading to its degeneration [8]. On the other hand, the success rate of repair with sutures strongly depends on the location and type of the tear. Treatment outcomes of tears located in highly loaded, avascular part of the meniscus are particularly poor [9, 10]. In order to improve treatment results, techniques aiming at inducing vascular supply, such as trephination and rasping have been employed [11, 12]. Nevertheless, it has been reported that up to 75% of repaired tears in the avascular zone failed to heal completely, and a further 20% required a secondary surgical intervention [13].

Therefore, there is an increasing interest in the development of alternative methods to both induce meniscus self-repair and provide mechanical stability to the tears. It has been shown that chemical modification of the meniscus tissue surface with collagenase *in vitro* resulted in decreased densities of ECM and increased numbers of cells in a close proximity to the tear edge [14]. Furthermore, tissue adhesives (glues) have been recognized as a promising alternative treatment to sutures, based on their ease of application, moldability, and possibility to deliver *in situ* biological compounds and degradability [15, 16]. However, the current clinically used adhesives are not suitable for this particular application, due to either their poor mechanical properties or their cytotoxicity [17, 18].

We recently developed two new tissue adhesives as potential materials for meniscus tear repair: biodegradable three-armed and hyper-branched block copolymeric structures [19, 20]. These adhesives attach to the tissue *via* covalent bonding of reactive isocyanate-end groups to proteins present on the surface of the tissue.

The first aim of the current study was to analyze the adhesive properties of the glues and the tissue response after application of the glues in an *in vitro* culture of meniscus

explants. The second aim was to investigate if modification of the tissue surface with collagenase improves the mechanical attachment of the glues to the tissue and increases the healing potential of the tissue itself.

Materials and Methods

Adhesive block copolymers

The isocyanate-terminated reactive three-armed- and hyper-branched adhesive block copolymers were synthesized as previously described in detail [19, 20]. The building blocks of the copolymers were either trimethylolpropane ethoxylate (TMPE, $M_n=450$ g/mol), trimethylene carbonate (TMC) and hexamethylene diisocyanate (HDI), or polyethylene glycol (PEG, $M_n=400$ g/mol), TMC, citric acid (CA) and HDI. The resulting products were labeled: TMPE-(TMC₂-HDI)₃ (three-armed) and CA-4PEG-(TMC₂)₂-HDI (hyper-branched), respectively. The adhesives were sterilized with gamma irradiation prior to use (25 kGy, SynergyHealth BV, The Netherlands). To ensure that the chemical structure of the copolymers was not affected by sterilization procedure, their ¹H NMR (Bruker 400 MHz NMR spectrometer) and FTIR (Perkin Elmer) spectra were recorded and compared before and after sterilization.

Preparation of meniscal explants

Knees from 12-18 months old cows were obtained from the local slaughterhouse. The medial menisci were removed from the joints under sterile conditions. Five cylindrical explants were harvested from the central part of each meniscus using an 8 mm biopsy punch as described by Hennerbichler *et al.* [21, 22]. The height of the explants was trimmed to 4 mm. Thereafter, to simulate a full-thickness meniscal tear, the explants were centrically cored with a 4 mm biopsy punch. The explants were randomly assigned to different experimental groups (n=6 per group). The cores were removed from the outer annulus and the explants were further processed and cultured *in vitro* as described in detail below.

Evaluation of the adhesive strength and biocompatibility of the glues

The repair constructs were created by gluing back the inner cylinder into the outer annulus of the explants using three-armed- or hyper-branched adhesive block copolymers, fibrin glue (mixture of fibrinogen (100 mg/mL) and thrombin (100 U/mL) in PBS, both isolated from bovine plasma, Sigma Aldrich, the Netherlands) or Dermabond® (Ethicon, Johnson&Johnson, the Netherlands) (Figure 1a). Repair constructs without applying glue were used as control. The repair constructs were cultured *in vitro* for 1 and 28 days in basal medium composed of Dulbecco's Modified Eagle's Medium (DMEM, Gibco®, UK) supplemented with 10% fetal bovine serum (FBS, Gibco®, UK), 1% penicillin/streptomycin/fungizone (PSF, Gibco®, USA) and L-ascorbic acid (Merck, Darmstadt, Germany) at a concentration of 25 µg/mL of medium.

At both time points, the adhesive strength of the glues to meniscal tissue (n=5 per group) was measured using a push out test on a BOSE® Electroforce® setup equipped with either a 20 or 200N load cell (BOSE Bose Corp. ElectroForce Systems Group, MN, USA). The setup was equipped with a metal rod, which was used to push out the inner cylinder of the explants. The inner cylinder (core) was displaced at a rate of 5 mm/min until it was completely separated from the outer annulus. In order to determine exact sizes of the constructs, pictures of explants were taken and their height and diameter were analyzed after each test. The adhesive strength was calculated by dividing the maximum peak force recorded during the adhesion test by the outer surface area P of the inner cylinder covered with the adhesive ($P = \pi \cdot d \cdot h$, where d is the diameter of the inner cylinder and h its height).

At the predetermined time points, repair constructs (n=1 per group) were fixated in 10% formalin, embedded in poly(methyl methacrylate) (PMMA), sectioned to 8 µm slides and stained with hematoxylin and eosin (H&E) to visualize cells and ECM. Sections were scored for the number of cells present inside and at the edge of the gap between the inner cylinder and outer annulus of the explants (3 random locations per explant using 200x field magnification).

Effect of tissue surface modification on adhesion strength and tissue response

In order to assess the influence of tissue surface pretreatment with collagenase, the explants (both annulus and the inner core) were incubated in basal medium containing 0.05 mg/mL collagenase (type IV from *Clostridium histolyticum*, ≥ 125 collagenase digestion units/mg solid, Sigma Aldrich) for 3 hours at 37 °C according to the protocol described by Qu *et al.* [23] (Figure 1b). Subsequently, the explants were washed 3 times with PBS and the repair constructs were prepared following the same protocol as described above. Constructs were cultured for 1 and 28 days in basal medium. Then, the constructs were subjected to the mechanical push out test (n=5) following the same protocol as described above. For histological analysis, samples (n=1 per group) were fixated in 10% formalin, embedded in poly(methyl methacrylate) (PMMA), sectioned to 8 μ m slides and stained with H&E, and Picrosirius Red (PR) to visualize changes in structure of collagen fibers after incubation in collagenase. Sections were scored for the number of cells in the same way as reported in the section above.

Figure 1. Overview of the experimental design. a) Setup used to assess the adhesive strength of and the tissue response to the glues; b) Setup used to evaluate the influence of collagenase pre-treatment of the explants on adhesion of the glues and tissue condition.

Statistical analysis

The adhesive strengths of the glues and differences in the number of cells in the gap between the groups were compared using a three-way ANOVA with Fischer's LSD post-hoc test. Data are presented as a mean $\pm$ standard deviation. P values <0.05

were considered as significant. All statistical analyses were performed using SPSS (version 20, IBM Corporation).

Results

The two block copolymeric tissue adhesives were successfully synthesized, which was confirmed by analysis of their ^1H NMR spectra as described previously in detail [19, 20]. Moreover, both ^1H NMR and FTIR measurements recorded before and after gamma sterilization showed nearly identical spectra indicating that their chemical structure was not changed due to the sterilization process.

Adhesive strength and biocompatibility of the glues

The glues were applied on the side surface area of the inner cylinder of the explants and reinserted into the annulus. Some of the glue, which leaked out due to the limited space between the gap edges, was removed before placing the repair constructs in the culture medium. The results of the adhesion tests showed that the adhesive strengths of the newly developed block copolymeric glues (mean of the adhesive strength after day 1 and day 28: 49.9 ± 31.7 kPa and 49.1 ± 37.8 kPa for three-armed and hyper-branched glue, respectively) were significantly higher than that of fibrin glue (8.4 ± 7.5 kPa) and of the control group without glue (7.1 ± 8.3 kPa) ($p < 0.05$; Figure 2). At the same time, there was no significant difference in adhesive strength between three-armed- and hyper-branched glues. Moreover, there were no significant differences in adhesive strength on day 1 and 28 within all experimental groups. Since Dermabond[®] had a much higher adhesive strength (471.1 ± 108.6 kPa) than the other glues, it was not included in the statistical analyses.

Figure 2. Adhesive strength of the glues to bovine meniscus tissue measured in a push-out test. Data are expressed as mean $\pm$ standard deviations. Asterisks indicate significant differences between means ($p < 0.05$). Dermabond® was not included in the statistical analysis.

The repair constructs were stained with H&E to visualize cells and ECM at day 1 and 28 of culture (Figure 3). Both the three-armed and the hyper-branched glues were present in the gap of the repair constructs after 1 and 28 days. After 1 day of explant culture, meniscus fibroblasts were seen in close proximity to the gap in the HE stained sections of all experimental groups (Figure 3). There were no differences in the number of cells between the different groups. After 28 days of culture similar results were observed and meniscus fibroblasts were detected in the gap and in close proximity to its edges in all groups except the Dermabond® group. An increased number of cells present in the gap was observed in explants glued with fibrin glue and in the control group.

Figure 3. H&E staining of the glued cylindrical explants cultured in vitro for 1 and 28 days. View on the gap between the inner cylinder and the annulus of the repair constructs.

Effect of tissue surface pre-treatment with collagenase on adhesion strength and tissue response

Incubating the meniscus explants in a collagenase solution resulted in a visible decrease in matrix density in PR stained sections and an increased number of cells after H&E staining in the area close to the gap between the annulus and inner cylinder of the explants (Figure 4).

Figure 4. H&E and PR staining of meniscus explants incubated in a collagenase solution for 3 hours (bottom) and a control (top). The arrows indicate cells present close to the gap edge (slides stained with H&E) and decreased density of collagen fibers at the gap edge (slides stained with PR). View on the gap between inner cylinder and annulus of the explants.

In the collagenase-treated explants the mean adhesive strengths after 1 and 28 days of the three-armed and hyper-branched glues (39.8 ± 28.4 kPa and 41.3 ± 25.0 kPa, respectively) were better than that of fibrin glue (14.2 ± 6.7 kPa) and the control group with no glue (6.8 ± 4.4 kPa) ($p < 0.05$) (Figure 5). There were no significant differences in adhesive strength between the three-armed and hyper-branched adhesives and no differences in adhesive strength between day 1 and 28. Only in the group glued with Dermabond[®], differences in adhesive strength between day 1 and day 28 were observed. Adhesive strength of Dermabond[®] (397 ± 109.2 kPa) was not included in the statistical test.

Figure 5. Adhesive strength of the glues to bovine meniscus tissue determined in a push-out test on explants incubated in a collagenase solution prior to the *in vitro* culture. Data are expressed as mean $\pm$ standard deviation. Asterisks indicate significant differences between means ($p < 0.05$). Dermabond® was not included in the statistical analysis.

All repair constructs were stained with H&E to visualize cells and ECM at day 1 and 28 of culture (Figure 6). Both the three-armed and hyper-branched glues could be seen in the gap of the repair constructs after 1 and 28 days. On the day 1, in all groups except Dermabond®, a slightly increased number of cells in close proximity to the gap was observed when compared to the previous experiment where explants were not incubated in collagenase prior to culture. However, at day 28, these differences in numbers of cells were not present anymore. Within the groups treated with collagenase, there were no differences in the number of cells between control, three-armed and hyper-branched glues groups, and a slightly increased number of cells in the fibrin glue group was observed. In the Dermabond® group no H&E stained cells could be detected.

Figure 6. H&E staining of the explants incubated in a collagenase solution prior to gluing and culturing *in vitro* for 1 and 28 days. View on the gap between inner cylinder and the annulus.

Discussion

The first aim of this study was to assess and compare the adhesive strength of two recently developed block copolymeric glues and to evaluate the tissue response after application of the glues in an *in vitro* meniscus explant culture. We employed a well-described meniscus explant model, which mimics full-thickness meniscal tears [21, 22, 24-26].

Both newly developed glues had a good adhesive strength to meniscus tissue (40-50 kPa), which was significantly higher than that of fibrin glue (approximately 11 kPa) and that of the control groups (approximately 7 kPa). The adhesion mechanism of the developed glues is the covalent attachment of reactive isocyanate end-groups to collagen fibers of meniscus tissue. However, as both the three-armed- and the hyper-branched glue had similar adhesion strengths, it seems that a further increase of the degree of branching of the copolymers will not improve on their adhesive properties. The decision which of the two compositions should be used in a future clinical application should be based on further mechanical studies and on currently ongoing *in vivo* biocompatibility evaluations of the glues.

Even though the adhesive strengths of our glues were lower than those of Dermabond[®], they do have clinical relevance. It has been shown that distraction

forces acting on a torn meniscus in a cadaver knee model did not exceed 5-10 N when a load of 300 N was applied [27, 28]. This means that the stress was not more than 50-100 kPa, during the recovery period after surgery the load on the knee might be even lower. Moreover, the glues stayed in place during the whole culture period and did not lose their bonding strength. This follows from the constant values of the adhesive strength observed after 1 and 28 days of culture for each particular composition. Histological staining of the slides revealed that our novel glues did not exhibit any toxicity, as fibroblast cells were seen after 28 days of culture *in vitro* in close proximity to the gap and tissue remained vital during the whole culture period. On the contrary, in the Dermabond[®] group, there were no cells present after 28 days, and the tissue started losing its integrity.

The meniscus is mostly an avascular tissue. Due to its limited cellularity and dense ECM, it has a very low capacity for self-repair [1]. Therefore, in clinical practice, surgeons use techniques such as mechanical rasping, trephination and the creation of access channels to stimulate neo-vascularization to the wound site, expose collagen fibers, refresh the wound and allow cells to enter the tear and stimulate healing [11, 29]. Moreover, roughening the surface of the tear and exposing the collagen fibers could potentially have a beneficial influence on the adhesive strength of the glues as well. As the available surface area is larger, the covalent interactions between the tissue and the glue will increase. Also the mechanical interlocking of the cured glue to the tissue would be enhanced.

Therefore, the second aim of this study was to assess the influence of surface modification on the adhesive strength of the glues to the tissue and the cellularity around the gap edges. The strategy we employed was analogous to the one described by Qu *et al.* [14], where the authors managed to re-program the wound environment to a more juvenile-like meniscus tissue with increased cellularity by incubating adult meniscus explants in collagenase solution. In our experiments, we incubated the explants in collagenase solution, after which the explants were glued and cultured *in vitro*. The results of the mechanical adhesion tests showed that the mean adhesive strength of all tested glues was slightly lower than those determined in the earlier experiments where no collagenase was used. However, the standard deviations were high and the differences were not statistically significant. This could be due to the non-homogenous distribution of the glues in the gap of the explants. Moreover, the

explants were from different donors and different locations of the meniscus. Such a design allowed excluding possible bias associated with differences in tissue structure and cellularity, but resulted in high standard deviations within the experimental groups. Nevertheless, it seems that treatment with collagenase did not improve the adhesive strength of the glues. Histological staining after 1 day of culture revealed that there was an increased number of cells in close proximity to the gap edge in all groups (except those glued with Dermabond[®]) when compared to the explants, which were not incubated in collagenase prior to culture. However, after 28 days the cellularity of the glued implants was comparable for both conditions. This could be due to the fact that the glues, which filled the whole gap between the inner cylinder and the outer annulus of the repair constructs, acted as a barrier for the cells and hindered them entering the tear gap. Moreover, the glue present in the gap might have blocked the access to nutrients present in the culture medium. Therefore, in future *in vivo* experiments and clinical application, only a few drops of the glue may be applied locally.

Conclusions

The two newly developed block copolymeric tissue adhesives are attractive materials to be used in repairing meniscal tears. An adhesive strength of 40-50 kPa should be sufficient to hold edges of meniscal tears together during the healing period. As there was no difference in adhesive strength between the two adhesive compositions, a decision on the most suitable adhesive to be used in future clinical application, should be made after investigating their mechanical properties, biodegradability and biocompatibility in an *in vivo* model in more detail. Incubation of the tissue explants in collagenase solution might improve tissue capacity for self-repair.

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Chapter 7

The Effect of Tissue Surface Modification with Collagenase and Addition of TGF- β 3 on the Healing Potential of Meniscal Tears Repaired with Tissue Glues *In Vitro*

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Abstract

The aim of the current *in vitro* study was to investigate if tissue surface modification with collagenase and addition of the TGF- β 3 can increase the number of cells present in meniscus tears repaired with the use of newly developed tissue adhesives based on isocyanate-terminated block copolymers.

Cylindrical explants were harvested from the inner part of bovine menisci. To simulate a full-thickness tear, the central core of the explants was removed and glued back into the defect, with or without incubation in collagenase solution prior to gluing. The repair constructs were then cultured with or without addition of TGF- β 3, and assessed for their histological appearance.

The histological staining of the constructs confirmed that both developed adhesives were not cytotoxic. After 28 days, meniscus cells were present in direct contact with the glues. The addition of TGF- β 3 to the culture medium resulted in the presence of cells that formed a sheath inside the simulated tear and in increased cell numbers at the edges of annulus of the explants. In the group in which the tissue was incubated in collagenase and cultured in medium containing TGF- β 3, thicker layers of cells were observed.

These results suggest that repairing the torn meniscus with tissue adhesives after pre-treatment of the tissue with collagenase and stimulation with a TGF- β 3 is a very promising treatment method, especially when treating the inner avascular part of the meniscus. Nevertheless, longer-term *in vitro* and *in vivo* studies are needed to confirm the beneficial effects of this combination therapy.

Introduction

Biodegradable tissue adhesives are an attractive class of materials with potential to be used in the repair of meniscus tears [1-3]. They are easy to apply, keep the torn parts of the tissue together during the period of healing, and degrade into harmless compounds that are then metabolized or excreted from the body.

However, meniscus tissue is largely avascular and its dense extracellular matrix (ECM) restricts the already relatively low number of cells in their mobility and

proliferation potential [4, 5]. Hence, the capacity of the meniscus for self-repair is very limited and tears, especially those located in the avascular zone, do not heal spontaneously. In clinical practice, rasping of the tissue and trephination to create access channels to stimulate neo-vascularization and new tissue ingrowth is an often-performed procedure [6-9]. Still, the success rate of repairing tears in the avascular zone is disappointing. The study of Rubman et al. showed that out of the 91 meniscal repairs evaluated arthroscopically, 23 (25%) were classified as healed, 35 (38%) as partially healed, and 33 (36%) as failed [10]. 20% of treated patients reported on in this study required a second intervention [10]. Therefore, there is a rapidly growing interest in the use of biological factors that could induce meniscus repair [11]. Qu *et al.* showed that partial digestion of adult meniscal explants with collagenase prior to culture *in vitro* resulted in a less dense ECM at the edges of the explants and in an increased cellularity [12]. A later *in vivo* experiment using ovine menisci confirmed that partial digestion of the tissue enhances repair by creating a more compliant and porous microenvironment that facilitates migration of cells to the tear margin and their proliferation [13]. Another approach to stimulate cellular migration and proliferation is the local delivery of growth factors [14-16]. Particularly, transforming growth factor TGF- β 3, which is an anabolic factor for meniscus fibroblasts, was shown to increase the integration strength in both adult and juvenile menisci *in vitro* [15].

We recently reported on the development of reactive three-armed and hyper-branched block copolymeric tissue adhesives that can attach to the tissue via covalent bonding [17, 18]. In a following study, meniscus explants were glued with these adhesives and cultured *in vitro*. It was shown that both developed materials have satisfactory bonding strengths to the tissue and did not exhibit toxicity to the cells, and therefore have the potential to be used in the treatment meniscal tears [19]. However, the continuous glue layer present in the repaired meniscal tear might limit cell migration and the transport of nutrients and waste products.

The aim of the current study was twofold. First, to evaluate if partial gluing of the repaired tissue allows cells to migrate into the simulated tear (gap). Second, to investigate if modification of the tissue surface with collagenase and the addition of TGF- β 3 can increase the number of cells present in the repaired meniscus tear and

thereby enhance healing of the tissue. We hypothesized that the optimal treatment for meniscus tissue repair would be a degradable glue to hold the edges of meniscal tear in close proximity to each other, in combination with additional biological factors to enhance the capacity of the meniscus for self-repair.

Materials and Methods

Tissue glues

Two different reactive isocyanate-terminated adhesive block copolymers were prepared: a three-armed TMPE-(TMC₂-HDI)₃ adhesive and a hyper-branched CA-4PEG-(TMC₂)₂-HDI adhesive. Their synthesis has been described in detail before [17, 18]. These adhesives were respectively prepared from trimethylolpropane ethoxylate (TMPE, $M_n=450$ g/mol), trimethylene carbonate (TMC) and hexamethylene diisocyanate (HDI), and from polyethylene glycol (PEG, $M_n=400$ g/mol), TMC, citric acid (CA) and HDI. Prior to use, the adhesives were sterilized by gamma irradiation (25 kGy, SynergyHealth BV, The Netherlands). The materials were characterized by ¹H-NMR (Bruker 400MHz NMR spectrometer) and FTIR (Perkin Elmer) before and after sterilization.

Preparation of meniscus explants

Meniscus explants were prepared in an analogous way as described by Hennerbichler *et al.* [20, 21]. Briefly, fresh knees from 12-18 months old cows were purchased from a local slaughterhouse. The medial menisci were removed from the joint under sterile conditions. Five cylindrical explants (8 mm-diameters) were obtained from the central part of each meniscus with a biopsy punch and randomly assigned to different experimental groups. Subsequently, their height was trimmed to 4 mm and an inner cylinder of 4 mm in diameter was centrally cored from each explant.

The influence of surface modification with collagenase and TGF- β 3 addition on cellular migration in glued meniscus tissue constructs

Explants (both outer annulus and inner cylinder) were incubated in basal medium containing 0 (control) or 0.05 mg/mL collagenase (type IV from *Clostridium*

histolyticum, ≥ 125 collagenase digestion units/mg solid, Sigma Aldrich) for 3 hours at 37 °C according to the protocol described by Qu *et al.* [13]. Basal medium was composed of Dulbecco's Modified Eagle's Medium (DMEM, Gibco®, UK) with 10% fetal bovine serum (FBS, Gibco®, UK), 1% penicillin/streptomycin/fungizone (PSF, Gibco®, USA) and L-ascorbic acid (Merck, Darmstadt, Germany) at a concentration of 25 μ g/mL of medium. Repair constructs were created by applying three lines of glue (approximately 5-10 μ L per line) onto the sides of the inner cylinder using a syringe equipped with a 23G needle and gently reinserting the inner cylinder into the outer annulus (Figure 1a). This gluing procedure allowed parts of the tissue from inner cylinder and the outer annulus to remain in direct contact (Figure 1b). Repair constructs were created using the three-armed adhesive and the hyper-branched one; constructs without any glue were used as controls.

Figure 1. a) An overview of the experimental design, b) Top view on the meniscal repair construct glued with the strips of the glue.

Constructs were cultured for 1 and 28 days in basal medium containing 0 (control) or 10 ng/mL of TGF- β 3. In total, four different culture conditions were employed (Table 1). After 1 and 28 days, the repair constructs (n=5 per group) were fixated in 10% formalin, embedded in poly(methyl methacrylate) (PMMA), sectioned into 8 μ m thick coupes and stained with hematoxylin and eosin (H&E) and picrosirius red (PR) to visualize cells, ECM and collagen fibers. For each group, H&E-stained sections were scored for the number of cells present inside and at the edge of the

simulated meniscal tear, which was defined as a gap between inner cylinder and outer annulus of the explants. Moreover, the number of cells and the thickness of the cell layers at the outer edge of the annulus of the explants (no cells, single cell layer or multiple cell layers) were assessed. Digestion of collagen fibers was visually assessed on the PR stained sections. For the scoring, 5 explants per group at 3 random locations of each explant were analyzed by light microscopy (200x field magnification).

Table 1. Conditions used in the in vitro culturing of glued meniscus tissue constructs.

Conditions	I	II	III	IV
Incubation in collagenase prior to gluing	no	yes	no	yes
Addition of TGF- β 3 to the culture medium	no	no	yes	yes

Statistical analysis

Differences in the number of cells in the different experimental groups were evaluated using a three-way ANOVA followed by Fischer's LSD post-hoc test. Data are presented as a mean value $\pm$ standard deviation. P values <0.05 were considered as significant. All statistical analyses were done using SPSS (version 20, IBM Corporation).

Results

The reactive three-armed TMPE-(TMC₂-HDI)₃ and hyper-branched CA-4PEG-(TMC₂)₂-HDI adhesives were successfully synthesized as confirmed by ¹H-NMR and FTIR analysis. No differences were observed before and after the sterilization procedure by gamma irradiation.

The H&E staining showed that the glues did not induce any toxic reaction to the tissue, the tissue remained vital during the whole culturing period. After 1 day of explant culture, vital meniscus fibroblasts were seen in close proximity to the gap in the H&E stained sections of all experimental groups. After 28 days cells could also be observed within the gap in all experimental groups. Within the same culturing conditions, there were no significant differences in cell numbers between the three-armed glue, the hyper-branched glue and the control group (Figure 2). There was also no difference in the number of cells between conditions I and II, indicating that

modification of the tissue surface by incubation in collagenase solution prior to culture did not have any beneficial influence on migration of the cells into the gap. However, there were differences between conditions IV, where explants were incubated in collagenase and TGF- β 3 was added to the culture medium, and the other culturing conditions. Although the differences in the number of cells were not statistically significant between these different culturing conditions (after 28 days respectively 3.6 ± 1.6 , 3.7 ± 1.9 and 4.1 ± 1.7 for the three-armed glue, the hyper-branched glue and the control), it could clearly be seen that when culturing under conditions IV the cells populating the gap between the inner cylinder and the outer annulus start to form sheaths in the spaces between the lines of glue. This was never observed when culturing under the other conditions.

At the outer edges of the annuli of the repair constructs there were no differences in cells numbers between the three-armed glue, the hyper-branched glue and the control group when cultured under the same conditions after 28 days (Figure 3). However, the influence of incubation in collagenase solution and the addition of growth factor TGF- β 3 was clearly visible (Figure 3). When non-treated explants were cultured in medium containing TGF- β 3 (III), single or double layers of cells aligned in an organized circumferential manner could be seen. When explants were incubated in collagenase and cultured in medium supplemented with TGF- β 3 (IV), even thicker multi-layered cell sheets were formed. This was not observed in explants cultured in the absence of growth factor (I and II), here only few cells at the outer edge of the repair constructs could be observed.

Figure 2. Representative light microscopy images of H&E stained meniscus tissue repair constructs cultured in vitro for 28 days under the different conditions (I-IV) described in Table 1. View on the partial gap between the inner cylinder and the annulus.

Figure 3. Typical images of the outer edge of the annulus of the repair constructs (H&E staining) cultured for 28 days under different conditions (I-IV) as described in Table 1. The figure shows images of the experimental three-armed glue group. In the other experimental groups (the hyper-branched glue and control groups), similar results were observed.

Visualization of the collagen fibers at the outer edges of the repair constructs after 1 day of culturing (after PR staining) revealed that in the groups incubated in collagenase (II and IV) the constructs were partially digested (Figure 4). After 28 days of culture, the influence of incubation in collagenase solution was even more pronounced. In the groups where TGF- β 3 was added to the culture medium (III and IV) circumferentially oriented collagen fibers were detected. This was not observed in experimental groups I and II. In the experimental group where the explants were incubated in collagenase and the medium contained TGF- β 3 (IV), the cellular layer and the collagen layer were thicker than in the group that only contained the growth factor (III).

Figure 4. Typical images showing the alignment of collagen fibers (PR staining) at the outer edge of the annulus of the repair constructs after 1 and 28 days of culture under the different conditions (I-IV) described in Table 1. Images of repair constructs prepared using the three-armed glue are shown. In the other experimental groups (the hyper-branched glue and the control group), similar results were observed.

Discussion

The two recently developed tissue adhesives appeared to be promising materials for meniscus tears treatment [19]. However, if the entire tear gap between the inner core and outer annulus of the explants is filled with glue, it could potentially limit cell migration. Therefore, in this study only partial gluing of the explants was performed. Such design will probably also be closer to a future clinical application, where only a few drops of glue would be applied to repair the tear [18]. In the present study, thin lines of the glues were applied to the tissue, and great care was taken to gently reinsert the inner cylinder into the outer annulus of the explants without spreading the glue over a larger surface area. As the glues could be seen in the histological slides after 1 and 28 days, partial gluing of the tissue, which stayed in place during the whole culture period, could be confirmed.

Histological evaluation of the gaps between the glued tissues in the repair constructs after 1 and 28 days showed the presence of fibroblasts in all experimental groups. Within 28 days of culture, cells were able to populate the gaps in between the lines of glue. There were no differences in cell numbers within the same culturing conditions, confirming the cell compatibility of the three-armed adhesive as well as that of the hyper-branched one.

The incubation of explants in collagenase and further addition of the TGF- β 3 to the culture medium resulted in a presence of sheets of cells in the gap of the repair constructs. Indeed, TGF- β 3 is known to be an anabolic chemotactic and mitogenic agent which attract cells and stimulates their replication [22]. Our findings are in accordance with data previously shown in literature that reprogramming the torn region of cartilaginous tissues such as the meniscus [12, 13, 15] and the articular cartilage [23, 24] with the use of enzymes *e.g.* collagenase or hyaluronidase increases the density of cells at the wound edges and promotes repair.

Although sheets of cells were formed in the gap and the cells were found to deposit new ECM, complete bridging of the tear gap was never observed. The reason for this could be that the growth factor was immediately absorbed in the peripheral part of the tissue. As this part was in a direct contact with the medium, the influence of the growth factor on the inner part of the repair constructs might not have been as pronounced. After longer culturing times of the explants (*e.g.* 6 or 8 weeks), the cell layers in the gap could possibly be more extensive [15]. Moreover, even though only partial gluing of explants was performed, the two edges of the tear stayed in very close proximity to each other, probably limiting the amount of nutrient containing medium that is available for the cells present in the gap. Furthermore, meniscus tissue is a tissue with very limited numbers of cells, especially in the inner region from where the explants used in this study were obtained. This might also be a reason for the low cell numbers in the gap and the limited differences between the various culture conditions.

Investigation of the outer edge of annulus of the explants, where the cells likely had better access to TGF- β and nutrients in the culture medium, confirmed the positive influence of incubation in collagenase solution and the addition of TGF- β 3 to the medium. Multi-layer cell sheets were present when the explants were both incubated in collagenase and cultured in medium containing growth factor. Slightly thinner

layers were seen when only TGF- β 3 was added to the medium. As the influence of both treatments was more pronounced at the outer edge of the annulus of the explants, it seems that the restricted access of medium to the gap is a limiting factor in our experimental setup. In clinical application, however, the glue will be applied as drops. Also, due to mechanical loading and fluid flow within the joint, the accessibility of nutrients to the cells is expected to be better.

Conclusions

Modification of the surface of meniscus tissue by incubation in collagenase and the presence of growth factor TGF- β 3 were shown to have a positive influence on the number of cells present in the gap of a meniscus repair construct glued with novel reactive isocyanate-terminated three-armed- and hyper-branched glues. The healing potential of a torn meniscus can possibly be enhanced by using a combined therapy which makes use of collagenase tear surface treatment, biodegradable glues and the presence of growth factors. Further longer-term *in vitro* and *in vivo* studies are needed to validate these findings.

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Chapter 8

***In Vitro* and *In Vivo* Characterization of Biodegradable Reactive Isocyanate-Terminated Three-Armed- and Hyper- Branched Block Copolymeric Tissue Adhesives**

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Abstract

Meniscus tears are common injuries of the knee joint. Currently a few treatment modalities are available, but these are not successful in all conditions. Tissue adhesives are an attractive class of biomaterials as they are easy to apply and cause minimal tissue trauma, their use could serve as an alternative treatment. We recently developed biodegradable reactive isocyanate-terminated three-armed- and hyper-branched block copolymeric adhesives (glues) that can potentially be used for the treatment of meniscal tears.

The aim of the current study was to evaluate the physicochemical- and adhesive properties of these novel glues, to study their degradation profile after curing both *in vitro* and *in vivo*, and to assess the tissue reaction after subcutaneous injection in rats.

The developed glues had sufficient adhesive strength to meniscus tissue after curing (66-88 kPa). After curing, networks based on the three-armed adhesive had tensile properties that were in the same range as those of human meniscus tissue. The networks degraded by hydrolysis. After 26 weeks networks based on the hyper-branched adhesive showed a faster mass loss (25.4 %) compared to networks prepared from the three-armed glues (5.5 %). Both glues induced an inflammatory reaction involving macrophages and PMN cells. However, no necrosis and only initial toxic effects on peripheral tissues were observed.

The proposed materials might be suitable candidates for the use as resorbable tissue adhesives for meniscus repair. Nevertheless, further studies in a biomechanically relevant animal model should still be performed.

Introduction

The meniscus is a frequently injured tissue with limited potential for self-repair [1, 2]. Current clinical repair techniques mostly make use of sutures or staples, but are not successful in all conditions [3, 4]. Surgical tissue adhesives (i.e. glues) are an attractive class of biomaterials that have the potential to be used for the treatment of meniscal tears [5]. They are easy to apply via a minimally invasive procedure [6], and their structure can be designed to degrade after the tissue is healed. Several tissue adhesives are already used in clinical practice. Fibrin glue is used in the treatment of

pulmonary leaks, Dermabond[®] for skin lacerations and the recently FDA-approved TissuGlu[®] for abdominoplasty [7-10]. However, none of these adhesives is suited for the treatment of meniscus tears due to inadequate mechanical properties or cytotoxic characteristics [11].

We have recently reported on the development of two novel biodegradable isocyanate-terminated three-armed and hyper-branched block copolymeric tissue adhesives with the potential to be used for the repair of meniscus tears [12, 13]. These materials have adequate adhesive strengths to the meniscus after curing. Moreover, previous *in vitro* experiments have shown that when using relevant amounts of these adhesives they are essentially non-toxic to meniscus cells [12]. The three-armed oligomeric adhesive has a well-defined and controlled molecular structure, in which the three isocyanate end-groups react to form a cured polyurethane network. The molecular structure of the hyper-branched adhesive is less well-defined. It has more reactive end-groups per molecule that can covalently attach to the tissue and cures to form a polyester-polyurethane network. [12].

The purpose of the current study was to evaluate the physicochemical- and adhesive properties of these three-armed and hyper-branched copolymeric tissue adhesives, to study their degradation profile after curing both *in vitro* and *in vivo*, and to assess the tissue reaction after subcutaneous injection in rats.

Materials and Methods

Materials

The isocyanate-terminated reactive three-armed- and hyper-branched adhesive oligomers (glues) were prepared as previously described [12, 13]. The building blocks of the three-armed block copolymeric adhesives were trimethylolpropane ethoxylate (TMPE, $M_n=450\text{g/mol}$), trimethylene carbonate (TMC) and hexamethylene diisocyanate (HDI). The building blocks of the hyper-branched adhesive were polyethylene glycol (PEG, $M_n=400\text{g/mol}$), TMC, citric acid (CA) and HDI. The prepared reactive adhesives oligomers are designated as: TMPE-(TMC₂-HDI)₃ (three-armed adhesive) and CA-4PEG-(TMC₂)₂-HDI (hyper-branched adhesive).

The adhesives were sterilized by gamma irradiation in a nitrogen atmosphere prior to use (25 kGy, SynergyHealth BV, The Netherlands). Their ^1H -NMR (Bruker 400MHz NMR spectrometer) and FTIR (Spectrum Two, Perkin Elmer) spectra were recorded before and after sterilization and compared.

Preparation and characterization of water-cured networks

Polyurethane networks films were obtained by casting the viscous isocyanate-terminated three-armed and hyper-branched copolymers with a 0.3 mm casting knife on glass plates, and subsequent curing in water as described previously [13]. To determine the gel content of the water-cured networks, they were dried in vacuum at 40 °C and their initial mass was determined (m_i). The specimens ($n=3$) were then swollen in chloroform for 24 hours to extract the sol fraction, and dried in vacuum until constant weight (m_{dc}). To assess their water uptake ($n=3$), they were placed in water for 24 hours and their wet mass (m_w) was determined. The networks were then re-dried until constant weight, and their dry mass was determined (m_{dw}).

The gel content (GC) was determined using:

$$GC = \left(\frac{m_{dc}}{m_i} \right) \cdot 100\%$$

The water uptake (WU) was determined using:

$$WU = \left(\frac{m_w - m_{dw}}{m_{dw}} \right) \cdot 100\%$$

The thermal properties of the networks in the wet state were assessed by differential scanning calorimetry (DSC, Perkin Elmer Pyris 1). Specimens weighing 5-10 mg were heated from -100 °C to 100 °C at 10 °C/min.

Adhesion of the reactive isocyanate-terminated block copolymers to bovine meniscus tissue

The reactive isocyanate-terminated oligomers were applied ($n=5$) onto strips of bovine meniscus tissue with sizes of approximately 0.5x10x25 mm. (The tissue strips had been kept hydrated in water and excess water was blotted from the surface

just before application of the adhesives). The adhesives were applied with a spatula and covered an area of approximately 1 cm². Subsequently, two strips were pressed together, immersed in water at room temperature and left to cure. The adhesive bonding strength to the tissue was assessed after 1, 4, 8 and 24 hours of curing in a lap shear adhesion experiment analogous to ASTM F2255-05. A Zwick Z020 universal tensile tester was used, the grip to grip separation was 25 mm and the crosshead speed was 50 mm/min. The shear adhesive bond strength (S) was calculated as the maximum shear force divided by the glued area.

***In vitro* degradation of water-cured networks**

The *in vitro* degradation of the water-cured networks (disc-shaped specimens with a diameter of 10 mm punched out from films with a thickness of 350-500 µm) was assessed in triplicate in phosphate buffer saline (PBS) solution (pH=7.4) and cholesterol esterase solution (CE, from porcine pancreas, 35 U/mL, Sigma Aldrich, The Netherlands) at 37 °C. Both solutions contained 0.02 wt% of sodium azide as bactericide. The samples were placed in 48 well-plates containing 1 mL of PBS or CE solution and let to degrade. The medium was changed twice per week. The specimens were recovered and their mass, thickness and the liquid uptake were determined in the wet state at predetermined time points and after drying in vacuum until constant weight.

***In vivo* biocompatibility of the reactive isocyanate-terminated block copolymers and *in vivo* degradation of water-cured networks**

To evaluate the biocompatibility of the isocyanate-terminated adhesive block copolymers *in vivo*, the reactive adhesives were subcutaneously injected in rats. Also, the degradation of the water-cured network films implanted subcutaneously was assessed. All procedures were approved by the Animal Ethics Committee of the Radboud University. In total 40 Sprague-Dawley rats weighing 200-250 g were operated in 5 groups based on time to sacrifice (4 male and 4 female per group). The animals were housed in a humidity- and temperature-controlled room with 12 hours light/dark cycle. The rats received water and pelleted diet *ad libitum*. The animals were operated under general anesthesia, which was induced using isoflurane. After shaving and disinfection, two subcutaneous pockets were made to the right and two

pockets to the left of midline incisions on the back of the rats. In two of these pockets 0.1 mL of the three-armed- or the hyper-branched isocyanate-terminated adhesive block copolymers were injected using a syringe, in the other two pockets films of water-cured networks based on the three-armed- and hyper-branched adhesives (20x10x0.3-0.6 mm) were implanted. The network films were decontaminated by washing in 70% ethanol (15 minutes), drying (30 minutes) and washing three times in PBS (5 minutes). This procedure was repeated three times. The implants (reactive adhesive or water-cured network film) were randomized among the pockets. Caregivers structurally monitored animal welfare during the first 7 postoperative days. After 1 day, 1, 4, 12 and 26 weeks, the rats were euthanized by CO₂ asphyxiation.

Histological evaluation of the tissue reaction towards the reactive adhesives

After subcutaneous injection of the adhesives, the *in situ* cured specimens together with the surrounding tissue were retrieved from the pockets. Additionally, two control samples (a piece of skin tissue without the cured glue from a female rat and a piece from a male rat) per time point were taken for analysis. Samples were fixated in formalin (10% solution in PBS), embedded in poly(methyl methacrylate) (PMMA), sectioned to 8 µm and stained with hematoxylin and eosin (H&E) to visualize the cells and the extracellular matrix (ECM). To visualize macrophages, immunohistochemical staining was performed as well. The samples were deplasticized (using chloroform/xylene 1:1) and cut to 5 µm thick sections. Endogenous peroxidase was then blocked by incubation in 3% H₂O₂ in methanol for 30 min and aspecific reaction sites were blocked by incubation in 10% normal horse serum for 30 min. Afterwards, the sections were incubated overnight at 4°C with mouse monoclonal anti-rat ED1 antibody (AbD Serotec®, 1:200 dilution in PBS, pH=7.4, with 1% bovine serum albumin (BSA)) and in biotinylated horse anti-mouse antibody (dilution 1:400 in PBS, pH=7.4) for 45 minutes at room temperature. Afterwards sections were incubated in Avidin Biotin Complex for 60 min at room temperature. Before, between and after each incubation step, the sections were washed 3 times with 3 minutes changes of PBS. The macrophages were visualized using dimethylaminoazobenzene and counterstaining with hematoxylin.

The histological slides were scored to assess the inflammatory reaction (cell type/response and tissue response) following a custom protocol based on ISO 10993-6 and the protocol described by Link *et al* [14] using a 200x microscope magnification field (Table 1). The histology scoring results are reported as median values with the observed range in parentheses.

***In vivo* degradation of water-cured networks**

The water-cured network films were explanted from the subcutaneous pockets, weighed in the swollen state, rinsed with water to remove any remaining debris or tissue and dried at 40 °C until constant weight. Their determined mass and thickness (m_d and d_d , respectively) were then compared with their initial mass and thickness (m_i and d_i) to assess the extent of degradation. The liquid uptake of the degrading networks was also determined. To observe changes in mechanical properties during implantation, explanted specimens of each material (n=6) were subjected to tensile testing in the wet state at room temperature (RT) according to ASTM D882. A Zwick Z020 universal tensile tester was used, the grip-to-grip separation was 11 mm and the crosshead speed was 50 mm/min. The properties of hydrated network films (n=6) which were not implanted were also determined.

At each time point, changes in the surface morphology of the different network films during *in vivo* degradation (n=1) were observed by Scanning Electron Microscope imaging (SEM, Zeiss Gemini Sigma 300).

Statistical analysis

To compare differences in mass loss, remaining height and degree of swelling of the *in vitro* and *in vivo* degrading water-cured networks in time, three-way ANOVA with Fischer's LSD post-hoc test was used. To evaluate differences in adhesive strength between the glues in time, a two-way ANOVA was used, followed by Fischer's LSD post-hoc test. P- values <0.05 were considered statistically significant. Data is presented as mean $\pm$ standard deviation (SD), unless indicated otherwise. Statistical analyses were performed using SPSS Statistics (version 20, IBM Corporation).

Table 1. Scoring criteria of the histological slide based on ISO 10993-6 standard and the protocol described by Link et al [13].

Evaluation (H&E)	Response	Score
Capsule quantitatively	Not applicable	0
	1-4 fibroblasts	1
	5-9 fibroblasts	2
	10-30 fibroblasts	3
	>30 fibroblasts	4
Capsule qualitatively	Cannot be evaluated because of infection or other factors not necessarily related to the material	0
	Capsule is fibrous, mature, not dense, resembling connective or fat tissue in the non-injured region	1
	Capsule tissue is fibrous but immature, showing fibroblasts and little collagen	2
	Capsule tissue is granulous and dense, containing both fibroblasts and many inflammatory cells	3
	Capsule consists of masses of inflammatory cells with little or no signs of connective tissue organization	4
Interface qualitatively	Cannot be evaluated because of infection or other factors not necessarily related to the material	0
	Fibroblasts contact the implant surface without the presence of macrophages or leucocytes	1
	Scattered foci of macrophages and leucocytes are present	2
	One layer of macrophages and leucocytes are present	3
	Multiple layers of macrophages and leucocytes present	4
Thickness of reactive layer	Narrow band	1
	Moderately thick band	2
	Thick band	3
	Extensive band	4
Neovascularization	None	0
	Minimal capillary proliferation, focal, 1-3 buds	1
	Groups of 4-7 capillaries with supporting fibroblastic structures	2
	Broad band of capillaries with supporting structures	3
	Extensive band of capillaries with supporting fibroblastic structures	4
Necrosis	None	0
	Minimal	1
	Mild	2
	Moderate	3
	Severe	4
Cytotoxic effect on the peripheral tissue	No	0
	Yes (e.g. giant cells attacking muscle cells)	3
Evaluation (Immunohistochemistry)	Response	Score
Macrophages	None	0
	Rare, 1-5/per high field	1
	5-10/per high field	2
	Heavy infiltrate	3
	Packed	4
Total maximum score		31

Results

Isocyanate-terminated three-armed- and hyper-branched block copolymeric tissue adhesives

Isocyanate-terminated three-armed- and hyper-branched block copolymers were successfully synthesized. Comparison of the areas under the peaks that correspond to PEG (3.5-3.7 ppm) or TMPE (3.5-3.75 ppm) with TMC (2.1 and 4.1-4.3 ppm) in the ^1H -NMR spectra of non-functionalized oligomers, showed that the conversion of TMC was 94% and 92% for the three-armed- and the hyper-branched oligomers, respectively. Analysis of the areas under the peaks that correspond to PEG or TMPE and HDI (3.1-3.35 ppm and 1.25-1.65 ppm) in the spectra of the oligomers functionalized with HDI confirmed that for both the three-armed- and the hyper-branched oligomers the degree of functionalization was 100%.

Comparison of the ^1H -NMR and FTIR spectra before and after gamma irradiation of the functionalized oligomers showed nearly identical spectra, indicating that their chemical structure was not affected by the sterilization process.

Characteristics of water-cured networks prepared from isocyanate-terminated three-armed- and hyper-branched block copolymers

The chemical composition and the physical properties of the water-cured networks prepared from the reactive adhesives are given in Table 2. Assuming complete incorporation in the networks, the mass ratio of the hydrophilic PEG or TMPE component to the hydrophobic TMC component in the functionalized block copolymeric oligomers was 0.73 and 0.98 for three-armed- and the hyper-branched reactive network precursor, respectively. The gel content of the cured networks was 98.7 ± 0.3 and 91.7 ± 6.7 after water-curing of the three-armed- and the hyper-branched adhesive, respectively. The water uptake of $25.3 \pm 8.7\%$ of networks based on the hyper-branched adhesives was significantly higher than that of the ones based on the three-armed adhesives, which was only $5.7 \pm 1.4\%$. In the hydrated state, the glass transition temperature (T_g) of both networks was below 37°C .

Table 2. Composition and physical properties of networks prepared by curing the three-armed- and hyper-branched adhesives in water.

	Three-armed adhesive-based network	Hyper-branched adhesive-based network
TMPE or PEG content ^{a)} [wt%]	28.7	36.1
TMC content ^{a)} [wt%]	39.1	36.8
Gel Content [%]	98.7 (0.3)	91.7 (6.7)
Water Uptake [%]	5.7 (1.4)	25.3 (8.7)
T _g ^{b)} [°C]	-27.2	-40.5

^{a)} Assuming the reactive oligomers are fully incorporated in the network

^{b)} Determined in the wet state

Adhesion of the reactive isocyanate-terminated block copolymers to bovine meniscus tissue

For both compositions, the adhesive strength of the glues to bovine meniscus tissue determined in lap-shear testing significantly increased with curing time in water (Figure 1) ($p < 0.05$). The mean adhesion of the hyper-branched adhesive was already 62 ± 56 kPa after 8 hours, which was close to the maximum value of 66 ± 5 kPa which was reached after 24 hours. The three-armed glue reached an adhesive strength of 65 ± 17 and 88 ± 51 kPa after 8 and 24 hours, respectively. There were no statistically significant differences in adhesive strength between the two glues.

Figure 1. The adhesive strength of reactive three-armed- and hyper-branched adhesives to bovine meniscus tissue determined in lap shear testing at different curing times.

***In vitro* and *in vivo* degradation of water-cured networks**

The mass loss and the liquid uptake of water-cured network films prepared from the reactive three-armed- and the hyper-branched block copolymers were determined after degradation *in vitro* in PBS and CE solutions, and *in vivo* after subcutaneous implantation. There were no behavioural changes or signs of physical impairment observed in the animals upon implantation of the water-cured network films. The remaining mass of the networks at the predetermined time points is depicted in the Figure 2. In all cases, networks prepared from the hyper-branched block copolymers degraded significantly faster than those prepared from the three-armed block copolymers ($p < 0.001$). The mass loss after 26 weeks of networks based on the three-armed adhesives was $5.5 \pm 3.2\%$, while that of networks prepared from the hyper-branched adhesives was $25.4 \pm 7.8\%$. The latter network films had lost their mechanical integrity after 26 weeks. Degradation in the different degradation environments, *i.e.* in CE, PBS or *in vivo*, did not lead to statistically significant differences in remaining mass.

Figure 2. The remaining mass (in the dry state) of networks prepared from reactive isocyanate-terminated three-armed- and hyper-branched block copolymers at different time points during a) *in vitro* incubation in CE, b) *in vitro* incubation in PBS, c) *in vivo* subcutaneous implantation in rats.

At all time points, the liquid uptake of networks prepared from the hyper-branched adhesives was significantly higher than that of networks prepared from the three-armed adhesives during incubation in the different environments ($p < 0.05$) (Supplementary Material, Figure S1). Moreover, there were no differences in the uptake of body liquids of a particular network in the different *in vitro* or *in vivo* degradation environments. Only after 26 weeks, the networks based on the hyper-branched adhesive degrading *in vivo* had a liquid uptake of $54.2 \pm 4.9\%$ that was significantly higher than that of the same networks degrading *in vitro* ($30.1 \pm 7.9\%$ and $29.1 \pm 8.3\%$ in PBS and CE, respectively).

For both networks, the thickness of the degrading films did not change significantly during the incubation in the different environments. The remaining thickness of the films in time is presented in the Supplementary Material, Figure S2.

The tensile properties of hydrated network films before and upon subcutaneous implantation were determined. The elastic modulus (E_{mod}) and the maximum tensile strength (σ_{max}) were considerably higher for networks prepared from the three-armed adhesives than for those prepared from the hyper-branched precursors (Table 3). The values of elastic modulus and tensile strength of networks based on the three-armed precursors did not change significantly during *in vivo* degradation. On the contrary, the properties of networks based on the hyper-branched adhesives did change considerably. The tensile strength decreased from 3.5 ± 2.1 to 1.3 ± 0.2 MPa, and the elastic modulus from 15.6 ± 11.7 to 3.8 ± 0.5 after 26 weeks *in vivo*. For both networks, the elongation at break decreased by nearly a factor two.

Table 3. Tensile properties of hydrated network films prepared from isocyanate-terminated three-armed- and hyper-branched block copolymers during subcutaneous implantation in rats.

Note that after 26 weeks the tensile properties of the networks prepared from the hyper-branched adhesives could not be determined due to loss of integrity of the specimens.

Time point	Three-armed			Hyper-branched		
	E [MPa]	$\sigma_{\max}$ [MPa]	$\varepsilon_{\max}$ [%]	E [MPa]	$\sigma_{\max}$ [MPa]	$\varepsilon_{\max}$ [%]
Control	102.2±9.6	10.4±3.3	71.7±53.2	15.6±11.7	3.5±2.1	87.0±46.9
1 day	113.1±18.8	11.2±3.0	52.2±23.7	19.0±1.7	2.7±0.5	27.4±7.0
1 week	76.0±14.1	10.6±3.3	83.0±41.6	5.1±0.6	1.4±0.6	45.7±15.7
4 weeks	73.7±13.0	6.4±2.4	28.5±22.0	5.8±0.7	1.6±0.4	38.7±7.4
12 weeks	81.5±22.0	8.5±3.5	45.3±30.0	3.8±0.5	1.3±0.2	49.5±13.6
26 weeks	131.3±8.8	12.4±1.2	44.5±18.0	-	-	-

The SEM images of the surfaces of the *in vivo* degrading network films showed changes in the surface morphology in time. Upon implantation, the initially smooth surfaces of the water-cured network films gradually became rough and irregular (Figure 3). This was especially evident in the case of network films prepared from the hyper-branched adhesive. Here it can be seen that already after 1 week the surface becomes rough, after 26 weeks the roughness was quite pronounced.

Figure 3. SEM images of the surfaces of network films prepared from isocyanate-terminated three-armed- and hyper-branched block copolymers degrading in vivo during subcutaneous implantation in rats.

Biocompatibility of the glues *in vivo*

Subcutaneous injection of the reactive adhesives resulted in the *in situ* curing and formation of the irregular-shaped polyurethane networks. During the process of network formation carbon dioxide was released, resulting in porous structured networks.

No behavioural changes or signs of physical impairment were observed in the animals during the course of the study.

A few rats had a rash appearing on the skin close to the injection pockets up to 1 week post implantation. However, the animals did not show any signs of discomfort and the rash disappeared spontaneously. The scoring results of the H&E-stained slides of the tissue samples into which the adhesives were subcutaneously injected are presented in Figure 4 and Figure 5. The median total scores of the histological slides at the different time points are given in Table 4. The total score of the control samples was 8 (6-10). The scoring results of each category at different time points are presented in Supplementary Material (Table S1 and Table S2 for the three-armed adhesive and hyper-branched adhesive, respectively).

There were no visible differences in the tissue reaction to the injected glues between female and male rats. There was, however, a large rat-specific variation in the tissue response to the adhesives: while in some of the rats a mild tissue reaction was observed, a more severe reaction to the adhesives was seen at the same time points in other rats.

At 1 day post injection, a tissue reaction with an immature, hydrated and slightly swollen fibrous interfacial tissue with low numbers of fibroblasts and immune cells was observed for both adhesives (Figure 4a and Figure 5a). The total histology score was 16 (3-23) and 15 (4-24) for the three-armed- and the hyper-branched adhesive, respectively. After 1 week the interface around the injected adhesive was much thicker, and more fibrous in nature with groups of sprouting vessels and larger numbers of immune cells present. Immunohistochemical staining (Figure 4b and Figure 5b, and Figure 6) indicate that these include macrophages and polymorphonuclear (PMN) cells. No differences between the two adhesives were

seen, the total histology scores were quite similar, 19.5 (7-29) and 20.5 (9-25) for the three-armed- and the hyper-branched adhesive, respectively. After 4 weeks post implantation, the number of PMN cells had decreased compared to the numbers at 1 week. For both glues, giant cells and clusters of macrophages could be seen in the interface (Figure 4c,d, Figure 5c,d and Figure 6b). After 4 weeks, the total score of the hyper-branched adhesive of 17 (6-21) was lower than that for the three-armed adhesive which was 20.5 (6-26). After 12 and 26 weeks there were pronounced differences in tissue response to the adhesives between individual rats (Figure 4e-h and Figure 5e-h). After 12 and 26 weeks, the total score of the hyper-branched glue (18 (3-23) and 17.5 (4-24), respectively) was similar that of the three-armed adhesive (16.5 (6-23) and 22 (13-23), respectively). There was a relatively mild response in some individual rats, while in other rats an increased number of PMN cells surrounding the degrading adhesives could be observed. Macrophages were seen only rarely (Figure 6c), and regarding neovascularization mainly small groups of capillaries with supporting fibroblastic structures were observed.

Table 4. Total histological evaluation scores of rat skin tissue samples after subcutaneous injection of the reactive isocyanate-terminated three-armed- and hyper-branched block copolymeric tissue adhesives. The data are presented as median values with the observed range in parentheses.

	1 day	1 week	4 weeks	12 weeks	26 weeks
Three-armed	16 (3-23)	19.5 (7-29)	20.5 (6-26)	16.5 (6-23)	22 (13-23)
Hyper-branched	15 (4-24)	20.5 (9-25)	17 (6-21)	18 (3-23)	17.5 (4-24)

Figure 4. Typical microscopy images of the rat subcutaneous tissue response (H&E stained) to the injected reactive three-armed adhesives after a) 1 day, b) 1 week, c, d) 4 weeks (mild and more severe response to the same glue in different rats), e, f) 12 weeks (mild and more severe response to the same glue in different rats), g, h) 26 weeks (mild and more severe response to the same glue in different rats).

Figure 5. Typical microscopy images of the rat subcutaneous tissue response (H&E stained) to the injected reactive hyper-branched adhesive after a) 1 day, b) 1 week, c, d) 4 weeks (mild and more severe response to the same glue in different rats), e, f) 12 weeks (mild and more severe response to the same glue in different rats), g, h) 26 weeks (mild and more severe response to the same glue in different rats).

Figure 6. Typical microscopy images of the rat subcutaneous tissue response (immunohistochemistry, macrophages are stained in brown colour and are indicated by arrows) to the injected reactive hyper-branched adhesive a) after 1 week clusters of macrophages were observed, b) after 4 weeks the number of macrophages had decreased and giant cells could be observed, c) after 26 weeks clusters of macrophages were only rarely seen.

Discussion

The successful synthesis of reactive isocyanate-terminated three-armed- and hyper-branched block copolymeric adhesives was confirmed by ^1H -NMR analysis. Moreover, gamma irradiation under nitrogen atmosphere appears to be a suitable sterilization method for these isocyanate-terminated glues as their NMR and FTIR spectra before and after gamma irradiation were almost identical. This is in agreement with reports in literature regarding the use of gamma irradiation for the sterilization of polyurethane precursors [15, 16].

Water-cured networks prepared from the reactive hyper-branched adhesives had a higher ratio of hydrophilic PEG components to hydrophobic TMC components and consequently higher water uptakes than networks prepared from the three-armed adhesives. The higher water uptake of networks prepared from the hyper-branched adhesives could also indicate that their cross-linking density was lower than that of networks prepared from the three-armed copolymers. For both networks, the high gel content values (above 90%) indicated successful curing upon reaction of the reactive oligomers with water. The gel contents of networks prepared from the three-armed adhesive were slightly higher than that of networks prepared from the hyper-branched adhesives. This could be due to the higher hydrophilicity of the hyper-

branched adhesives and networks which could lead to dissolution of the reacting block copolymeric oligomers before their incorporation into the (swollen) network.

The glass transition temperature of both networks in the wet state was below the physiological temperature of 37 °C. This implies that in the intended application conditions, the cured adhesives will be flexible and rubber-like in nature.

The shear adhesion strength to meniscus tissue of both the three-armed- and the hyper-branched glues increased with curing time, respectively reaching maximum values of 88 kPa and 66 kPa after 24 hours. These adhesive strengths should be sufficient for application in repairing meniscal tears, as it was reported in literature that distraction forces acting on a sutured meniscus did not exceed 5N when a load of 300N was applied to human cadaver knees [17, 18]. These values correspond to stresses of approximately 50 kPa. In recovery/rehabilitation period after surgery, the loads applied to the knee may even be lower.

The curing rate of the hyper-branched adhesive appeared to be faster than that of the three-armed adhesive, as bonding strengths close to maximum values were reached after 8 hours and 24 hours, respectively. The differences in curing rate might be due to the different numbers of reactive isocyanate end-groups per molecule in the different adhesive compounds. It is expected that these numbers are higher in the case of the hyper-branched adhesives [12]. The hyper-branched adhesive was also more hydrophilic, and may have attracted more water leading to faster curing. Its adhesive strength was not significantly higher than that of the three-armed adhesive. This can be due to partial dissolution of the adhesive in water before curing, also the higher water uptake of the formed network might have limited the extent of covalent bonding to the tissue.

The degradation of the water-cured networks *in vivo* could be due to the activity of macrophages which excrete degradative enzymes, or by plain hydrolysis. Therefore, *in vitro* degradation experiments in PBS (to assess the extent of plain hydrolysis) and in CE solution (which is an enzyme excreted by macrophages that is known to degrade polyurethanes and poly(trimethylene carbonate) [19-22]) were also performed.

The rate of mass loss of networks prepared from the three-armed adhesives was significantly lower than that of networks based on the isocyanate-terminated hyper-branched oligomers in all degradative environments. These differences are due to the different hydrophilicities of the materials, as it is known that degradable hydrophilic polymeric materials hydrolyse faster [23]. The networks resulting from the hyper-branched adhesive absorb significantly more degradation medium. Furthermore, the ester linkages that are present in these networks are more susceptible to hydrolysis than urethane bonds [24].

As there were no statistically significant differences in rate of mass loss of the degrading networks in the different *in vitro* and *in vivo* degradation environments, it can be concluded that plain hydrolysis is the main mode of degradation. Enzymatic degradation of PTMC apparently does not play a role. The thickness of the degrading network films in the different environments did not change significantly in time (Supplementary Material, Figure S2). Thus, as the mechanical properties of the networks decreased with the loss of mass in time (some networks had disintegrated at 26 weeks), it can be concluded the networks degrade via a bulk erosion process.

The values of the elastic modulus (E) and the maximum tensile stress ($\sigma_{\max}$) of networks prepared from the three-armed adhesives did not change significantly during *in vivo* degradation and were in the same range as those of the human meniscus ($\sigma=10\text{-}19$ MPa, $E=60\text{-}150$ MPa) [25, 26]. The somewhat low values of E and σ determined at 1, 4 and 12 weeks may be due to irregularities, defects or air bubbles in the prepared network films. In contrast, the elastic modulus and maximum tensile stress values of networks based on the hyper-branched adhesives were significantly much more affected by the degradation process. The networks had become brittle in time and were fragmented at 26 weeks.

SEM images of the surfaces of network films degraded *in vivo*, showed that the surface of network films prepared from the hyper-branched adhesives already became rough and irregular after 1 week of implantation. This roughness was even more pronounced after 26 weeks. Changes of the surface topographies of networks prepared from the three-armed adhesives were visible only after 12 weeks of degradation, and only after 26 weeks their surface had become somewhat rougher. This is in line with the observations of mass loss of the networks during degradation, which was significantly faster for networks based on the hyper-branched adhesives.

Subcutaneous injection of the glues resulted in the presence of inflammatory reaction. The three-armed adhesives showed a slightly more severe tissue reaction than the hyper-branched ones. Inflammation involving infiltration of macrophages was observed, however there was no necrosis, and inflammation was seen only in tissue in direct contact with the glue. The cytotoxic reaction of the peripheral tissue was observed only in few rats after 1 day and 1 week post injection. At the later time points this was not seen anymore. This initial inflammatory reaction can be due to the isocyanate groups, these are known to be skin irritants [27]. A different application site, *e.g.* in intramuscular-, bone- or meniscus tissue, could possibly result in a less extensive reaction [16]. The observation that the hyper-branched adhesives showed a less severe tissue reaction may be due to their faster curing times compared to the three-armed compositions, the tissue may be exposed to the unreacted isocyanate groups for shorter times. At the same time, the wide variations in the histological scoring results after injection of these adhesives can be the result of the fact that part of the material had not been entirely incorporated into the network. As described, the gel content of networks prepared from hyper-branched adhesives, was slightly lower than that of those prepared from the three-armed adhesives.

The inflammatory reaction, which could still be observed at the later time points post injection, was probably a reaction to the presence of degradation products of the *in situ* cured adhesives. Networks based on the three-armed adhesives ultimately degrade into water-soluble TMPE, 1,3-propane diol, CO₂, water and 1,6-hexanediamine. Networks based on the hyper-branched adhesives degrade into PEG, 1,3-propane diol, CO₂, water, 1,6-hexanediamine and CA. These water-soluble compounds can be metabolized or excreted by the kidneys. However, these compounds can also lead to changes in local pH which can be the cause of the inflammation.

The porous degrading networks formed after subcutaneous injection of the adhesives attracted many PMN cells, these are normal host defense mechanisms and inflammatory reactions to both the surgical procedure and the presence of implanted porous and degrading materials [28, 29]. The erosion rate, and the size and shape of implanted materials can influence the intensity of the tissue response to degradable

polymers [30-32]. In cases where the injected adhesives had cured forming porous spheres, many PMN cells were observed in the histological slides. In other cases, where the adhesives had formed more elongated and flat porous films, considerably less PMNs were seen. There were large variations in tissue response observed after injecting the adhesives. These were found to be rat-specific and not much dependent on the follow-up period. Analysis of the histological slides showed that while the same rats showed more severe responses to both of the injected glues, other individuals showed a very mild inflammatory response towards the glue. This could be due to the fact that isocyanates can induce contact sensitivity [33]. In our case, probably not all animals had become sensitized, explaining the variability in the results.

In our experiments, we injected 100 μL of the adhesives subcutaneously, as this amount allowed to easily detect the glues under the skin of rats. However, in clinical application this amount would be significantly lower (only a few 5-10 μL drops). As the target application of our glues is repairing the torn meniscus, which is mostly an avascular tissue, the inflammatory reaction is expected to be much less extensive. Furthermore, a mild inflammatory reaction would be desired as this could induce healing of the meniscus tissue.

Conclusions

The two newly developed isocyanate-functionalized three-armed- and hyper-branched tissue adhesives are promising materials for the repair of meniscal tears. They reach satisfactory adhesive strengths to meniscus tissue after curing. Their degradation profiles depend significantly on their chemistry, particularly on the presence of ester linkages in their network structure as these are prone to hydrolytic degradation. Histological analysis revealed that the glues caused an inflammatory reaction upon subcutaneous injection. This reaction is expected to be less prominent when the adhesives are applied in lower amounts to avascular meniscal tissue. To minimize tissue exposure times to free isocyanate groups, current studies are aimed at minimizing curing times of the adhesives.

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Supplementary Material

Figure S1. Uptake of liquids by network films prepared from reactive isocyanate-terminated three-armed- and hyper-branched block copolymers at different time points during a) *in vitro* in CE, b) *in vitro* in PBS, c) *in vivo* subcutaneous implantation in rats. In all degradation environments, the liquid uptake was significantly higher for networks prepared from hyper-branched adhesives than from three-armed ($p < 0.05$).

Figure S2. Thickness of network films prepared from reactive isocyanate-terminated three-armed- and hyper-branched block copolymers at different time points during a) in vitro in CE, b) in vitro in PBS, c) in vivo subcutaneous implantation in rats. There were no significant differences in thickness between the degrading networks in time.

Note that after 26 weeks it was not possible to determine the thickness of network films prepared from isocyanate-terminated hyper-branched block copolymers degrading in vivo due to loss of integrity of the specimens.

Table S1. Histological evaluation scores of rat skin tissue samples after subcutaneous injection of the reactive isocyanate-terminated three-armed copolymeric tissue adhesives. The data are presented as median values with the observed range in parentheses.

	Capsule quantitatively	Capsule qualitatively	Interface qualitatively	Thickness of reactive layer	Neovascu- larization	Necrosis	Cytotoxic effect on the peripheral tissue	Macrophages
1 day	2 (0-3)	2 (1-4)	2 (1-4)	2 (1-3)	2 (0-3)	3 (0-3)	3 (0-3)	0
1 week	0 (0-4)	3 (2-4)	4 (1-4)	3.5 (2-4)	3 (2-4)	1 (0-3)	3 (0-3)	2 (0-3)
4 weeks	1.5 (0-4)	3.5 (2-4)	4 (1-4)	4 (2-4)	3.9 (1-3)	0	1.5 (0-3)	3 (0-4)
12 weeks	3.5 (0-4)	2.5 (1-4)	4 (2-4)	3 (2-4)	2.5 (1-4)	0	0	1 (0-3)
26 weeks	4 (0-4)	4 (3-4)	4	4 (3-4)	2	0 (0-3)	0	3 (2-3)

Table S2. Histological evaluation scores of rat skin tissue samples after subcutaneous injection of the reactive isocyanate-terminated hyper-branched copolymeric tissue adhesives. The data are presented as median values with the observed range in parentheses.

	Capsule quantitatively	Capsule qualitatively	Interface qualitatively	Thickness of reactive layer	Neovascularization	Necrosis	Cytotoxic effect on the peripheral tissue	Macrophages
1 day	3 (2-4)	2 (1-3)	1.5 (0-5)	2 (1-4)	2 (0-2)	1.5 (0-3)	3 (0-3)	0 (0-1)
1 week	0	4 (2-4)	4 (2-4)	4 (3-4)	3 (2-4)	0 (0-3)	3 (0-3)	2.5 (0-3)
4 weeks	4 (2-4)	1.5 (1-3)	3.5 (1-4)	3 (1-4)	2.5 (1-3)	0	0	2.5 (0-3)
12 weeks	4 (0-4)	3 (1-4)	3.5 (1-4)	3.5 (1-4)	3 (0-3)	0	0	1 (0-3)
26 weeks	4 (0-4)	3 (1-4)	3.5 (1-4)	3.5 (1-4)	2 (1-3)	0 (0-2)	0	1.5 (1-3)

Chapter 9

Development of a Fast Curing Tissue Adhesive for Meniscus Tear Repair

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Abstract

Isocyanate-terminated adhesive amphiphilic block copolymers are attractive materials to treat meniscus tears due to their tuneable mechanical properties and good adhesive characteristics. However, a drawback of this class of materials is their relatively long curing time. In this study we evaluate the use of an amine cross-linker and addition of catalysts as two strategies to accelerate the curing rates of a recently developed biodegradable reactive isocyanate-terminated hyper-branched adhesive block copolymer prepared from polyethylene glycol (PEG), trimethylene carbonate, citric acid and hexamethylene diisocyanate.

The curing kinetics of the hyper-branched adhesive alone and in combination with different concentrations of spermidine solutions, and after addition of 2,2-dimorpholinodiethylether (DMDEE) or 1,4-diazabicyclo [2.2.2] octane (DABCO) were determined using FTIR. Additionally, lap-shear adhesion tests using all compositions at various time points were performed. The two most promising compositions of the fast curing adhesives were evaluated in a meniscus bucket handle lesion model and their performance was compared with that of fibrin glue. The results showed that addition of both spermidine and catalysts to the adhesive copolymer can accelerate the curing rate and that firm adhesion can already be achieved after 2 hours. The adhesive strength to meniscus tissue of 3.2-3.7 N was considerably higher for the newly developed compositions than for fibrin glue (0.3 N).

The proposed combination of an adhesive component and a cross-linking component or catalyst is a promising way to accelerate curing rates of isocyanate-terminated tissue adhesives.

Introduction

Traumatic meniscal tears are common injuries of the knee. They result in pain, swelling and locking of the knee joint, and may eventually result in the development of osteoarthritis [1]. Bucket handle tears are among the most frequently occurring types of tears resulting from the injury [2, 3]. These are extended, longitudinal tears

that are not likely to heal spontaneously due to the displacement of part of the torn tissue.

Tissue adhesives are attractive biomaterials to be used for treating meniscus tears [4]. They are already employed in clinical practice for various applications. Cyanoacrylates are used clinically for gluing skin lacerations, fibrin glue for pulmonary leaks and repair of cardiovascular defects, and TissueGlu® for abdominoplasty [5-7]. However, these materials are not suitable for the repair of meniscal tears either due to their cytotoxicity or their insufficient mechanical- and adhesive properties. Therefore, there is a growing interest in the development of new tissue adhesives, which are strong, non-toxic, biodegradable and fast-curing.

Isocyanate-terminated polymers are of particular interest, since they have easily tuneable mechanical properties, they attach to the tissue via formation of strong covalent bonds and degrade to non-toxic products [4]. However, a drawback of this class of materials is the relatively long curing time: it can take up to several hours before the adhesive reaches maximum bonding strength. Few strategies have been employed to speed up the curing rates of isocyanate-terminated polymers. Sternberg et al. engaged the use of polymers such as hyaluronic acid, gelatin, chitosan acetate, and chitosan chloride, which were mixed with the isocyanate-terminated adhesive component prior to its application [8, 9]. Another strategy was chosen by Beckman et al. and Smith et al. who added catalysts such as 2,2-dimorpholinodiethylether (DMDEE) and 1,4-diazabicyclo [2.2.2] octane (DABCO) to isocyanate-terminated adhesive compounds [10, 11]. They reported that attachment to the tissue can already be achieved after several minutes.

We have recently developed a series of isocyanate-terminated amphiphilic block copolymers for the repair of meniscal tears [12, 13]. These adhesive materials have satisfactory adhesion strengths to meniscus tissue, adequate mechanical properties of the networks after curing, in addition they are biodegradable and compatible with meniscus cells and tissue when used in physiological amounts. However, it takes between 8 to 24 hours for these materials to completely cure. This may cause leakage into surrounding tissues and limit their potential clinical application.

The aim of the current study was to develop a fast curing tissue adhesive (glue) based on a previously developed hyper-branched adhesive block copolymer [13, 14]. We describe two ways of accelerating the curing rate of this isocyanate-terminated copolymeric glue. One strategy was to employ spermidine, a polyamine that acts as a cross-linker. The other strategy was based on the addition of a catalyst to the isocyanate-terminated copolymer, which will accelerate the curing process in the presence of surrounding body fluids. The resulting adhesive compositions were evaluated in terms of their curing rates, and their adhesion strengths to meniscus tissue.

Materials and Methods

Adhesive components

The reactive isocyanate-terminated hyper-branched adhesive block copolymer was synthesized as previously reported in detail [13]. The adhesive was prepared from polyethylene glycol (PEG, $M_n=400\text{g/mol}$), trimethylene carbonate (TMC), citric acid (CA) and hexamethylene diisocyanate (HDI). The final product was labeled CA-4PEG-(TMC₂)₂-HDI. After each synthesis step, the chemical structures of the intermediate reaction products were confirmed by ¹H NMR spectroscopy (Bruker 400MHz NMR spectrometer) using CDCl₃ as the solvent.

Two-component adhesive with cross-linkers

The two-component adhesive (glue) was composed of the reactive isocyanate-terminated hyper-branched block copolymer (adhesive component) and a cross-linking component. The cross-linking component was a mixture of spermidine (Sigma Aldrich, the Netherlands) and a linear copolymer HO-TMC₂-PEG₄₀₀-TMC₂-OH, which was prepared as described previously [12]. The glue was applied to the tissue with a double-chamber syringe (Sulzer, Switzerland) in which the volume ratio of the adhesive component to the crosslinking component was 4:1 (see Fig. 1a). The amount of spermidine in the cross-linking solution was adjusted so that the ratio of the isocyanate groups of the adhesive component to the amine groups in the cross-linking component was 5:1 (spermidine 5:1) or 10:1 (spermidine 10:1) (assuming that the degree of functionalization of the hyper-branched block copolymer with

HDI was 100%). The molar ratio of HO-TMC₂-PEG₄₀₀-TMC₂-OH to spermidine in the cross-linking component was 2.14 for spermidine 5:1, and 4.65 for spermidine 10:1.

Catalysed single-component adhesive

The effect of adding a catalyst to the adhesive component on the curing and the adhesive properties of the cured network was evaluated. The adhesive component (which is a viscous liquid) was placed in a round bottom flask at room temperature (RT) and kept under argon. Subsequently, 2,2-dimorpholinodiethylether (DMDEE, Sigma Aldrich, the Netherlands) or 1,4-diazabicyclo [2.2.2] octane (DABCO, Sigma Aldrich, the Netherlands) was added and the mixture was stirred for 2 hours to activate the terminal isocyanate groups of the adhesive component. The amount of added DMDEE was 0.1 wt% of the adhesive component, while the amount of DABCO was equal to 0.034 of the number of isocyanate groups of the adhesive component (assuming 100% degree of functionalization with HDI). The mixtures of the adhesive component and the catalysts were applied using a conventional 2 mL syringe equipped with a 23G needle (Fig. 1b).

Figure 1. Schematic drawing of the syringe systems used to apply the fast curing adhesives. a) Double-chamber syringe equipped with a static mixer where the adhesive component and the cross-linking component are mixed during their application, b) Conventional syringe used to apply a mixture of the adhesive component and a catalyst.

Curing kinetics of the adhesives

The curing kinetics of the hyper-branched adhesive component and after mixing with a cross-linking component or a catalyst were determined by FTIR using a Perkin Elmer Spectrum Two Spectrometer. As the free isocyanate groups react to form a network, the peaks at 2255 cm^{-1} disappear. A drop of the adhesive composition ($n=1$) was placed on the prism and covered with demineralized water, the spectra were recorded after 0, 5, 15 and 30 minutes, and after 1, 2, 4, 8 and 24 hours. The conversion of isocyanate groups was calculated from the areas of the peaks relative to the peak area at $t=0$.

Lap-shear adhesion strength of the adhesive compositions to meniscus tissue

Lap-shear adhesion testing of the adhesives to meniscus tissue was also performed at different curing times. Bovine lateral menisci from a local slaughterhouse were frozen and cut with a cryotome (Microm GmbH, Germany) into strips measuring approximately $0.5 \times 10 \times 25\text{ mm}$ parallel to the direction of alignment of the circumferential collagen fibers. The tissue strips were thawed to RT and kept hydrated in water until the gluing experiment. The surfaces of the tissue strips were blotted dry, and the different adhesives were applied to the strips of tissue. The adhesives covered an area of approximately 100 mm^2 . The strips of tissue were then pressed together and immersed in water. The strength of the adhesive bond was measured in a lap shear adhesion test using a Zwick Z020 universal tensile tester in analogy to ASTM F2255-05. The grip to grip separation was 22 mm and the crosshead speed was 50 mm/min. The measurement was performed after 30 minutes and after 1, 2, 4, 8 and 24 hours of applying the glues ($n=3$). The shear adhesive bond strength (S , expressed in kPa) was determined from the maximum shear force divided by the glued area.

Evaluation of the adhesive strength of the adhesives in a meniscus bucket-handle tear model

The adhesive strength of the glues was evaluated in a simulated meniscus bucket-handle tear. Fresh frozen bovine menisci were thawed in water and the outlines of the bucket-handle tears were drawn along the central part of the menisci ending 1

cm from posterior and anterior horns. Subsequently, the menisci were mounted in a custom-made clamp using needles (see Fig. 2a) and placed in a BOSE[®] Electroforce[®] setup equipped with a 200N load cell (BOSE Bose Corp. ElectroForce Systems Group, MN, USA). The outer part of the meniscus was fixed using three needles and the inner part was fixed with a single needle. Then, bucket-handle tears were cut along the drawn outline with a scalpel. The inner part of the meniscus was pulled down at a rate of 5 mm/min to pre-condition the tissue (10 repetitions). The maximum displacement that could be applied with this setup was 6 mm.

The two outer needles fixing the upper (outer) part of the meniscus were removed, the meniscus was now fixated with only one needle holding the upper (outer) part and one needle holding the bottom (inner) part (Fig. 2b). The tissue was then again pre-conditioned by opening and closing the tear (5 repetitions). The meniscal tear was opened again and the tear surface was rasped using a surgical rasp (diamond rasp 45°, Acufex, Smith & Nephew). The different adhesive compositions (100µL) were applied to the surface of the tears using the syringe systems pictured in Fig. 1. In this setup, the performance of the hyper-branched adhesive, the hyper-branched adhesive mixed with spermidine 5:1 and the hyper-branched adhesive containing DABCO was evaluated (n=5). As a control, the performance of fibrin glue (a mixture of fibrinogen (100 mg/mL) and thrombin (100 U/mL) in PBS (pH=7.4), both isolated from bovine plasma, Sigma Aldrich, the Netherlands) was also evaluated (n=3). An experiment in which no adhesive was applied was also performed (n=1). After application of the adhesives, the tear was closed and the meniscus was wrapped with a moist tissue and left to cure at 37 °C.

After approximately 18 hours (overnight), bucket-handle tear of the meniscus was opened to evaluate the adhesive strength of the glues. The hyper-branched adhesive component needed longer curing time than other compositions, therefore to have the same protocol during the experiments, the adhesion of all adhesives was tested after overnight curing. Force-displacement curves at 5 mm/min were recorded. After the glue failed, a second force-displacement curve was determined. This curve was used as a baseline for the non-glued meniscus (n=5). The force-displacement curve for a cut meniscus to which no glue was applied (n=1) was also obtained.

The maximum adhesive strength of the glues (the maximum force before failure of the adhesive bond) and corresponding displacement were determined. Additionally, the energy required to open the glued meniscus tears was determined from the area under the force-displacement curve up to the force at failure and the corresponding displacement. The energy to rupture the adhesive bond between the glue and the tissue was calculated as the difference between the energy required to open the glued tear and the area under the baseline curve up to the force at failure and the corresponding displacement (Matlab, The MathWorks, USA).

Results

Analysis by ^1H -NMR after each synthesis step and FTIR confirmed that the adhesive hyper-branched block copolymers were successfully synthesized [12, 13].

Curing kinetics of the adhesive compositions

The curing kinetics of the hyper-branched adhesive component and those of the hyper-branched adhesive component after mixing with a cross-linking component and a catalyst were determined by FTIR spectroscopy. The disappearance of the peak corresponding to free NCO groups was followed in time, and the conversion of free isocyanate groups upon curing with water was determined (see Fig. 3).

In the hyper-branched adhesive component, 76.3% and 23.3% unreacted isocyanate groups remained after 4 and 8 hours reaction with water, respectively. After 24 hours, all isocyanate groups had reacted. By mixing the hyper-branched adhesive with the cross-linking compositions, the rate of curing accelerated significantly: after 4 hours the remaining unreacted isocyanate groups were 32.5% and 20.1% for the compositions with spermidine 1:10 and spermidine 1:5, respectively. After 8 hours these values were 3.5% and 6.7%, respectively. For all compositions, all isocyanate groups had reacted after 24 hours.

Figure 2. Fixation of a bovine lateral meniscus tissue in a custom-made clamp. a) Meniscus with a drawn bucket-handle tear outline mounted in the clamp using 4 needles, b) The two outer needles holding the upper (outer) part of the meniscus were removed, and the bucket-handle tear was opened to allow application of the glues.

When the DMDEE catalyst was added to the hyper-branched adhesive component, its curing rate was similar to that after adding the cross-linking composition. The remaining unreacted isocyanate groups were 20.6% and 0.4% after 4 and 8 hours, respectively. Addition of DABCO to the hyper-branched adhesive component resulted in a much faster curing rate. Already after 2 hours the amount of remaining isocyanate groups was only 5.5%, while after 8 hours all isocyanate groups had reacted.

Figure 3. Changes in the amount of unreacted isocyanate groups determined by FTIR during curing by reaction with water of a) the hyper-branched adhesive component to which cross-linking compositions were added and b) the hyper-branched adhesive component to which catalysts were added.

Lap-shear adhesion strength of the adhesive compositions to meniscus tissue

The shear adhesion strength of the hyper-branched adhesive component and that of the hyper-branched adhesive component after addition of the cross-linking component and the catalysts was determined at different curing times (Fig. 4). The adhesive strength of the hyper-branched adhesive was already close to its maximum value after 8 hours (62.6 ± 56.4 kPa) and reached 65.7 ± 5.2 kPa after 24 hours. After mixing the hyper-branched adhesive component with spermidine 5:1, the determined adhesive strength was 48.7 ± 23.2 kPa and 99.9 ± 57.7 kPa after 8 and 24 hours, respectively. The addition of the spermidine 10:1 resulted in a lower ultimate adhesive strength of 15.3 ± 9.3 kPa after 24 hours.

When the DMDEE catalyst was added to the hyper-branched adhesive, the adhesive strength was relatively low initially; after 4 and 8 hours values of 10.9 ± 2.3 kPa and 11.5 ± 12.8 kPa, respectively, were determined. However, after 24 hours, the adhesive strength reached 61.5 ± 46.9 kPa. Addition of the DABCO catalyst to the reactive hyper-branched oligomer resulted in already reaching relatively high adhesive strengths after 2 hours of application (50.5 ± 14.9 kPa), ultimately reaching 64.4 ± 14.3 kPa after 24 hours.

Figure 4. Evolution of the shear-adhesion strength of the different adhesive compositions to meniscus tissue during curing by reaction with water of a) the hyper-branched adhesive component to which cross-linking components were added and b) the hyper-branched adhesive component to which catalysts were added. The data is presented as mean $\pm$ standard deviations.

Adhesion in a bucket-handle meniscal tear model

The adhesive properties of the hyper-branched adhesive component, and the composition after mixing with spermidine 5:1 and DABCO were evaluated in a meniscal bucket-handle tear model. The average length of the tear was 45.0 ± 3.8 mm and the average tear surface area was 353.4 ± 53.3 mm². A characteristic example of the recorded force-displacement curves is shown in Fig. 5.

The maximum adhesive strength of the adhesive component and that of the adhesive to which cross-linking composition and a catalyst were added was between 3.2 ± 0.47 N for the composition with DABCO, and up to 3.7 ± 0.96 N and 3.7 ± 1.5 N, respectively, for the hyper-branched adhesive component alone and the hyper-branched adhesive component to which spermidine 5:1 was added (Table 1). The displacement at which the maximum force was reached was between 1.6 ± 0.4 mm for the composition with spermidine 5:1, and up to 2.8 ± 0.6 mm for the composition with DABCO. It was not possible to determine a maximum adhesive strength of the fibrin glue, as its force-displacement curve was similar to that of a non-glued torn meniscus and it did not show a clear maximum. Thus the highest force recorded during

opening the bucket-handle tear glued with fibrin glue was the force of 0.3 ± 0.04 N reached at the maximum displacement of 6 mm. A single meniscus with a simulated tear, but without application of glue was tested as well. It was found that the force-displacement curve was very similar to the baselines determined after failure of the glues in the glued menisci.

The energy necessary to open a glued meniscus tear was determined from the area under the force-displacement curves of the glued menisci. The highest values were determined for the hyper-branched adhesive component (11.0 ± 5.2 mJ). The energies calculated for compositions with spermidine 5:1 and DABCO were slightly lower: 5.5 ± 3.7 mJ and 10.3 ± 2.1 mJ, respectively. For all compositions, the required energy to open the glued tear was pointedly higher than that for the fibrin glue and the non-glued control.

By subtracting the areas under the force-displacement curves of the baseline curves from the force-displacement curves of the glued tears, the energy required to break the adhesive bond between the glue and meniscus could be determined (Fig. 5). The highest values were determined for the hyper-branched adhesive component (6.1 ± 3.5 mJ), followed by the values for the composition with DABCO (4.8 ± 1.1 mJ) and spermidine 5:1 (3.2 ± 2.1 mJ). For the fibrin glue this energy was determined to be 0, as its force-displacement curve overlapped the baseline curve.

Figure 5. Characteristic force-displacement curves obtained during evaluation of the adhesive strength of the adhesives in a bucket-handle meniscus tear model. The figure shows the behaviour of the reactive hyper-branched adhesive component during opening of the glued tear and that of the reference baseline curve obtained after the adhesive failed.

Discussion

Two methods to accelerate the curing rates of isocyanate-terminated hyper-branched copolymers were evaluated. In one of the employed strategies a two-component adhesive formulation consisting of a hyper-branched adhesive component and a cross-linking mixture with an amine group-containing compound was used. It is known that amine groups have up to 50 times higher reactivity towards isocyanate-groups than hydroxyl groups, and using such composition will allow the glue to cure within significantly shorter times [8, 9, 15]. The adhesive was applied using a double-chamber syringe equipped with a static mixer. In this way, the two components can be mixed immediately before application on the tissue. For this to occur efficiently, similar viscosities of the components are required. As it is also necessary to maintain the correct (excess) stoichiometric balance between isocyanate groups and amine groups, the spermidine cross-linker was diluted with a

low molecular weight hydroxyl group-terminated HO-TMC₂-PEG₄₀₀-TMC₂-OH block copolymer. In this manner correct viscosities and ratios of isocyanate groups to amine groups were obtained.

Table 1. Adhesion testing of different compositions of the reactive isocyanate-terminated hyper-branched adhesives using a bucket-handle meniscus tear model.

Glue	Maximum adhesive force [N]	Displacement at maximum force [mm]	Energy required to open glued tear [mJ]	Energy required to detach adhesive [mJ]
Hyper-branched adhesive	3.7±0.97	2.4±0.96	11.0±5.2	6.1±3.5
Hyper-branched adhesive + spermidine 5:1	3.7±1.5	1.6±0.4	5.5±3.7	3.2±2.1
Hyper-branched adhesive + DABCO	3.2±0.47	2.8±0.6	10.3±2.1	4.8±1.1
Fibrin glue	0.3±0.04*	6.0*	1.6±0.1**	0

* The value is the force at the maximum displacement of 6 mm, a maximum force was not reached.

** These values are the areas under the curve for displacement from 0 to the maximum displacement of 6 mm.

A second strategy to accelerate the curing rate of the adhesive component was the use of catalysts DMDEE or DABCO. These catalysts have been shown to significantly increase the curing rates of isocyanate-terminated adhesives [10, 11].

Both the addition of a cross-linking component and catalysts accelerated the curing rates of the hyper-branched adhesive component (Fig. 3). The curing rate was most increased when DABCO was added to the hyper-branched adhesive, after only 2 hours nearly 95% of the isocyanate groups had already reacted to form a polyurethane network. Formulating the reactive hyper-branched adhesive with a cross-linking component resulted in slightly lower curing rates. However, the extruded reacting mixture could be precisely placed on the tissue as a result of its high viscosity. Once placed, it did not flow and would probably not leak into the surrounding tissues when used in a clinical application.

The values of the adhesive strength of the adhesive component and those of the mixture of the adhesive component with spermidine 5:1 determined in lap shear tests were similar. However, when the reactive hyper-branched adhesive was mixed with spermidine 10:1, the resulting ultimate adhesive strength was lower. It seems that in the latter case the diluent concentration was too high and hampered the adhesion process. When catalysts were added to the adhesive component, the ultimate tensile strengths of the different cured compositions were similar. This indicates that the faster network formation did not hinder the attachment of the adhesives to the tissue, and that the number of remaining unreacted isocyanate groups available was sufficient to allow adhesion of the curing glues to the tissue.

The adhesive composition to which spermidine 5:1 had been added, and the adhesive component mixed with DABCO showed the most promising results in lap-shear adhesion testing and their curing times were considerably shorter than for the adhesive component alone. Thus, these two compositions were further evaluated as adhesive formulations in the bucket-handle meniscus tear model, as bucket handle tears are among the most commonly occurring types of traumatic meniscus injuries. They are of great clinical relevance, since they result in locking of the knee joint [3]. The performance of the two new adhesive compositions was compared with that of the hyper-branched adhesive component only and fibrin glue. The torn meniscus was preconditioned, glued, kept moist and left overnight to allow the glues cure, after which the adhesive test was performed.

It was observed that during the several hours the meniscus was mounted in the clamp, its mechanical properties had changed and the force needed to open the tear decreased (data not shown). This was probably due to relaxation, which is well-known to occur in biological tissues [16-18]. Also, even though the tissue was wrapped with a moist fabric, it could have been not sufficiently hydrated [19, 20]. Therefore, to be able to assess the effect of gluing the meniscus tears with the different adhesive compositions, comparisons were made with 'baseline' force-displacement curves obtained directly after testing the glued menisci to failure. We showed in a single experiment that a meniscus in which a tear was cut (but not glued) and evaluated under the same conditions, displayed similar mechanical properties as

those that had been glued and tested to failure. This implies that application of the glues did not affect the mechanical characteristics of the meniscus tissue.

The force necessary to rupture the adhesive bond between the glue and the meniscus and open the bucket-handle tear for the isocyanate-terminated hyper-branched component alone was similar to that after mixing with spermidine 5:1 or DABCO. The determined forces were considerably higher than when fibrin glue was employed. Apparently, mixing-in of a cross-linking composition and catalyst to the adhesive do not substantially hamper the adhesive strength of the cured network.

The adhesive strength of the adhesive compositions (3.2-3.7 N) should be sufficient to hold the torn meniscus together during the period of healing, as it has been reported that in a cadaver knee model the distraction forces acting on a torn meniscus do not exceed 5-10 N when a load of 300N is applied [21, 22]. During the recovery period after surgery, the load on the knee would be even lower. From this it follows that the developed adhesive compositions should be able to perform adequately in the clinic. The simulated lesion was made with a scalpel and even though a mechanical rasp was used to refresh the wound and expose collagen fibers as done in clinical practice [23, 24], a meniscus tear that occurs spontaneously will be much rougher and more fibrillated. In gluing such a tear, better attachment of the adhesive due to larger surface areas and more extensive mechanical interlocking can be expected [25].

The energy required to open the glued meniscus tears was pointedly higher for the developed adhesive formulations than for fibrin glue and the control. The force-displacement curves indicated that these adhesives did not fail immediately when forces close to the maximum values were reached, but were able to maintain an adhesive bond for several more millimeters of applied displacement. The force-displacement curve of the meniscus tear model in which fibrin glue was used was identical to that of the baseline, indicating that fibrin glue had no effect in keeping the edges of meniscus tears together.

Conclusions

New, rapidly curing adhesive systems composed of a reactive isocyanate-terminated hyper-branched adhesive component and a cross-linking composition or catalyst are promising biomaterials for the repair of meniscal tears. The addition of a cross-linking composition or a catalyst to the hyper-branched adhesive component resulted in considerably accelerated curing rates, without notably compromising the adhesive properties to the tissue. The adhesive strengths evaluated in a meniscus bucket-handle lesion model were found to be much higher than that of fibrin glue. Further studies are needed to confirm the biocompatibility of these systems with cells and tissues *in vitro* and *in vivo*.

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Summary

The aim of the work described in this thesis was to develop biocompatible and biodegradable tissue adhesives for the repair of meniscus tears. The chosen strategy was to use isocyanate-terminated three-armed and hyper-branched block copolymers. The building blocks of these reactive copolymers were trimethylolpropane ethoxylate (TMPE), trimethylene carbonate (TMC) and hexamethylene diisocyanate (HDI), and polyethylene glycol (PEG), TMC, citric acid (CA) and HDI, respectively.

The aim and general structure of the thesis were described in **Chapter 1**

In **Chapter 2** the anatomy, physiology and biomechanics of the meniscus were discussed. Current repair techniques were reviewed and requirements for tissue adhesive materials were formulated. The ideal candidate for a tissue adhesive has to fulfil several general requirements, *i.e.* it has to be biocompatible, allow easy application and has to be biodegradable. Moreover, the tissue adhesive material should have specific properties, such as an adequate tensile strength, elastic modulus and degradation rate, in accordance with the tissue of interest. In **Chapter 2** it was defined that the ideal adhesive for meniscus repair has to withstand stresses in the knee of at least 50 kPa. It also should have tensile properties close to those of the human meniscus in circumferential direction, as this is the direction in which the majority of the meniscus tears occur. The material should hold the torn tissue together during the period of healing, and then degrade into non-toxic low molecular weight products that can be excreted by the kidneys. Polymer-based materials have been found to be promising candidates to be used as tissue adhesives, as the properties of these well-defined materials are easily tuneable.

In **Chapter 3** a first study on the development of amphiphilic block copolymeric adhesives was described. Three-armed star-shaped isocyanate-terminated block copolymers based on TMPE and TMC varying in composition were designed and synthesized. The characteristics of these networks were compared with those prepared from analogous linear structures based on PEG and TMC. Tensile testing showed very good mechanical properties of the cured copolymers. These properties were found to depend on the length of the PTMC chains in the structure. In lap-shear adhesion tests, very good bonding strengths to chamois leather, which was used as

a tissue model, of up to 600 kPa were obtained. These strengths were much higher than those of analogous linear structures. The obtained results indicate that branching can enhance both the mechanical and the adhesive properties of these glues.

Chapter 4 addressed the problem of choosing right tissue models when performing adhesion experiments using newly developed tissue adhesives. The adhesive properties of three different tissue adhesives, namely Dermabond[®], fibrin glue, and the newly developed three-armed isocyanate-terminated adhesive, were evaluated using five different biological substrates: bovine Achilles tendon, meniscus tissue, skeletal muscle, chamois leather and collagen films. It was found that the strength of the adhesive bond depends both on the used glue as well as on the surface roughness of the substrates, their hydrophilicity and biochemical composition. It was concluded that standardized procedures and models should be developed to allow direct comparison between different tissue adhesives, and to enable extrapolation of the results towards the specific targeted human tissues.

Chapter 5 presented a series of amphiphilic hyper-branched copolymers based on PEG (molecular mass $M_n=200$ g/mol or $M_n=600$ g/mol), TMC, CA and end-functionalized with HDI. The adhesive strength of the networks to bovine meniscus tissue determined in a lap-shear adhesion test ranged between 20 - 80 kPa, which was significantly higher than the adhesion strength of fibrin glue. The mechanical properties of the copolymeric networks were found to be highly dependent on the molecular mass of PEG in the molecule. The elastic modulus of the networks containing PEG₂₀₀ was determined to be in the same range as that of the human meniscus in circumferential direction. The degradation rate of the networks was also dependent on the molecular mass of PEG, and was found to be significantly faster for the networks based on PEG₆₀₀. After incubation with different amounts of the glues for 7 days, the viability of bovine meniscus cells was not affected when physiologically relevant amounts of the adhesives were added to the medium.

The networks based on the PEG₂₀₀ had mechanical properties close to the values of human meniscus, but their degradation rate was low. The networks based on PEG₆₀₀ had inferior mechanical properties, but faster degradation rates. For further experiments, networks based on PEG₄₀₀ were chosen.

In **Chapter 6** the adhesive properties of- and the tissue response to three-armed- and hyper-branched tissue adhesives were assessed and compared in a meniscus explant model. Additionally, the surface of the tissue was modified using collagenase to assess if it improved the attachment of the adhesives and increased the healing rate of the tissue. Cylindrical explants were glued with the three-armed or hyper-branched adhesives and cultured *in vitro* for 28 days. Their performance was compared with that of fibrin glue and Dermabond[®]. The adhesive strength of the three-armed- and hyper-branched glues was 40-50 kPa, which was significantly higher than that of fibrin glue. Additionally, according to the requirements stated in **Chapter 2**, this adhesive strength was sufficient to be used in the treatment of meniscus tears. The adhesion strength to explants treated with and without collagenase was similar, therefore it was concluded that collagenase treatment did not enhance adhesion. Cells were observed to be in direct contact with the glues, and the tissue remained vital during the whole culture period. Increased cellularity around the tear in the collagenase-treated explants was observed after the first day. However, at day 28, the cellularity around the tear was similar to the group that was not incubated in collagenase. By filling the whole tear gap, the glues might have acted as a barrier and blocked access to the nutrients present in the medium thereby limiting the migration of cells to the tear.

Due to these limitations, it was proposed in **Chapter 7** to use a modified experimental setup which introduced only partial gluing of the explants. The focus of this study was to investigate if tissue surface modification with collagenase and the addition of TGF- β 3 to the culture medium can increase the number of cells present in meniscus tears glued with the three-armed- and hyper-branched adhesives. Histological staining of the repair constructs confirmed that both developed adhesives were not cytotoxic and that meniscus cells were present in direct contact with the glues. The addition of TGF- β 3 to the culture medium resulted in the presence of cells that formed a sheath within the simulated tear. Based on these results, it was concluded that the healing potential of a torn meniscus can possibly be enhanced by using a combined therapy which makes use of tear surface treatment using collagenase, biodegradable glues and the addition of growth factors.

In **Chapter 8** a more detailed characterization of the three-armed- and hyper-branched adhesives *in vitro* and *in vivo* was performed. Their physicochemical and adhesive properties, the degradation profile of the water-cured network films and the tissue reaction after subcutaneous injection of the adhesives in rats were tested. Both developed glues showed to have sufficient adhesive strengths (80-100 kPa) to meniscus tissue after curing. The main mechanism of degradation was found to be hydrolysis and after 26 weeks the networks based on hyper-branched adhesives showed a faster mass loss (approximately 25.5 %) compared to the networks prepared from three-armed glues (approximately 5.5 %). Both glues induced a mild to moderate inflammatory reaction involving macrophages and PMN cells. However, no necrosis or toxic effects on peripheral tissues were observed. As the intended application is treatment of damaged meniscus tissue, it is expected that the application of only a few drops of adhesive will result in a minimal tissue reaction.

Acceleration of the curing rates of the adhesives is desired, as it takes up to 24 hours for the materials to fully cure. This could potentially limit their clinical application. In **Chapter 9** it was proposed to employ an amine cross-linker and to add a catalyst to the adhesive components to increase their curing rate. The curing kinetics of the hyper-branched adhesive alone, the adhesive in combination with different concentrations of spermidine solutions, and after addition of 2,2-dimorpholinodiethylether (DMDEE) or 1,4-diazabicyclo [2.2.2] octane (DABCO) to the adhesive, were determined using FTIR. Additionally, lap-shear adhesion tests were performed after application of the adhesives onto strips of bovine meniscus tissue and to simulated bucket-handle lesions in the bovine meniscus. It was found that addition of both spermidine and catalysts accelerated the curing rate of the adhesive copolymer, resulting in firm adhesion already after 2 hours after application of the glue. The adhesion strength to meniscus tissue determined in a bucket handle tear model (3.2-3.7 N), was considerably higher for the newly developed compositions than for fibrin glue (0.3 N).

The work presented in this thesis describes the development of novel tissue adhesives for the repair of meniscus tears. The developed materials have satisfactory adhesive strengths to meniscus tissue. They attach to the tissue surface via formation of covalent bonds and simultaneously cure in the presence of body fluids. Their

tensile strength, elastic modulus and degradation profile can be tuned by varying the PEG molecular weight, PTMC chain length and the ratio between the particular components in the adhesives. These adhesive materials showed good biocompatibility with meniscus cells and tissue *in vitro*, and a mild to moderate tissue reaction after subcutaneous injection *in vivo*. The obtained results pave the way for further *in vivo* studies in larger animal models to assess the efficacy of these glues in treating meniscal tears.

Samenvatting

Dit proefschrift beschrijft de ontwikkeling van biocompatibele en biodegradeerbare weefsellijmen om meniscusscheuren te repareren. Hierbij werd gekozen voor het gebruik van drie-armige en vertakte copolymeren. De bouwstenen van deze reactieve co-polymeren waren respectievelijk trimethylolpropan ethoxylaate (TMPE), trimethyleen carbonaat (TMC) en hexamethyleen diisocyaanate (HDI) en polyethyleen glycol (PEG), TMC, citroenzuur (CZ) en HDI.

Het doel en de algemene structuur van dit proefschrift zijn beschreven in **Hoofdstuk 1**.

Hoofdstuk 2 beschrijft de anatomie, fysiologie en biomechanica van de meniscus. Er werd een overzicht gegeven van de huidige reparatietechnieken en de eisen voor weefsellijmen werden geformuleerd. De ideale weefsellijm heeft een aantal algemene eigenschappen. Het moet bijvoorbeeld biocompatibel en biodegradeerbaar zijn en het moet gemakkelijk toe te passen zijn. Bovendien moet de weefsellijm specifieke eigenschappen hebben welke overeenkomen met het weefsel waarin het toegepast wordt zoals adequate treksterkte, elasticiteitsmodulus en degradatiesnelheid. In **Hoofdstuk 2** werd vastgesteld dat de ideale lijm voor meniscusreparatie mechanische spanningen van minstens 50 kPa aan moet kunnen. Daarnaast moet de lijm een treksterkte hebben die overeenkomt met de circumferentiele treksterkte van een menselijke meniscus, omdat de meeste meniscus scheuren zich in die richting bevinden. Het materiaal zou het gescheurde weefsel bij elkaar moeten houden gedurende de herstelperiode om vervolgens af te breken in gifvrije producten met een laag moleculair gewicht die uitgescheiden kunnen worden door de nieren. Materialen gebaseerd op polymeren worden gezien als veelbelovende kandidaten voor weefsel lijmen, omdat de eigenschappen van deze materialen gemakkelijk aan te passen zijn.

Hoofdstuk 3 beschrijft een eerste studie met betrekking tot de ontwikkeling van amfifiele blokcopolymerische lijmen. Drie-armige, ster-vormige isocyaanate getermineerde blokcopolymeren gebaseerd op TMPE en TMC met verschillende composities werden ontworpen en gesynthetiseerd. De eigenschappen van deze materialen zijn vergeleken met gelijksoortige lineaire structuren gebaseerd op PEG

en TMC. Uit de trekproeven bleek dat de copolymeren zeer goede mechanische eigenschappen bezaten. Deze eigenschappen waren afhankelijk van de lengte van de PTMC ketens in de structuren. De overlapsafschuivings experimenten lieten zien dat de materialen zeer goed hechtten aan zeemleer met spanningen tot 600 kPa. Zeemleer werd gebruik als weefselmodel. Deze spanningen waren veel hoger dan die van de gelijksoortige lineaire structuren. De resultaten geven een indicatie dat vertakking zowel de mechanische eigenschappen als ook de hecht eigenschappen van deze strcuturen kan verbeteren.

Hoofdstuk 4 behandelt de problemen bij de keuze voor de juiste weefselmodellen bij het testen van de ontwikkelde wefsellijmen. De lijmeigenschappen van Dermabond[®], fibrinelijm en de nieuw ontwikkelde drie-armige isocyanaat getermineerde lijm werden geevalueerd op vijf verschillende weefsels: achillespeesweefsel van een koe, meniscusweefsel, skeletspieren, zeemleer en collagene films. De sterkte van de binding hing af van zowel het type lijm als de ruwheid, hydrophiliciteit en biochemische samenstelling van het substraat. Hieruit is geconcludeerd dat er gestandaardiseerde procedures en modellen ontwikkeld dienen te worden zodat er een directe vergelijking tussen de verschillende lijmen gemaakt kan worden en extrapolatie van de resultaten naar specifieke humane weefsels mogelijk wordt.

Hoofdstuk 5 presenteerde een serie van amfifiele vertakte copolymeren gebaseerd op PEG (moleculaire massa $M_n=200$ g/mol of $M_n=600$ g/mol), TMC, CZ en gefunctionaliseerd met HDI. De lijmsterkte van de netwerken op meniscusweefsel van koeien was tussen de 20 – 80 kPa en werd bepaald met behulp van overlapsafschuivings experimenten. Dit was significant hoger dan de lijmsterkte van fibrinelijm. De mechanische eigenschappen van de copolymeer netwerken bleken zeer afhankelijk van de moleculaire massa van de PEG in het molecuul. De elasticiteitsmodulus van de netwerken met PEG₂₀₀ bleek vergelijkbaar met die van de humane meniscus in circumferentiele richting. Ook de degradatiesnelheid bleek afhankelijk van de moleculaire massa van PEG waarbij de netwerken met PEG₆₀₀ significant sneller degradeerden. In de aanwezigheid van verschillende hoeveelheden lijm, gedurende 7 dagen, bleek dat de levensvatbaarheid van rond

meniscus cellen niet beïnvloed werd door fysiologische hoeveelheden lijm in het medium.

The netwerken gebaseerd op PEG₂₀₀ hadden mechanische eigenschappen die dicht in de buurt zaten van die van de humane meniscus, maar ze degradeerden langzaam. Netwerken gebaseerd op PEG₆₀₀ hadden slechtere mechanische eigenschappen, maar degradeerden sneller. Voor toekomstige experimenten werd daarom gekozen voor netwerken gebaseerd op PEG₄₀₀.

Hoofdstuk 6 beschrijft het onderzoek naar de lijmeigenschappen van de drie-armige en vertakte wefsellijmen en de weefselreactie daarop. Deze werden vergeleken met een model van een meniscus explantaat. Daarnaast was het oppervlak van de weefsels behandeld met collagenase om te bekijken of dit de hechting van de lijmen en de snelheid van weefselgenezing kon verbeteren. Cylindrische explantaten werden gelijmd met drie-armige of vertakte lijmen en vervolgens *in vitro* gekweekt voor 28 dagen. Ze werden vergeleken met fibrinelijm en Dermabond®. De lijmsterkte van de drie-armige en vertakte lijmen was 40-50 kPA. Dit was significant hoger dan die van fibrinelijm. Bovendien is dit sterk genoeg voor gebruik voor meniscusreparatie volgens de richtlijnen die opgesteld werden in **Hoofdstuk 2**. De lijmsterkte van explantaten die met collagenase waren behandeld waren vergelijkbaar. Hierdoor werd geconcludeerd dat behandeling met collagenase geen invloed had op de aanhechting.

Cellen hadden direct contact met de lijmen en het weefsel bleef vitaal gedurende de gehele periode. Bij explantaten die behandeld waren met collagenase was rond de scheur een verhoogde celactiviteit te zien op de eerste dag. Echter, op de 28^e dag was de celactiviteit rond de scheur gelijk voor beide groepen. Door de gehele ruimte van de scheur te vullen met lijm, zouden deze een barriere gevormd kunnen hebben voor voedingsstoffen in het medium en daarmee de celmigratie verhinderd kunnen hebben.

Vanwege deze limitaties werd in **Hoofdstuk 7** voorgesteld om een gemodificeerde experimentele setup te gebruiken waarbij explantaten slechts gedeeltelijk werden gelijmd. Het doel van deze studie was om te onderzoeken om weefsel modificatie met collagenase en de toevoeging van TGF-β3 aan het kweekmedium zou leiden tot

een toename van het aantal cellen in de meniscusscheuren die waren gelijmd met de drie-armige en vertakte lijmen. Histologische kleuring van de constructen toonde aan dat de ontwikkelde lijmen niet giftig waren en dat de meniscuscellen in direct contact waren met de lijmen. De toevoeging van TGF- β 3 aan het kweekmedium zorgde voor cellen in de scheur. Gebaseerd op deze resultaten werd geconcludeerd dat het heelproces van de meniscus verbeterd kan worden door een combinatie van weefselbehandeling met collagenase, bioafbreekbare lijmen en de toevoeging van groeifactoren.

In **Hoofdstuk 8** werd een gedetailleerdere *in vitro* en *in vivo* karakterizatie verricht van de drie-armige en vertakte lijmen. Er werd gekeken naar de fysiochemische- en hechtingseigenschappen, het degradatie profiel van de in water gecrosslinkte netwerken en de weefselreactie na onderhuidse injectie van de lijmen in ratten. Beide ontwikkelde lijmen lieten zien dat ze voldoende lijmsterkte hadden aan meniscus weefsel (80-100 kPa). De degradatie verliep met name via hydrolyse en na 26 weken bleek dat de vertakte netwerken een hoger massaverlies hadden (ongeveer 22.5 %) in vergelijking met de drie-armige netwerken (ongeveer 5.5 %). Beide lijmen hadden een lichte tot gemiddelde ontstekingsreactie tot gevolg welke gekenmerkt was door de aanwezigheid van macrofagen en PMN cellen. Er werden echter geen necrose van, of giftige effecten op het omliggende weefsel geconstateerd. Omdat de behandeling van gescheurd meniscusweefsel de beoogde applicatie is, kan worden verwacht dat het gebruik van een paar druppels lijm slechts zal leiden tot een minimale weefsel reactie.

Omdat het 24 uur duurt voordat de materialen volledig gecrosslinked zijn, is een versnelling van het crosslinkproces gewenst. De klinische toepassingen kunnen hierdoor namelijk gelimiteerd zijn. In **Hoofdstuk 9** werd voorgesteld om een amine-crosslinker te gebruiken en een katalysator toe te voegen om het crosslinkproces te versnellen. Met behulp van FTIR werd de crosslinkkinetiek bepaald van vertakte lijmen, vertakte lijmen in combinatie met verschillende concentraties spermidine en de lijmen met als toevoeging van 2,2-dimorfoline diethylether (DMDEE) of 1,4-diazabicyclo [2.2.2] octaan (DABCO). Daarnaast werden er overlapsafschuivings experimenten uitgevoerd na het aanbrengen van de lijmen op plakjes

meniscusweefsel en gesimuleerde scheuren in de rundmeniscus. De toevoeging van spermidine en een katalysator zorgden beide voor een versnelling van het crosslinkproces van de copolymeren, met volledige crosslinking na 2 uur. De lijmsterktes in het model met de gesimuleerde meniscusscheuren waren aanzienlijk hoger (3.2-3.7 N) voor de nieuw ontwikkelde lijmen in vergelijking met fibrinelijm (0.3 N).

Het werk dat beschreven wordt in deze thesis beschrijft de ontwikkeling van nieuwe weefsellijmen voor de reparatie van meniscusscheuren. De ontwikkelde materialen hebben voldoende lijmsterkte aan meniscusweefsel. Ze binden aan het oppervlak via covalente bindingen en crosslinken tegelijkertijd in de aanwezigheid van lichaamsvocht. De treksterkte, elasticiteitsmodulus en het degradatieprofiel kunnen worden beïnvloed door de moleculaire massa van PEG, PTMC ketenlengte en de verhouding tussen de componenten van de lijm te variëren. Deze lijmen waren compatibel met meniscuscellen en weefsel *in vitro*, en hadden een milde tot gemiddelde ontstekingsreactie tot gevolg *in vivo*. De behaalde resultaten openen de deur naar *in vivo* studies in grotere dieren om de doelmatigheid van deze lijmen bij de behandeling van meniscusscheuren te onderzoeken.

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198

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Aga

List of publications

1. **Bochyńska AI**, Hannink G, Grijpma DW, Buma P. Tissue adhesives for meniscus tear repair: an overview of current advances and prospects for future clinical solutions. *Journal of Materials Science: Materials in Medicine* 2016;27:1-18.
2. **Bochyńska AI**, Van Tienen TG, Hannink G, Buma P, Grijpma DW. Development of biodegradable hyper-branched tissue adhesives for the repair of meniscus tears. *Acta Biomaterialia* 2016;32:1-9.
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5. Wennink JWH, Niederer K, **Bochyńska AI**, Moreira Teixeira LS, Karperien M, Feijen J, et al. Injectable hydrogels by enzymatic co-crosslinking of dextran and hyaluronic acid tyramine conjugates. *Macromolecular Symposia* 2011;309-310:213-21.
6. **Bochyńska AI**, Hannink G, Verhoeven R, Grijpma DW, Buma P. Evaluation of novel biodegradable three-armed- and hyper-branched tissue adhesives in a meniscus explant model. *Manuscript submitted to Journal of Biomedical Materials Research Part A*.
7. **Bochyńska AI**, Hannink G, Verhoeven R, Grijpma DW, Buma P. The effect of tissue surface modification with collagenase and addition of TGF- β 3 on the healing potential of meniscal tears repaired with tissue glues in vitro. *Manuscript submitted to Biomedical Materials*.
8. **Bochyńska AI**, Hannink G, Janssen D, Buma P, Grijpma DW. Development of a fast curing tissue adhesive for meniscus tear repair. *Manuscript submitted to Journal of Materials Science: Materials in Medicine*.
9. **Bochyńska AI**, Hannink G, Rongen JJ, Grijpma DW, Buma P. *In vitro* and *in vivo* characterization of biodegradable reactive isocyanate-terminated three-armed- and hyper-branched block copolymeric tissue adhesives, *in preparation*.
10. **Bochyńska AI**, Hannink G, Buma P, Grijpma DW. Adhesion of tissue adhesives to different biological substrates, *in preparation*.

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