GROWTH FACTOR RELEASING SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING

JÉRÔME SOHIER

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in Dakar, Senegal

Promoters:	prof. dr. C. A. van Blitterswijk
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Assistant promoter:	dr. J. M. Bezemer

The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka!", but

"That's funny..."

Isaac Asimov (1920, 1992)

A Papa, Maman

et Cajinette

This thesis was based on the following publications:

Peer reviewed papers

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M. Cucchiarini, J. Sohier, K. Mitosch, K. G., D. Zurakowski, J.M. Bezemer, D. Kohn, H. Madry. Effect of transforming growth factor-ß1 (TGF-ß1) released from a scaffold on chondrogenesis in an osteochondral defect model in the rabbit. *Central European Journal of Biology,* 2006; 1(1).

J. Sohier, T.J.H. Vlugt, N. Cabrol, C. van Blitterswijk, K. de Groot, J.M. Bezemer. Dual release of proteins from porous polymeric scaffolds. *Journal of Controlled Release,* 2006; 111(1-2): 95-106.

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J. Sohier, K. de Groot, J.M. Bezemer. Evaluation of methods to both incorporate and release proteins from porous scaffolds, *Controlled Release Society*, Glasgow, United Kingdom, 2003.

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Patent

J. Sohier, JM Bezemer, Coating for medical devices, US2005101692

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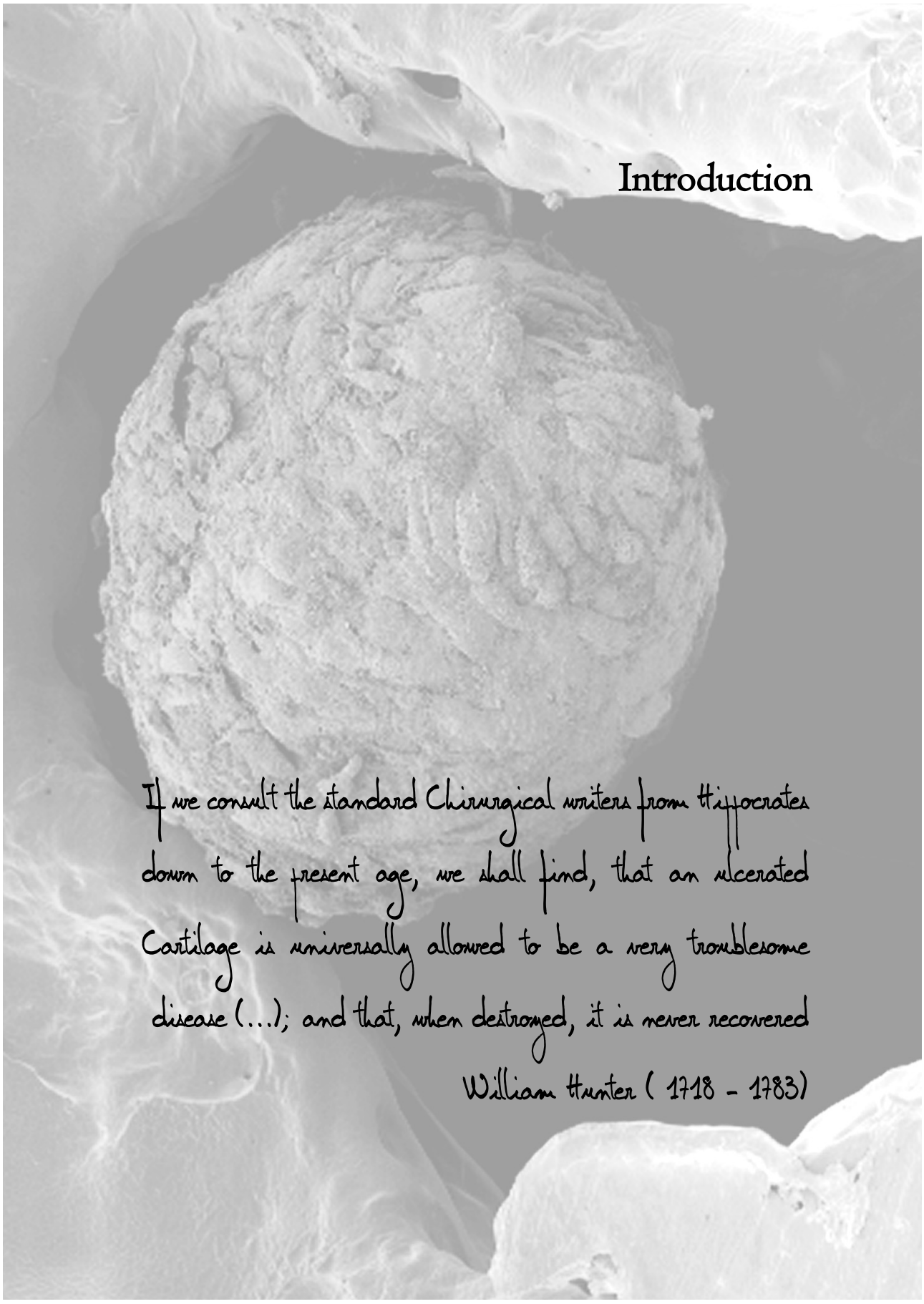


Introduction

If we consult the standard Chirurgical writers from Hippocrates down to the present age, we shall find, that an ulcerated Cartilage is universally allowed to be a very troublesome disease (...); and that, when destroyed, it is never recovered

William Hunter (1718 - 1783)

Chapter I

General introduction

Cartilage, tissue engineering and growth factors

Some 3.8 billion years ago, the first unicellular organisms appeared on earth. Step by step, they multiplied, colonized the oceans and evolved. This slow but nonetheless fruitful evolution led to the emergence of the first vertebrate, the first mammals and finally the first hominids from which we all descend. Along the path of this evolution, our body acquired its current organization and complexity, adapted to our environment.

Over time, Nature has dictated the functions and limitations of each of our organs, giving the ability to repair spontaneously to some of them and to others a limited regenerative capacity. Cartilage is among the latter. This apparent limitation was probably not problematic as long as the longevity of our ancestors was shorter than the functional life span of their cartilage. However, during the last century, the life expectancy of human populations has increased at a pace far too rapid for our body to adapt, and the prevalence of disorders linked to damaged cartilage has increased accordingly.

Articular cartilage is an avascular and non-innerved tissue which has the important functions to assure the freedom of movement of the joints and to bear loads and dissipate stresses. Its smooth and frictionless surfaces combined with viscoelastic properties allow a stable movement of our skeleton [1]. It is therefore a key component of our body which once degenerated by diseases or injuries induces pain and morbidity. Considering this, considerable efforts have been displayed to repair this tissue and restore the capacity of movement using various surgical techniques [2-4]. However, the success of the different therapeutic approaches has been so far limited on the long term and sufferers are still longing for more reliable solutions. Tissue engineering aims to fulfill this need by providing ways not only to repair cartilage but to fully regenerate it. This recent approach proposes to reconstruct or reconstitute tissues both structurally and functionally by combining cells, biomaterials mimicking extracellular matrix (scaffolds) and regulatory signals such as growth factors [5-7]. As illustrated in Figure 1, different strategies can be envisaged within the frame of tissue engineering in which the role of the scaffolds is central.

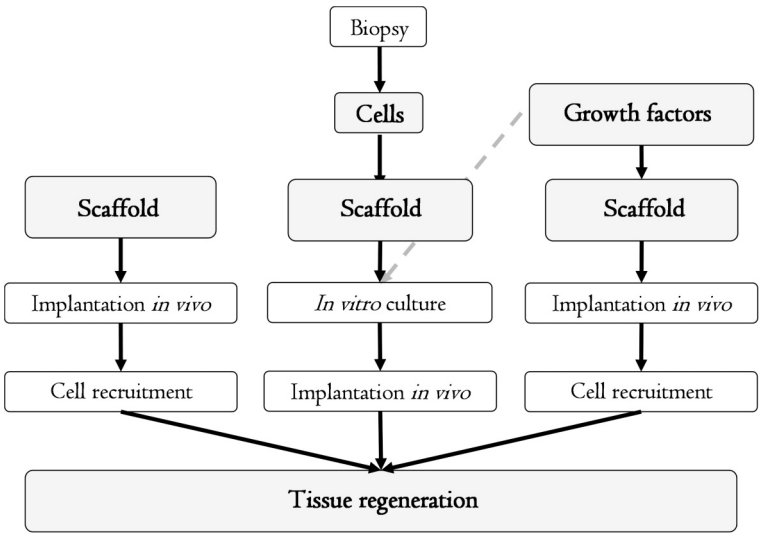


Figure I: schematic representation of the different tissue engineering approaches. Scaffolds can be designed such that they will recruit cell once implanted in the body to regenerate a new tissue. Alternatively, autologuous cells of interest can be isolated from a patient, combined with the scaffold and cultured in vitro to generate a tissue which will be later implanted. Finally, scaffold can be associated with growth factors to help the recruitement differentiation and proliferation of recruited cells after implantation.

Scaffolds provide the three dimensional template in which the newly formed tissue can form and grow. In order to support an adequate cell attachment, proliferation and differentiation it should comply with numerous defined requirements regarding its architecture, chemical, mechanical and surface properties [8-11]. Ideally, a porous scaffold should possess interconnected pores so that cells can migrate and proliferate within the interstices. Its surface should promote cell adhesion or support chondrogenic phenotype. Biocompatibility is important to avoid immunological reactions. Additionally, the material should degrade to be replaced by newly-formed extracellular matrix and its mechanical characteristics should match the surrounding tissue. Although various materials and processing techniques have been investigated that create scaffolds, it is not possible, so far, to combine all these ideal parameters [12]. Therefore, the opportunity of the scaffolds to carry and provide signaling molecules, such as growth factors, to the site of implantation is of utmost interest as it could compensate or potentiate some of the parameters to achieve the desired tissue formation [13,14].
Growth factors are proteins involved in the cellular communication system which modulate cell activity in a concentration and time dependent fashion [15,16]. Hundreds of growth factors have been identified that inhibit or stimulate proliferation, differentiation, migration, or gene expression of various cell types. With regard to cartilage, several growth factors have regulatory effects on cartilage metabolism among which the most investigated are transforming growth factor-β_1 (TGF-β_1), bone morphogenetic proteins (BMPs) and insulin growth factor-I (IGF-I) [17-20]. These molecules play a role in the maintenance of the chondrogenic

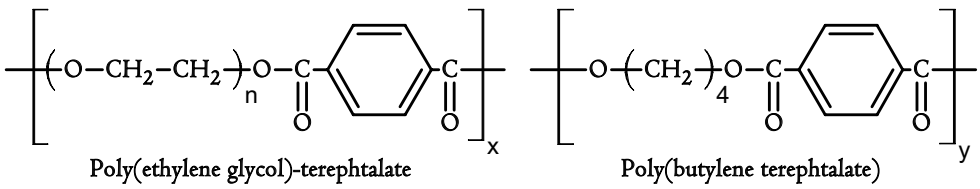
phenotype, the proliferation of chondrocytes and the differentiation of pluripotent progenitor cells towards cartilage. Accordingly, they are promising candidates to be associated with scaffolds to support, induce or enhance the growth and differentiation of different cells types towards the chondrogenic lineage and to orchestrate the cartilage repair.

Growth factor release and scaffolds

To exert their action, each growth factor requires different dosages and length of exposure to the cells. Consequently, they can potentially induce undesired side effects when presented in wrong fashion and if present at systemic levels [21,22]. Therefore, scaffolds associated with growth factors should provide the means to precisely control their doses and supplementation rate at a local level. In addition, growth factors are labile and have a short half-life in the body [23]. These different characteristics and requirements logically lead to the development of controlled release approaches for the delivery of growth factors from scaffolds. By offering a sustained release of the growth factor to the site of implantation, one can expect to induce a longer and more stable tissue response. Optimally, multiple growth factors should be released independently from the scaffold to orchestrate the repair.

Controlled release of drugs and proteins is an important field of research in the pharmaceutical industry. It aims to deliver organic compounds, peptide or proteins at a defined and controlled rate from various matrices to obtain a well defined pharmacokinetic profile in the body. A variety of methods has been investigated to reach this aim, among which the encapsulation of proteins within a polymeric matrix. This promising approach allows to protect the drug from rapid clearance and to provide a sustained release [24]. The continuous release occurs either by diffusion of the drug from the matrix, by degradation of the polymer, or by a combination of the two mechanisms. In general, the requirements of the polymer containing the protein should comply with the following requirements: it should be biocompatible, biodegradable at a defined rate and be non-toxic. In addition, it should provide a safe environment for the encapsulated protein to prevent denaturation and loss of biological activity. Within this frame, the main biodegradable polymers that have been studied for drug delivery are poly(lactic-co-glycolic acid) copolymers (PLGA). Their biocompatibility, tailorable degradation rate and ability to release small peptides has brought them to commercial applications [25]. However, there is concern about their suitability to incorporate and release growth factor in a controlled way. Their bulk degradation leads to the formation of acidic degradation products which lower the pH within the polymeric matrix [26] and might cause denaturation of sensitive proteins such as growth factors. More over, the ability of PLGA to manipulate protein release rate is limited and characterized by an initial burst and often incomplete release [27].

To overcome these drawbacks, other polymers have been investigated among which poly(ether-ester) multiblock copolymers (Figure 2). Theses amphiphilic copolymers composed of repeating blocks of hydrophilic poly(ethylene glycol)-terephtalate (PEGT) and hydrophobic poly(butylene therephtalate) (PBT) offer interesting properties. They have shown to be extensively biocompatible both *in vitro* and *in vivo* [28-31] and reached clinical



aPEGTbPBTc a = PEG MW b = PEGT weight % c = PBT weight %

Figure 2: Chemical structure and nomenclature of PEGT/PBT copolymers.

applications as cement stoppers and bone fillers in orthopedic surgery [32,33]. In addition, they have been used successfully as scaffolds of various shape for cartilage tissue engineering [34,11,35].

One major advantage of these copolymers is that, by varying the PEG molecular weight (MW) and the weight ratio (wt%) of PEGT and PBT blocks it is possible to tailor-make properties such as wettability [36], swelling [37-39], biodegradation rate [39], mechanical properties [34,40] and release rate of embedded proteins [37,41]. PEGT and PBT blocks form different domains in the copolymers in which PEGT is amorphous and PBT mainly crystalline. As a result, increasing PEG MW and wt% of soft amorphous PEGT blocks usually result in copolymers of increasing swelling and of decreasing mechanical properties [37]. Conversely, copolymers of high PBT wt% have lower swelling properties but higher mechanical strength and stiffness. The degradation of PEGT/PBT copolymers occurs via both hydrolysis of the ester bonds (especially between PEG and terephtalate) and oxidation of the ether bonds (scission of the PEG chain in the presence of radicals) [42,39]. Accordingly, PEG MW and wt% allow to manipulate the degradation rate of the copolymers, as higher values result in higher water uptake and more accessible PEGT domains. The release of incorporated proteins is due to a combination of protein diffusion and matrix degradation, which allows zero-order release profiles over long time periods. Due to matrix degradation, the diffusion coefficient of the proteins increases as a function of time and compensate the reduced drug concentration in the matrix. The protein release rate can be controlled by varying the copolymer composition. Increasing PEG MW and wt% result in increasing polymeric network mesh size and degradation rate, which allows an easier diffusion of the protein and faster release rates [37,41]. Moreover, the proteins are prevented form denaturation when entrapped within the amphiphilic matrix [41,43]. A further modulation in degradation rate and protein release profile can be achieved by substituting part or all of the terephtalate groups with succinate blocks during the copolymerization reaction, resulting in PEG(T/S)/PB(T/S) copolymers (Figure 3) [44-46]. Higher amounts of aliphatic succinate result in higher degradation rates of the copolymers due to a more important swelling and to the easier hydrolysis of the aliphatic esters compared to terephtalate (aromatic esters) [44,45]. Similarly to PEGT/PBT copolymers, the release mechanism of embedded proteins is based on a combination of diffusion and matrix degradation and the release rate can be tailored by varying PEG MW and wt%, and the degree of substitution by succinate groups

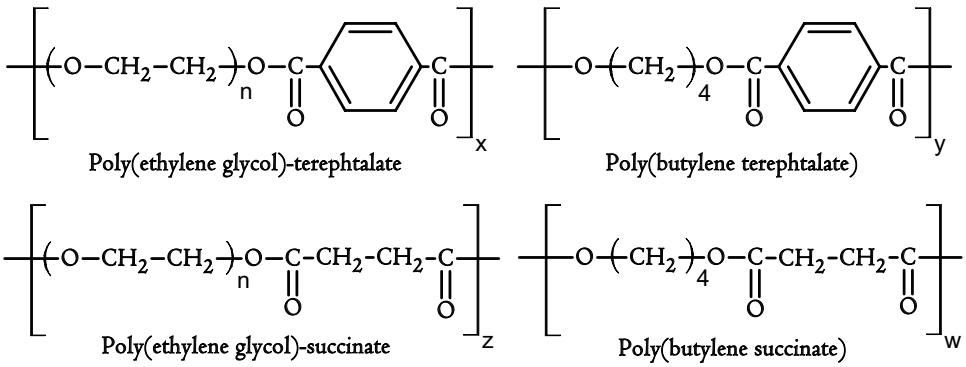


Figure 3: Chemical structure and nomenclature of PEG(T/S)/PB(T/S) copolymers.

[46]. In addition, the higher swelling and degradation rate of the substituted copolymers allows to release proteins of high molecular weight such as bovine serum albumin (67 kD). However, although PEGT/PBT and PEG(T/S)/PB(T/S) copolymers have been demonstrated as successful delivery systems for various proteins, they have never been evaluated and used to release growth factors.

In the context of growth factor release from scaffolds in tissue engineering applications, the requirements of the release system and those of the scaffolds should meet. The most logical approach to reach this goal is to use polymeric scaffolds that encapsulate growth factors and acts as matrix to control their release. However, the current scaffolds processing methods available are not suitable for the incorporation of unstable proteins such as growth factors due to the use of heat, pressure or organic solvents. As a result, the majority of the attempts to release growth factors from scaffold for cartilage engineering has been limited to the adsorption of the signaling proteins on the surface of prefabricated scaffolds [47-49]. This method is not optimal as it offers only a limited control on the growth factor release rates, based on the specific affinity between protein and scaffold surface. There is therefore a need to invent other ways of associating scaffolds and growth factors controlled release.

Aim and outline of the thesis

Although the combination of growth factors with porous scaffolds is appealing, this approach is recent and important knowledge is still missing. A better understanding of the relations between growth factor delivery rate and cartilage regeneration would be an important tool to successfully enhance the success of cartilage tissue engineering with this strategy. Therefore, the aim of this thesis is to investigate the relation between growth factor release

from scaffolds and cartilage regeneration. To achieve this goal the following questions need to be answered:

A - Can proteins be combined with porous scaffolds of defined properties and released in a well controlled way without losing their biological activity ?
The tool to study the influence of growth factor delivery from scaffold on tissue formation is so far missing. Conventional processing techniques to prepare scaffolds are not suitable for protein incorporation and small opportunity to control the release rates. A suitable method should be found.

B - Are PEGT/PBT and PEG(T/S)/PB(T/S) copolymers suitable for the incorporation and controlled release of growth factors ?
Previous investigations on PEGT/PBT and PEG(T/S)/PB(T/S) copolymers as matrix for controlled release application focused on model and therapeutic proteins of relative stability. However, growth factors are highly unstable and labile compounds. Therefore, the suitability of PEGT/PBT copolymers to act as matrix for the release of growth factor should be evaluated.

C - What is the impact of growth factor controlled release on cartilage formation and is a sustained delivery a real benefit ?
Due to the short half life and high potency of growth factors, the idea that their effect would be enhanced by a sustained release comes naturally. However, growth factors regulate cells proliferation and differentiation by complexes mechanism which might not necessarily require long term stimulation. The real impact of a sustained release should therefore be investigated.

D - Can two different proteins be released from a single scaffold in an independent and controlled fashion ?
The physiological process of tissue repair does not involve a single growth factor but many that are present at different doses and time frame to orchestrate healing. A releasing scaffold which follows the same principles might be of benefit to achieve optimal tissue regeneration. Accordingly, the opportunity to prepare scaffolds releasing different proteins in a separate pattern would be interesting.

To answer these questions, PEGT/PBT compression molded/salt leached scaffolds relevant for cartilage tissue engineering [50,35,11] were used as model. TGF-β1 was selected as growth factor considering its positive effects on extracellular matrix synthesis [51-53], chondrocyte proliferation [54,20] and chondrogenic differentiation of progenitor cells [55-57].
The results obtained to the above questions are addressed in this thesis, as follows. Chapter 2 provides an overview of the current research focusing on the release of growth factors from porous scaffolds in cartilage tissue engineering. The concept of scaffold in tissue engineering

is detailed regarding the necessary requirements, the materials available and the processing techniques developed. The potential ways to combine growth factors with scaffolds are discussed with regard to the instability of these proteins and the attempts to deliver growth factors are evaluated. This review as well identifies parameters that should be investigated in the future.

Chapter 3 and 4 introduce two novel methods to incorporate and release proteins from porous scaffolds based on PEGT/PBT copolymers and water-in-oil emulsions techniques. The methods are evaluated with regards to the resulting scaffold structures and mechanical properties, to the ability to release the models proteins in a controlled fashion, and to the stability of these proteins. In chapter 3, an emulsion-coating method is presented in which the inner pores of prefabricated compression molded/salt leached scaffolds are coated with an emulsion containing different model proteins (lysozyme and bovine serum albumin). The mechanisms to control the release of proteins from PEGT/PBT copolymer coated onto scaffolds are compared with reported studies. In chapter 4, a paraffin leached method is investigated with the use of lysozyme-containing emulsion as polymer phase and compared to emulsion-coated scaffolds.

In Chapter 5, the most suitable method is evaluated with TGF-β_1 as loaded growth factor. The ability of PEGT/PBT emulsion coated scaffolds to precisely control the release of the growth factor is presented and its stability discussed. In addition, the potential effect of the released TGF-β_1 is considered with regard to cartilage formation *in vitro*.

Chapter 6 and 7 investigates the relative effect of TGF-β_1 release on cartilage formation in a model system for chondrogenesis *in vitro* and *in vivo*. Chapter 6 presents the influence of different delivery profiles of the growth factor from scaffolds relatively to the cartilage differentiation of pluripotent stem cells. The interest of a sustained delivery is discussed extensively in relation with the intrinsic physiological properties of TGF-β_1. In chapter 7, a scaffold releasing the growth factor in a sustained way is implanted in a rabbit osteochondral defect and the cartilage regeneration examined to evidence a potential improvement of the repair.

Finally, chapter 8 presents a novel approach to associate and release multiple proteins from scaffold in a controlled manner. The release mechanisms taking place in these release systems is thoroughly investigated and modeled to provide efficient tolls to design and control the release of two model proteins (lysozyme and myoglobin).

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Chapter 2

Cartilage, scaffolds and growth factor release: a review

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Introduction

ARTICULAR cartilage has the important functions to assure the freedom of movement of the joints and to bear loads and dissipate stresses. This unique tissue provides smooth and frictionless surfaces which, combined with its viscoelastic properties, allow a stable movement of our skeleton over a lifespan [1]. However, in many cases, articular cartilage degenerates and loses its structure and function, causing pain, loss of motion and morbidity. This is either the result of joint diseases (predominantly osteoarthritis), metabolic and genetic conditions (such as Paget's disease and Stickler syndrome), or traumatic lesions [2]. The problem is aggravated by the fact that impaired cartilage has low capacity to self-repair structural damages resulting from injuries or diseases, as was already reported by William Hunter more than two centuries ago [3].

Considering the high prevalence of articular disorders in societies where the proportion of middle aged and elderly populations increases, surgeons and scientists have displayed considerable efforts to repair or regenerate this tissue [2]. Different surgical techniques have been evaluated and used to relieve patients from pain and restore the capacity of movement [4,5]. However, the success of the different therapeutic approaches has been so far limited and a traumatic complete replacement of the joint by a prosthesis is often the best compromise offered to the sufferers.

Over the last decade, a novel approach bridging biotechnology and materials science has gained interest in view of repairing deficient cartilage more efficiently [6,7]. Tissue engineer-

ing proposes to reconstruct or reconstitute tissues both structurally and functionally by combining cells, biomaterials mimicking extracellular matrix (scaffolds) and regulatory signals [8]. Different approaches have been considered for each of these aspects, for instance regarding the cell source [7,9] (chondrocytes, dedifferentiated chondrocytes or pluripotent mesenchymal stem cells), the scaffolds chemistry and architecture [10,11] or the type of growth factors to be used. The latter is of high importance as growth factors have the potency to support, induce or enhance the growth and differentiation of different cell types towards the chondrogenic lineage and orchestrate the tissue repair. However, each growth factor requires different dosages and delivery rates to the cells *in vitro* or *in vivo*. Therefore, the porous scaffolds should offer the possibility to control the release of one or more growth factors in a defined manner. The aim of this review is to highlight the potential techniques to create scaffolds containing and releasing growth factors and evaluate the relative interest of growth factor release for cartilage tissue engineering.

Cartilage structure and repair

Although the human body contains three types of cartilage (elastic, fibrous and hyaline), most current research involving porous scaffolds and growth factor release is centered on hyaline cartilage. Therefore, hyaline cartilage is presented in more details hereafter.

Hyaline cartilage is the predominant form of cartilage in the body and coats the surface of articulating joints. For this reason, often it is referred to as articular cartilage. The constituents of articular cartilage are water containing gases, small proteins, metabolites, and a high concentration of ions such as Na^+, Ca^{++}, and Cl^- (60-80 weight %); chondrocytes (2 volume %); and extracellular matrix (40-20 weight %) [1,12,13]. The extracellular matrix is mainly composed of collagen fibrils (from which over 90 % is of type II), non-collagenous proteins and proteoglycans. The collagen matrix has a complex anisotropic organization which provides much of the mechanical integrity of cartilage. The proteoglycans are formed by negatively charged glycosaminoglycans polysaccharides (hyaluronic acid, chondroitin sulfate, keratan sulfate and dermatan sulfate) covalently attached to a central protein. The major proteoglycans in the cartilage (90 % of the total proteoglycans) have a large number of chondroitin and keratan sulfate and are called aggrecan. They associate non-covalently with hyaluronic acid of high molecular weight to form large aggregates. Due to the high polarity of the glycosaminoglycans, the proteoglycans interact strongly with water and swell. Water is drawn into the tissue because of the osmotic imbalance caused by their negative charge and mobile counter ions such as Na^+. The hydratation is restricted by the collagen fibrillar network, resulting in a swelling pressure that provides the compressive strength and elastic properties of cartilage [14,15].

Although in low number, the chondrocytes continuously remodel and organize the surrounding matrix in a unique and complex anisotropic structure, as schematically shown in Figure 1. The cartilage can be divided in 4 zones from joint cavity to subchondral bone: superficial, middle, deep and calcified. The cellular organization and density varies between zones. In the superficial zone, the chondrocytes are of high density (24000 cell/mm^3 [13]), flattened and

aligned parallel to the surface. In this zone, the content of aggrecan is at its lowest and collagen fibers of small diameter (20 nm) run tangential to the joint surface, thus providing resistance to the tensile forces generated in the joints. In the middle zone, cells of lower density (10300 cells/mm^3) have the typical morphology of hyaline cartilage. They are rounded and surrounded by a narrow pericellular region of low collagen fibrils content (about 2 μm) [16]. The collagen fibers, of increasing diameter, weave in an oblique fashion and the content of aggrecan augment. The deep zone consists of large and spherical cells clustered in column (chondron, in average of 6-7 cells). The cell density is at its lowest (7700 cells/mm^3) but aggrecan content is maximal. Collagen fibrils of important diameter (120 nm) are oriented in a vertical pattern, perpendicular to the joint surface. A zone of calcified cartilage follows, where chondrocytes are hypertrophic and synthesize type X collagen which can calcify the extracellular matrix. This interface provides excellent integration with the subchondral bone. Articular cartilage contains no vasculature, nerves or lymphatic vessels. Therefore, it must remain relatively thin (2.4 mm in average [13]) to allow sufficient nutrient and waste diffusion. Under loading of the joint, the compression will cause seeping of the fluid from the matrix and redistribution within. After load, cartilage regains its original shape by resorbing the exuded fluid. These exchanges between tissue and synovial fluid allow cell sustenance.
There are two potential mechanism of cartilage repair that rely on the depth of the lesion. Intrinsic repair concerns lesions limited to the cartilage alone, termed partial thickness or

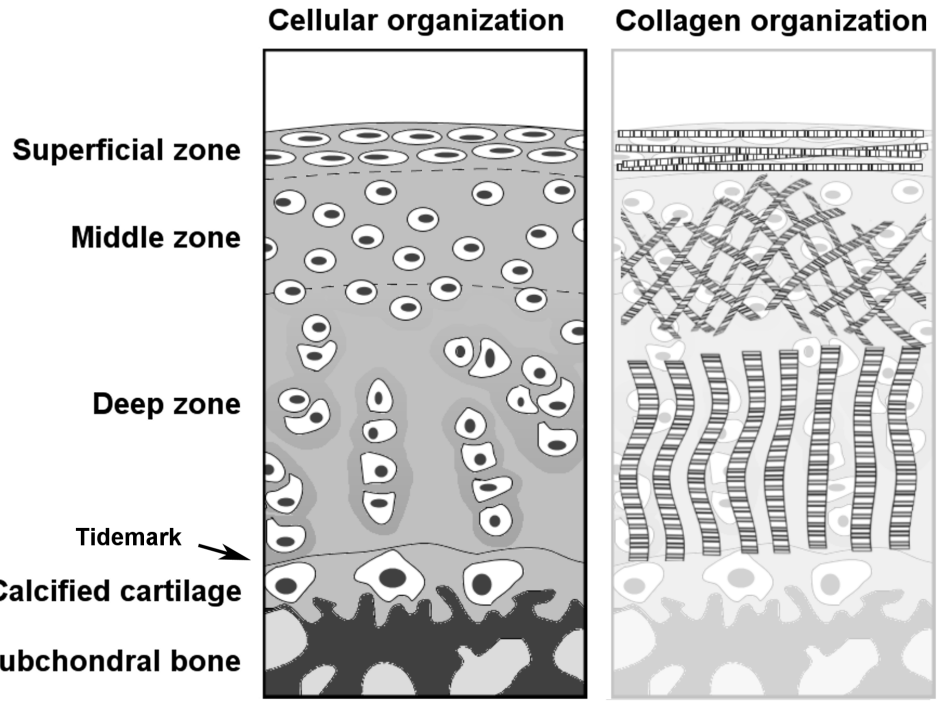

Figure 1: Schematic representation of the general structure of human articular cartilage, showing the anisotropic distribution and orientation of collagen fibers and chondrocytes within the different cartilage zones.

chondral defects. Such defects do not penetrate the subchondral bone and therefore cannot be accessed by the host blood supply, macrophages or stem cells originating from the bone marrow. The repair solely relies on the limited mitotic capabilities of the chondrocytes and is rarely effective [17,2,4,7]. Conversely, extrinsic repair concerns lesions reaching the subchondral bone (full thickness or osteochondral defect). In this case, the access to mesenchymal cells and blood vessels from the bone marrow allows a limited repair [18]. The defect is filled with a fibrocartilagenous tissue of heterogeneous composition and inferior mechanical competence [5]. As a result, it usually degenerates within 6 to 12 months [18,4]. Despite the poor outcome of this natural repair, it is still the basis of numerous orthopaedic treatments, such as microfracture and osteochondral drilling, which have been reviewed extensively elsewhere, together with other clinical approaches [19-21,5]. If these different repair techniques allow a temporary improvement of the patients quality of life, satisfactory functional results are seldom in the long term and clinician are still longing for ways not only to repair but to fully regenerate impaired cartilage.

A first attempt to regenerate cartilage was reported in 1989 by Grande *et al.*, using a cell based therapy: autologous chondrocyte implantation (ACI) [22]. In this approach, healthy chondrocyte are isolated and expanded *in vitro* prior to implantation in a defect sutured with a periosteal flap [23]. The positive outcome, as a whole, of this regenerative concept [24] has given rise and stimulated the new field of tissue engineering in orthopedic research.

Articular cartilage tissue-engineering is generally based on a central scaffolding matrix around which other strategies circumvolve, such as cells, signaling molecules and mechanical stimulation [25]. The cells of various origins are either cultured on the scaffold *in vitro* prior to implantation or recruited from the site of implantation (in the case of an osteochondral defect). The scaffold therefore acts as an extracellular matrix where the cells can organize themselves and populate an empty space. If cells are cultured on the scaffolds, mechanical stimulation can be applied on the scaffold to orientate or fix the cells towards the cartilage phenotype [15]. Similarly, the scaffolds can contain and deliver signaling molecules such as growth factors to recruit or orientate undifferentiated cells toward the chondrogenic lineage. As this review focus on the combination of scaffolds and growth factors, the cells and mechanical aspects of the scaffolds will not be touched upon. Excellent reviews on these topics can be found elsewhere [9,26,11,25].

Scaffolds for cartilage tissue engineering

General requirements

The crucial role of the scaffold in cartilage tissue engineering implies a number of requirements, based on the biological structure and repair mechanism of cartilage [20,11,5,25]. These requirements are illustrated in Table 1. Ideally, a porous scaffold should possess interconnected pores so that loaded or recruited cells can migrate and proliferate within the interstices. Its surface should promote cell adhesion or support chondrogenic phenotype.

Biocompatibility of the scaffold material is important to avoid immunological reactions within and around the defect. Additionally, the material should degrade to be replaced by newly-formed extracellular matrix, without inducing cytotoxic, nephrotoxic or other undesirable effect due to degradation products. Physical characteristics such as compressive strength and elasticity, or structural stability must also be considered accordingly to the surrounding cartilage or bone.

Each of these scaffold aspects is important to guide cell attachment, proliferation and differentiation into the tissue to regenerate [27-30]. Nonetheless, it appears difficult to combine all of them successfully. Therefore, the ability of the scaffolds to act as carrier and release system for signaling molecules, such as growth factors, appears of utmost significance, as it

Table I: Scaffold requirements related to the regeneration of cartilage in chondral or osteochondral defects.

Scaffold requirement	Biological basis
Biocompatibility	To allow a good contact with the native tissue, cell survival, and prevent inflammatory and immune responses.
Porosity of defined size	High ratio surface/volume for effective cell seeding, cell migration, proliferation and extracellular matrix production.
Inter-pore connection/permeability	Maximize nutrient/waste exchange, limit oxygen gradient and allow ingrowth of bone marrow cells in the case of ostechondral defect.
Carrier for signaling molecules	Contains and release growth factors and/or cytokines in a defined and controlled way to sustain, induce or maximize cartilage formation.
Cell attachment	To optimize cell seeding and to optimally retain or promote chondrogenic phenotype.
Biodegradability	Allow remodeling of the newly formed tissue while avoiding inflammatory response
Structural stability and cohesion	Prevent the matrix outflow from the defect or too early deliquescence.
Bonding and integration	Support integration between newly formed tissue and surrounding native tissue
Mechanical properties	Match the native tissue to insure homogeneity of implant response.
Structural anisotropy	Promotion of native tissue structure.
Size and shape	Reproducible sizes and shapes, relevant f or clinical applications.
Matrix property linked to defect type or surgical application	Minimally invasive techniques, using injectable matrices solidifying in situ for chondral defects or preformed and stiff matrix that can be easily reshaped by the surgeon for osteochondral defects.

could compensate or potentiate the other parameters to achieve the adequate cell proliferation or differentiation [31,32]. A number of studies lately showed that the ability of scaffolds to control-release at least one biological signal is determinant to the formation of improved tissues *in vitro* and *in vivo*, despite of the favorable physicochemical properties of the biomaterial [33,34]. However, ways to prepare scaffolds that combine the highest number of requirements mentioned above and contain growth factors are rare. The signaling molecules can either be incorporated directly in the scaffold matrix, or added to a prefabricated scaffold by mean of microspheres [35-37] or coatings [38,39]. Current techniques to prepare scaffolds of different properties and materials are presented hereafter, while their advantages and drawbacks are discussed relative to the scaffold properties and growth factor incorporation.

Materials and Fabrication Methods

Synthetic Polymers

Within synthetic polymers, linear aliphatic polyesters such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and copolymers (PLGA) have been broadly used, as they are biocompatible and approved by the Food and Drug Administration (FDA). By varying their copolymer ratio, the biodegradation rate and the mechanical properties can be tailored. They have already been studied for drug delivery [40-44,36,45,46] and are suitable for tissue engineering applications [47-51], as the degradation products (lactic and glycolic acids) obtained by hydrolysis are normally present in the metabolic pathways of the human body. The release rate of incorporated proteins is linked to the degradation rate of the polymer. However, their bulk degradation leads to the build-up of acidic degradation products inside the matrix lowers the pH within the polymeric matrix. This might result in local inflammation in tissues [52] and denaturation of proteins in the matrix [53-55]. Another linear aliphatic polyester commonly used in tissue engineering is poly(α-caprolactone) (PCL). This polymer has found many applications for its good biocompatibility and mechanical properties, but it degrades at a much lower rate than PLA, PGA, and PLGA, which makes it attractive when long-term implants and controlled release applications are desired [56-59].
Another family of thermoplastic polymers that has been recently studied for drug delivery and tissue engineering is poly(ethylene glycol)-terephtalate-co-poly(butylene terephtalate) (PEGT/PBT). These poly(ether-ester) multiblock copolymers belong to a class of materials known as thermoplastic elastomers which exhibit good physical properties like elasticity, toughness and strength [60]. These characteristics result mainly from a phase separated morphology in which soft, hydrophilic PEG segments are physically cross-linked by the presence of hard and semi crystalline PBT segments at environmental temperatures. In contrast to chemically cross-linked materials, these cross-links are reversible and will be disrupted at temperatures above their glass transition or melting point, which results in the material good processability. This family of copolymers has already been of great interest for tissue engi-

neering and drug delivery applications.
By varying the molecular weight of the starting PEG segments and the weight ratio of PEGT and PBT blocks it is possible to tailor-make properties such as wettability [61], swelling [60,62,63], biodegradation rate [63], protein adsorption [64], mechanical properties [65,66], and release rate of embedded proteins [67]. The release mechanism is due to a combination of protein diffusion and matrix degradation, which allows zero-order release profiles over long time period. Furthermore, PEGT/PBT block copolymers have shown to be extensively biocompatible both *in vitro* and *in vivo* [68-71] and reached clinical applications as cement stoppers and bone fillers in orthopedic surgery [72,73]. Being polyether-esters, degradation occurs in aqueous media by hydrolysis and oxidation, the rate of which varies from very low (high PBT contents) to medium and high (larger contents of PEGT and longer PEG segments) [60,63]. A further modulation in degradation rate and protein release profile can be achieved by substituting part or all of the terephtalate groups with succinate blocks during the copolymerization reaction [74-76].
Among the multitude of other synthetic polymer investigated for controlled release and tissue engineering applications, interesting classes are polyphosphoesters [77,78], polyphosphazenes [79-82], polyanhydrides [83] and polyortho-esters [84], as they have shown a surface erosion degradation mechanism [85,86], which is also known affect the stability of the scaffolds in the long term lo a lesser extent and to elicit a lower immune reaction. Injectable polymers are also very attractive as they can be used in minimally invasive surgery such as arthroscopy, resulting in a decrease in patient discomfort. Furthermore, they can fill irregularly shaped tissue defects [87-89], and cells and bioactive agents can be easily incorporated into them [90-92]. In particular, photopolymerizable systems like poly(propylene fumarate)-diacrylamide (PPF-DA) and poly(ethylene glycol)-diacrylamide (PEG-DA) based polymers, or poly(ethylene oxide)-dimethacrylate (PEODM) and poly(ethylene glycol) (PEG) have been investigated since they can entrap cells and be transdermally hardened by applying a light source [93-97]. Alternatively, chemically curable polymers (also based on PPF) have also been studied as they eliminate the need for light. In these materials, the double bonds available along PPF backbone are crosslinked through the use of a vinyl monomer, N-vinyl pyrrolidinone, and an initiator, benzoyl peroxide [98-100], with minimal temperature rise. However, the incorporation of proteins and growth factors in such *in situ* polymerisable hydrogels might be hampered by the exposure to ultraviolet light and crosslinking agents which can induce protein denaturation or aggregation and decrease the activity of encapsulated proteins [101].

Natural Polymers

Natural polymers also offer a broad selection of materials used for tissue engineering. Since they are naturally present in the body, their biocompatibility and degradation is less problematic than synthetic materials. These systems are typically in a gel-like phase and are easy to process. In addition, cells or biological agents can be readily incorporated during the gel formulation. Collagen, for instance, has been used for various tissue regenerations [102-106]. In

particular crosslinked collagen type I and type II scaffolds alone, or in combination with glycosaminoglycans have been considered for bone and cartilage repair [107,104,108,106]. Collagen matrices allow the release of proteins and growth factors by diffusion, degradation of the matrix and affinity between protein and collagen [109-111]. However, their gel nature seems to prevent the cell migration within the matrix, reducing so the tissue repair [112]. A further possibility is to use denatured collagen (gelatin) [105], fibrin [113,114] or demineralized bone matrix (DBM) [115-117]. The latter has found clinical applications either as an injectable gel or as a foamy porous scaffold.

Another group of natural polymers that have been investigated for this purpose comprises polysaccharides like alginate [118,119], chitosan [120,121], and hyaluronate [122,123]. Alginate consists of two repeating monosaccharide units, L-gluronic and D-mannuronic acids, which are water soluble and jellify when exposed to calcium ions. This provides a suitable system to include growth factors directly during gel formation. The protein is then released by a mix of diffusion and matrix degradation [124,33,125]. Chitosan is structurally similar to GAG and is composed of â linked D-glucosamine residues. It has been recently attracted more and more attention because of its non-toxicity, bioresorbability, and wound healing abilities [126]. Furthermore, it was shown successful to release sensitive growth factors [127-131,34,132]. Hyaluronic acid is also abundantly present in the human body articulation, within the synovium fluid. However, in its natural form this material lacks some desirable properties (too high water solubility, fast resorption and tissue clearance times) to consider it as a polymer for scaffold fabrication [133]. A change of its chemical structure through an esterification reaction allows the generation of a new set of biomaterials, hyaluronates, with improved properties, increased biocompatibility and fine-tunable degradation rates [134]. These materials have been studied for cartilage and skin regeneration [135,136,122] and reached clinical applications for the treatment of deep skin wounds and cartilage [137,138]. Nevertheless, only a few studies consider it as a suitable candidate to release growth factors [139].

Although these materials seem suitable to prepare scaffolds of defined properties, concerns over natural polymers are still present due to the potential pathogen transmission, immune reactions, poor handling and less controlled degradability with respect to synthetic polymers.

Fabrication Methods

Many different methods have been developed to fabricate scaffolds of various structures for tissue engineering applications. In general, the incorporation of growth factors in the scaffolds can be achieved by dispersing the protein in the polymer phase prior scaffold processing, using two main approaches. The simplest way consists of adding the signaling molecule directly to the polymer solution or powder [40]. Alternatively, a water phase containing the protein can be mixed with a polymer dissolved in an organic solvent to form a water-in-oil (w/o) emulsion [42,35]. In addition to the scaffold processing methods discussed hereafter, this first step to associate growth factors with scaffolds might induce a loss of activity of the protein due to contact with organic solvents or w/o emulsions [55].

Conventional scaffold fabrication techniques include fiber meshes and bonding, gas foaming, phase separation, freeze drying, and particulate leaching, among others.
Fibrous non-woven, woven or knitted scaffolds can be fabricated from polymeric fibers manufactured with standard textile technologies [140]. These scaffolds, however, lack of structural stability and consequently they can experience high deformations due to cells contractility and motility [141]. To improve the mechanical properties a fiber bonding technique has been developed [142], where by applying a heat treatment, the fibers of the scaffolds are joined at the cross-points.
In gas foaming the polymer is saturated with carbon dioxide (CO_2) at critical pressures to achieve high solubility of the gas in the polymer. When the gas pressure is brought back to the atmosphere pressure, the solubility of the CO_2 in the polymer rapidly decreases, resulting in the formation of gas bubbles or cells of variable size [143,42]. A similar approach is applied in phase separation, where a polymer solution is quickly cooled at low temperatures to generate a liquid-liquid phase separation. The solution is then quenched and a two-phase solid is formed. The solvent is then removed by sublimation to fabricate the porous scaffold [144,40]. Freeze drying is slightly different than phase separation, since the polymer solution is directly frozen or freeze-dried to yield porous scaffolds [35,145].
Particulate leaching can be achieved in two ways. One consists of incorporating particles of a specific size (salts crystal or other polymeric particles with defined shape and geometry) into a polymer solution, where the solvent used is a non-solvent for the particles. After evaporation of the solvent, a porous scaffold can be produced by leaching out the particles in a medium that is non-solvent for the polymeric scaffold [146-148,43,149-151]. Another approach, denominated compression molding, consists of mixing porogen particles with polymer granules and applying heat and pressure to melt the polymer and form a dense block. The particles are then leached as mentioned above to produce a porous scaffold [152,153,30]. This method can as well be used with protein-loaded microspheres instead of polymer granules, to incorporate proteins in the scaffold. The microspheres are fused around the porogen particles (usually salt crystals) by compression and gas quenching [44,36,45].
These fabrication techniques have been broadly used to fabricate scaffolds for tissue engineering and drug release applications. However, a number of drawbacks can be outlined in their use for an optimal control of tissue formation and protein incorporation. In particular, the pore size and shape of these matrices is often not controllable, resulting in tortuous and not completely interconnected pathways for the nutrients and biological signals that are to be released from the scaffolds. Pore tortuosity biases the distribution of viable cells within the scaffolds, which is limited mostly to 500 μm in depth [154]. Recent studies found that this phenomenon is also associated to a drop in the oxygen concentration from the outside to the center of the scaffold [155,156]. In addition, the incorporation or association of signaling molecules such as growth factors or other biological agents to the porous scaffolds is hampered by the processing conditions. In textile technologies the high temperatures involved in the manufacture can induce denaturation of the compound to be integrated. In solution-based techniques the solvents used can hinder the stability of the desired biological factor and cause aggregation and loss of activity [40,55]. In gas foaming and particulate leaching fur-

ther arising problems are connected to the use of pressure and heat which might induce protein denaturation. In addition, in particulate leaching, the efficiency of the agent incorporation might be lowered during the porogen washing step part of the compound is similarly washed away.

Among novel scaffold fabrication techniques currently available, rapid prototyping systems appear to be the most promising to satisfy the many requirements of a porous scaffold. They can process a wide number of biomaterials [11,157,158] in a custom-made shape and with matching mechanical properties in comparison with the specific application considered [159-161]. The outcomes are three-dimensional (3D) scaffolds that normally possess fine tunable porosity, pore size and shape, and have a completely interconnected pore network, which allows a much proficient cell migration and nutrient perfusion than scaffolds fabricated with conventional techniques [154,155]. Within rapid prototyping systems, 3D fiber deposition (3DF) has lately been investigated by our group to fabricate custom-made scaffolds and to modulate their mechanical properties for tissue engineering applications showing encouraging results [65,162,66]. In contrast, scaffolds fabricated with conventional techniques can be still shaped with custom-made molds, but it is more difficult to control their mechanical properties, pore size, shape and interconnectivity, resulting in nutrients limitations and cells apoptosis in the center of the construct as previously explained. Briefly, 3DF is a fused deposition modeling (FDM) technique, where a molten polymeric filament is extruded from a CAM controlled robotic unit on a stage. Filaments are deposited to form a layer and a porous scaffold is built with a layer-by-layer strategy, following a CAD pattern. Many other FDM tools have been developed to fabricate scaffolds, comprising also multi-dispensing systems that allow depositing different materials at the same time to produce constructs with different physico-chemical properties [163]. This possibility is quite appealing to study the release of multiple compounds from a single scaffold and to exploit the different interactions of the polymers with different cell populations in order to regenerate a more complicated hierarchical structure. However, FDM techniques still have the disadvantage of applying high temperatures during fabrication. Thus, the direct incorporation of a biological factor remains problematic.

Other direct printing technologies include solid free form (SFF) techniques like 3D printing™, selective laser sintering (SLS), and laser ablation (LA). 3D printing was one of the first rapid prototyping devices to be developed for tissue engineering applications. Here, a 3D scaffold is fabricated by depositing in a CAD/CAM controlled manner a jet of solvent on top of a polymer powder-bed. The solvent binds the powder, thus forming patterned fibers and building the scaffold layer-by-layer [164,165]. In a similar way, selective laser sintering consists in projecting a laser beam on a polymeric powder-bed. The laser beam sinters the powder due to the local increase of the temperature above the glass transition temperature of the polymer. The porous scaffolds are still fabricated in a CAD/CAM fashion [166,167]. Laser ablation works in the opposite way, as from a solid block of material the porous structure is formed through the fusion of the material hit by the laser beam in specific locations [168]. If the ablation process is conducted in all of the three directions, a scaffold can be built. These techniques allow the fabrication of periodic structures with well

defined, controlled and completely interconnected porosity, but still have as a disadvantage the use of solvents or the production of heat (although here localized to the spot where the laser beam hit the polymer, as in SLS and LA), which will affect or compromise the direct incorporation of proteins. A promising modification of SLS that can release active compounds like ribonuclease is surface selective laser sintering (SSLS) [169], although ribonuclease is known to be an exceptionally stable enzyme.

Biocompatible and biodegradable photosensible polymers that can be used in rapid prototyping techniques like stereolithography are also investigated. Stereolithography is normally used to produce a negative replica that is filled typically with ceramic or metallic slurries and burnt away during sintering [170]. This step still includes the use of high temperature. Therefore, the use of photosensible polymers in this system would allow the direct fabrication of the scaffold. Incorporation of any biological compounds, however, depends on their sensibility towards the light source used to start the polymerization (typically UV or blue light).

As can be seen from the different scaffold preparation methods, the incorporation of growth factors in the scaffold matrix is problematic due to potential denaturation by the preparation process. A schematic representation of the different potential causes of protein denaturation by the various preparation methods is given in Figure 2. A possible way to circumvent this difficulty consists of dissociating the scaffold preparation step from protein incorporation. This can be done by applying growth factor-loaded microspheres [131,34] or polymer coatings [38,39] to prefabricated scaffolds of defined properties or by incorporating micospheres, or liposomes in hydrogels [113,171]. Microspheres and coatings are usually prepared using water-in-oil-in-water (w/o/w) or w/o emulsion techniques. In both cases, a w/o emulsion containing the growth factor is prepared and either applied on a prefabricated scaffold or poured in a stirred second water phase to form a w/o/w emulsion. Upon solvent evaporation, a polymeric coating or microspheres are formed. The latter are then introduced in a scaffold. Although more steps are involved during the scaffold preparation, these approaches allow a better control of both scaffold properties and growth factor stability.

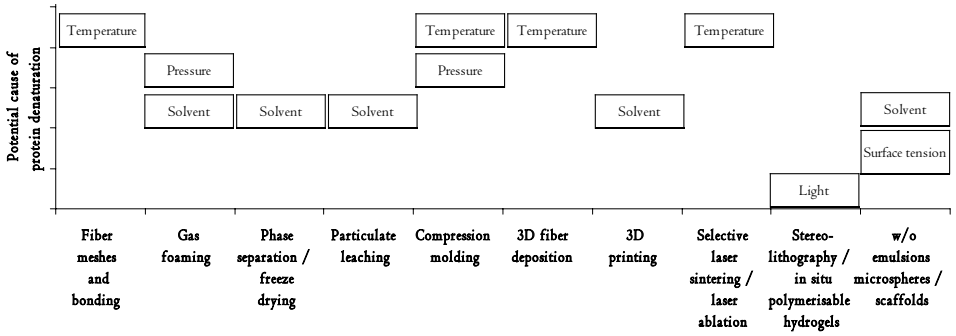

Figure 2: Potential causes of protein denaturation intrinsic to various scaffold preparation methods.

Growth factor release for cartilage tissue engineering

Growth factors are polypeptides involved in the cellular communication system [172]. They transmit signals that modulate cellular activity, by either inhibiting or stimulating proliferation, differentiation, migration, or gene expression [173]. In general, growth factors are pleiotropic, meaning that the same growth factor may act on different cell types to induce similar or distinct effects. Additionally, different growth factors can induce the same effect for a given cell type (redundancy). They exert their effect on target cells either by endocrine (released in the blood stream), paracrine (diffusion to nearby target cell) or autocrine (source and target cell are the same) fashion. They initiate their action by binding to specific receptors located on the target cell membrane [174]. When a sufficiently large number of receptors has been activated, a signal transduction process takes place that results in a specific cellular activity [175]. Consequently, growth factors effects are concentration and time dependent. Hundreds of growth factors have been described and grouped by homology in families and superfamilies [176]. Some have been more extensively characterized and are now readily available by mean of recombinant technology, which allows a thorough investigation of their potential in various tissue engineering applications.

Growth factors are usually produced by cells as inactive or partially active precursors. These precursors are often more stable than the active molecule. Upon proteolytic cleavage or binding to extracellular matrix molecules, the growth factors are activated and rapidly degraded. In general, their biological half life time is short (in the range of minutes) [172,177]. This important factor, combined with the potential toxicity of the growth factors at systemic level, naturally led to the sustained release of these proteins to enhance their efficacy. In combination with a porous scaffold, this approach offers a localized supply of signaling molecules aiming to enhance the proliferation or differentiation of cells towards the desired phenotype *in vivo*. With regard to cartilage, several growth factors that have regulatory effects on cartilage metabolism have been identified and are summarized in Table 2.

To better understand their potential, a brief description of the most relevant growth factors for cartilage regeneration is given hereafter.

Table 2: Growth factors of interest for articular cartilage regeneration.

Regulatory effects	Growth factor	References
Chondrogenic differentiation of progenitor cells	TGF-β_1	[178-183]
	IGF-I	[184]
	BMPs	[185-191]
Chondrocyte proliferation	TGF-β_1	[192-195]
	IGF-I	[196,171,195,197]
	FGF	[198,199]
Matrix synthesis	TGF-β_1	[200-206]
	BMPs	[207-211]
	IGF-I	[212-214]

TGF-β$_1$

Transforming growth factor-β$_1$ (TGF-β$_1$) is a 25 kilodalton (kD) homodimeric protein, member of a super family of over 100 different related proteins which include the bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDF) [215]. In addition to TGF-β$_1$, two highly homologous isoforms (TGF-β$_2$ and TGF-β$_3$, 70-80 % sequence homology) have been identified in all mammalian species and are less abundant in the body [216,217]. Although the three different isoforms are investigated for cartilage regeneration, TGF-β$_1$ was the first to be discovered and is the most studied.
Most cells can express TGF-β$_1$ receptors and secrete TGF-β$_1$. As a result, its cellular activities are numerous and play an important role in cell proliferation and differentiation, bone formation [218,219,111], angiogenesis [220,221], neuroprotection [222] and wound repair [223,224,215]]. The half life of TGF-β$_1$ in the body is short (less than 30 minutes [225,221,172]) due to a rapid binding to extracellular matrix components which activates, inhibates or buffers its activities [172]. Of high interest for cartilage tissue engineering, TGF-β$_1$ controls the production of extracellular matrices by stimulating the synthesis of collagens, fibronectin [200,201] and proteoglycans [202,204] and it has positive effects on cartilage differentiation and repair, as is detailed in Table 2 [192,178,179,114,181-183,193,194,195]. Nevertheless, TGF-β$_1$ can also induce undesired side effects such as inflammatory responses and osteophyte formation in articular cartilage defects if present in the knee joint for too long periods [226].

IGF-I

Insulin-like growth factor I (IGF-I) is a 70 amino acids polypeptide structurally related to insulin. It is synthesized primarily in the liver under the regulation of growth hormone. IGF-I controls the DNA synthesis of multiple cell types among which chondrocytes and accounts for most of the chondrocyte stimulating activity found in serum [176,227]. The half life of IGF-I in the body is short (10-12 min) [228]. To provide long-term growth stimulation, it associates with IGF binding proteins (IGFBPs) which form a more stable reservoir of the growth factor. A number of these binding proteins are secreted by chondrocytes to regulate IGF-I activity [197] and seem to be associated with components of the chondrocytes pericellular matrix [229,230].
In addition to its positive effect on chondrocytes proliferation [196,195], IGF-I increases proteoglycan and collagen type 2 synthesis [213,214,231]. *In vivo*, it has been reported to enhance the chondrocyte-based repair of osteochondral defects [232].

BMPs

Bone Morphogenetic Proteins, originally identified as inducers of bone and cartilage formation in ectopic tissues [233], compose a subfamily of the TGF super family. Almost 30 mem-

bers have been identified which regulate the growth and differentiation of chondroblasts and osteoblasts [234]. For instance, BMP-2, BMP-4 and BMP-7 maintain chondrocyte phenotype and stimulate proteoglycan synthesis in culture [207,235,208,209,211]. Many BMPs can direct mesenchymal stem cells towards the chondrogenic lineage as was demonstrated for BMP-2, 3, 4 and 9 [185-187,189]. Recently, BMP-2 and 7 were demonstrated to be the most effective in this respect [236,191]. *In vivo*, BMP-2 was shown to be effective in regenerating hyaline cartilage in osteochondral defects, with or without autologuous chondrocytes [237,238]. However, BMP-2 might induce the formation of osteophytes (due to dose and length of exposure) and appears less potent than TGF-β_1 in promoting proteoglycan synthesis in the joint [210].

Scaffolds, growth factor release and interest for cartilage regeneration

The short half life of IGF-1, TGF-β_1 and BMP-2, the potential side effects of the latter two, combined with their chondrogenic potential make these growth factors promising candidates for sustained delivery from porous scaffolds in cartilage tissue engineering. To reach this aim, different methods and materials have been evaluated. A summary is given in Table 3. The majority of the efforts to design drug delivery systems for cartilage applications where conducted with TGF-β_1. It should be noted that numerous papers describe release of BMPs from scaffolds. However, these studies have been conducted in view of bone tissue engineering applications, which is outside the scope of this review.

Most of the methods evaluated to combine growth factor delivery and supporting structures are based on the use of hydrogels of diverse materials. This approach is interesting as it allows to encapsulate cells easily in the releasing matrix prior to implantation, which optimally can be done by non-invasive injection [171,37]. However, the limited mechanical properties and stability of hydrogels might ultimately hamper their use in articular cartilage repair [25]. The association of growth factors to porous structures is usually achieved by separating the scaffold preparation step from the protein incorporation, to reduce the detrimental effect of scaffold processing on protein. Growth factor loaded microspheres or liposomes were incorporated in hydrogels or prefabricated scaffolds [114,171,131], polymer coating applied on compression molding scaffolds [39] or, more often, prefabricated matrices were soaked with growth factor solutions [248,139,246]. Although adsorption of the growth factors by soaking seems the easiest and less harmful approach, it limits the possibility to control the release of the growth factors from the scaffolds. In addition, it was demonstrated that adsorption could as well result in protein denaturation [251]. The activity of the released protein from the scaffolds, although an essential factor to take into consideration, is not always evaluated or in an incomplete way .This renders the evaluation the scaffolds processing methods difficult.

Most studies confirmed the potential of growth factors release. *In vitro*, the sustained delivery of TGF-β_1 and IGF-1 supported cartilage repair and maintenance. The fast release of TGF-β_1 over 1 or 3 days from chitosan microspheres embedded in collagen or chitosan scaffolds induced the proliferation and GAG production of co-encapsulated chondrocytes over

Table 3: Strategies elaborated to release growth factors from scaffolds and resulting delivery rates

Growth factor	Scaffold type	Growth factor incorporation	Material	*In vitro / in vivo*	Release rate (80 % completion, in days)	Remaining activity	References
TGF-β_1	Hydrogels	Soaking	Gelatin	*In vivo*	1 to 14	n.a.	[239]
			Dextran	*In vitro*	2	70 – 80 %	[240]
		Dispersion in hydrogel solution	Alginate	*In vivo*	>5	n.a.	[33]
	Composite hydrogel - microspheres	W/o/w	PEO - PLGA	*In vitro*	15	n.a.	[171]
		Soaking	OPF -gelatin	*In vitro*	3 to 24	n.a.	[241,242,37]
				In vivo	?		[243]
	Composite hydrogel -liposome	liposome	Fibrin	*In vivo*	25	n.a.	[113,114]
	Freeze dried scaffold	Soaking	Collagen	*In vivo*	8 to 35	n.a.	[111]
	Composite Freeze dried scaffold - microspheres	W/o/w	Collagen and/or chitosan - chitosan	*In vitro*	1 to 3	n.a.	[131,34]
	Compression molded	Polymer-coating	PEGT/ PBT	*In vitro*	12 to 50	85 %	[244,39]
				In vivo	12	85 %	[245]
IGF-I	Decellularized bladder	Soaking	Collagen	*In vivo*	1	n.a.	[246]
	Composite hydrogel - microspheres	W/o/w	PEO - PLGA	*In vitro*	5 to 15	n.a.	[171]
	Composite hydrogel -liposome	liposome	Fibrin	*In vivo*	?	n.a.	[114]
BMPs	Phase separated	Dispersion in polymer solution	PLGA	*In vitro*	6	n.a.	[247]
	Hydrogel	Soaking	gelatin	*In vivo*	1	n.a.	[248]
	Phase separated/salt leached	Soaking	Hyaluronate	*In vitro*	10	n.a.	[139]
	Helistat ©	Soaking	collagen	*In vivo*	5 to 10	n.a.	[249,238]
	Composite hydrogel -liposome	liposome	Fibrin	*In vivo*	?	n.a.	[114]
	Microspheres	W/o/w	PLGA	*In vivo*	10	n.a.	[250]

Abbreviations: PEO, pol y (ethylene oxide); w/o/w, water -in-oil-in-water emulsion; OPF, oligo(poly(ethylene glycol) fumarate); n.a., not available

21 days [131,34]. Similarly, the release over 7 days from gelatin microparticles embedded in hydrogels allowed the multiplication of chondrocytes over 28 days with maintenance of their phenotype [37]. Such approaches could be of interest for the regeneration of chondral defects as they allow the preparation of the cell-containing scaffolds, prior to implantationA more elaborated strategy consists of embedding gelatin microparticles simultaneously acting as porogen and TGF-β_1 delivery system, in hydrogels without cells. Due to the natural presence of collagenase in the injured knee, the particles release the growth factor while being digested. The voids, created by the degraded gelatin microspheres allow the ingrowth of progenitor cells [242]. Similar to TGF-β_1, the release of IGF-1 over 5 days from PLGA microspheres co-encapsulated with chondrocytes in a hydrogel induced the proliferation and enhanced the GAG production of chondrocytes embedded in a hydrogel [171].

In the same study [171], the opportunity to combine the release of different growth factor from the same supporting structure was as well investigated by mixing IGF-1 and TGF-β_1-containing PLGA microspheres. This approach appeared promising as the two growth factors had synergistic effects on the enhancement of chondrocytes proliferation and on the maintenance of their phenotype. In a broader view, it is likely that the release from scaffolds of different growth factors with different release profiles would be beneficial. For instance TGF-β_1 or BMP-2 could be released in a first step to induce chondrogenic differentiation of progenitor cells while IGF-1 release over longer time periods would maintain and enhance the obtained phenotype at a later stage. Other methods have been considered for this purpose. For example, mixing two populations of gelatin microparticles releasing IGF-1 and TGF-β_1 within an hydrogel or adsorbing TGF-β_1 to the hydrogel directly allowed to control independently the release profiles of the two proteins [252]. Another approach reported consists of applying multiple gelatin coatings containing BMP-2 and IGF-1 on flat surfaces to control the release of each growth factor independently by diffusion through the superposed layers [253,254]. Similarly, the successive coating of PEGT/PBT copolymers containing different model proteins on prefabricated compression-molded scaffolds allowed a tailored and independent release [255]. However these techniques are yet preliminary and still have to be tested in relevant articular cartilage defects.

In vivo, the beneficial effect of growth factors sustained release was as well demonstrated. In rabbit osteochondral defects, the release of TGF-β_1 over at least 5 days from alginate microparticles [33] or a release of BMP-2 within 10 days from collagen sponges were evaluated [237,238]. An improvement of the tissue repair after 6, 12 or 24 weeks was measured, in comparison to defects filled with unloaded matrix or left empty. The BMP-2 delivery showed a similar cartilage restoration as compared to the implantation of autologuous chondrocytes in the defect. This indicates the potency of the released growth factors to differentiate progenitor cells present at the implant site, which may eliminate the need of extra cell source. Similarly, in minipig chondral defects a 25 days release of TGF-β_1 or BMP-2 from fibrin hydrogels induced a successful healing by differentiating migrating synovial cells [114,113]. However, recent studies in rabbit ostechondral defects with scaffolds releasing TGF-β_1 at similar concentrations showed either only a limited improvement of cartilage restoration [243] or no improvement when release over 12 days [245]. The same negative

result was found in chondral defects exposed to IGF-1-releasing liposomes, possibly because of a wrong dosage or release rate which was not evaluated or to the lack of suitable progenitor cells [113].
Although the delivery of growth factor from supporting scaffolds appears overall beneficial, different aspects of the release still need to be examined to further enhance cartilage regeneration, especially *in vivo*. For instance, the amount of growth factor released is of importance to achieve the optimal effect while avoiding side effects. This parameter has been evaluated with different TGF-β_1 concentrations released in osteochondral and chondral defect and revealed a concentration dependency of chondrogenesis between 200 and 900 ng/ml. Above 900 ng/ml, adverse effects such as osteophytes formation, synovitis and cartilage erosion were observed [114,33]. However, such studies were not performed with other growth factors and the optimal dose ranges of BMP-2 and IGF-1, for example, are still unknown for chondral or osteochondral defects. In addition, the integrity and activity of the released growth factor is seldom considered or evaluated. Often, if the protein released from a scaffold still elicits a biological response *in vitro* or *in vivo*, it is considered as fully active, even though only a small part of the protein might actually be active. Considering the high potency of growth factors and their high costs, this point requires more attention. An exact determination of the ratio of protein effectively active would allow to further select the most suitable scaffold preparation methods. Optimally, the release of highly active growth factors would permit the reduction of the amount of growth factor needed for a similar effect and a more precise management of potential side effects. Finally, the influence of growth factor release rate on cartilage reconstruction is rarely investigated. This is surprising as research focusing on the controlled release of BMP-2 from porous scaffolds for bone tissue engineering clearly showed that this parameter was as important as dosage [249,256,257]. Recently, we evaluated the influence of release profiles of TGF-β_1 from porous scaffolds on the chondrogenic differentiation of bone mesenchymal stem cells *in vitro* [244]. The most effective stimulation was found for a burst delivery of the growth factor. Although this should be further confirmed *in vivo*, it suggests that a sustained release over days might not be necessary to induce cartilage formation, which would additionally minimize side effects. With regard to BMP-2 and IGF-1, the influence of the release rate should be as well evaluated to better understand the requirements for an optimal scaffold delivery system. Such knowledge would allow to improve more effectively the regeneration of articular cartilage by mean of supporting structure and growth factor release.

Conclusions and future considerations

In the current cartilage tissue engineering research, the role of the scaffold is crucial. Different strategies and approaches have been considered, both cell-based and cell-free. Over time, the requirements of the scaffolds have been defined and refined, and many materials, processing methods and designs are now available. However, even though positive results were obtained, none of these techniques resulted so far in complete and functional repair.
In parallel, over the last years, various growth factors have been identified that regulate carti-

lage homeostasis and induce the chondrogenic differentiation of progenitor cells. Logically, the use of these signaling molecules in combination with scaffolds is being investigated for cartilage tissue engineering. The local release of selected growth factors from scaffolds is aimed at attracting pluripotent cells, stimulating their differentiation and maintaining their acquired phenotype – which has shown great potential.

Although this concept seems logical and appealing, the intrinsic properties of the growth factors limit the number of materials and preparation methods that can be used to prepare growth factors releasing scaffolds. In addition, the physiological mechanisms of growth factors should be taken into account. More than just sustained, the release from scaffold should be precisely controlled as cells react in a concentration and time dependent fashion to growth factors. Optimally, the delivery of multiple growth factor should mimic the endogenous profile of growth factor production during tissue morphogenesis or repair. Therefore, a greater understanding of the required therapeutic doses and release kinetics will be important to obtain the benefit resulting from the association of growth factor and scaffolds.

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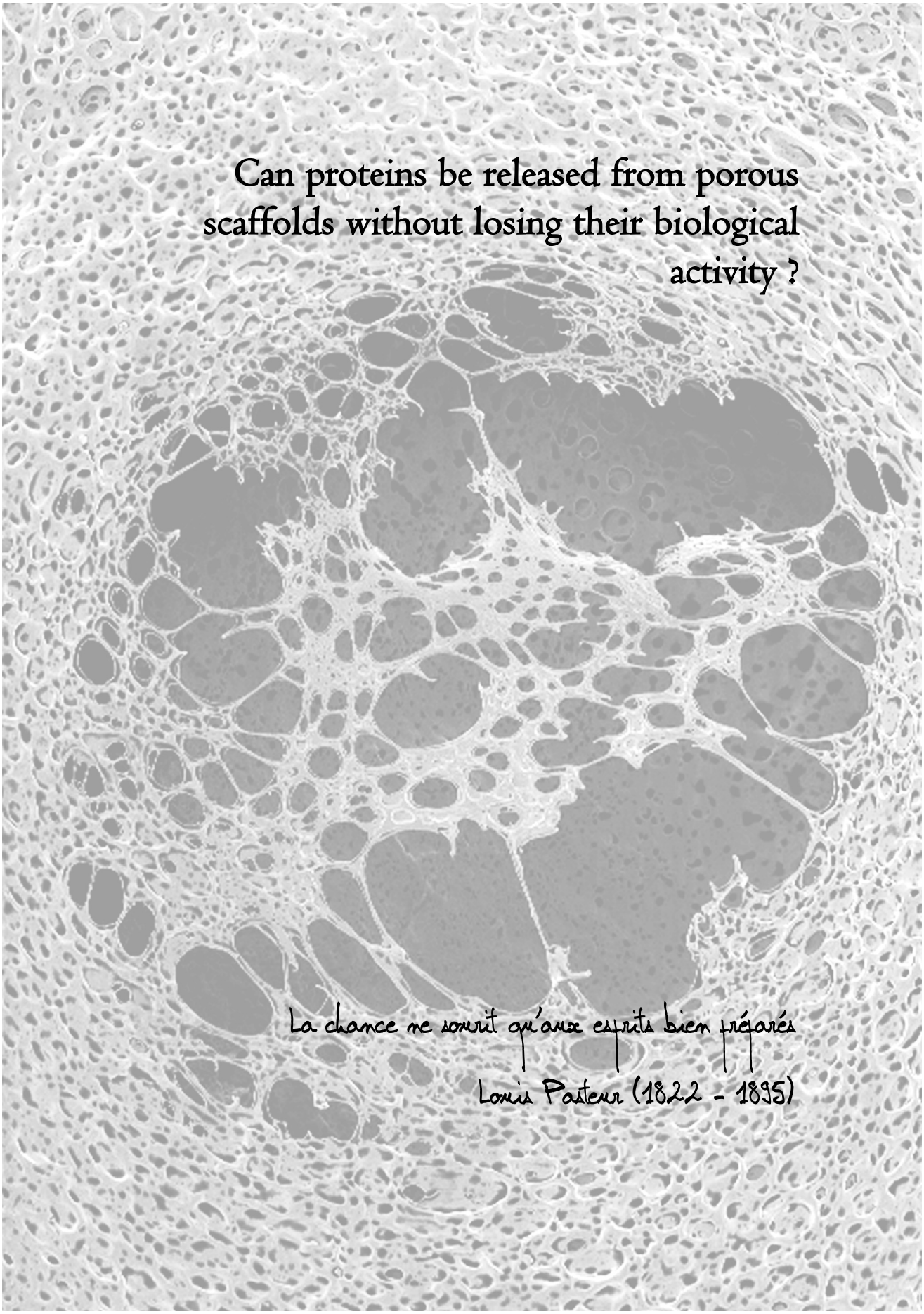
Can proteins be released from porous scaffolds without losing their biological activity ?
La chance ne sourit qu'aux esprits bien préparés
Louis Pasteur (1822 - 1895)

Chapter 3

A novel method to obtain protein release from porous polymer scaffolds: emulsion coating

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Abstract

To obtain a controlled release of proteins from macro-porous polymeric scaffolds, a novel emulsion-coating method has been developed. In this process, a water-in-oil emulsion, from an aqueous protein solution and a polymer solution, is forced through a prefabricated scaffold by applying a vacuum. After solvent evaporation, a polymer film, containing the protein, is then deposited on the porous scaffold surface. This paper studies the effect of processing parameters on emulsion-coating characteristics, scaffold structure, and protein release and stability. Poly (ether-ester) multiblock copolymers have been chosen as polymer matrix for both scaffolds and coating. Macro-porous scaffolds, with a porosity of 77 volume % and pores of approximately 500 μm were prepared by compression moulding/salt leaching. A micro-porous, homogeneous protein-loaded coating could be obtained on the scaffold surface. Due to coating, the scaffold porosity was decreased whereas the pore interconnection was increased. A model protein (lysozyme) could effectively be released in a controlled fashion from the scaffolds. A complete lysozyme release could be achieved within 3 days up to more than 2 months, by adjusting the coated emulsion parameters. In addition, the coating process did not reduce the enzymatic activity. This new method looks promising for tissue engineering applications.

Introduction

Modern tissue engineering combines materials science with biotechnology and biology to repair and replace damaged or worn out tissues. It has been shown that new tissues can be engineered from living cells and three-dimensional scaffolds [1-3]. The success of these approaches is largely dependent on the scaffold properties. For instance, it is well-recognized that the scaffolds should provide sufficient mechanical strength (depending on the type of tissue targeted) and have a high porosity and pore interconnection to ensure nutrient diffusion, cell ingrowth and elimination of waste [4]. In addition, the scaffold should degrade in non-toxic products in a controlled fashion to prevent long-term physical hindrance and unwanted tissue reactions. Various ways have been investigated to produce such scaffolds with different porosity and surfaces structures, using a range of materials [5,6]. For example, poly(α-hydroxy acid) polymers have been employed successfully in gel casting [7-9], solvent casting and particulate leaching [10], and gas saturation methods [11].

A critical issue in tissue engineering is local and well-timed delivery of the various cell-signalling molecules that are crucial in tissue development. For cartilage and bone for instance, it has been reported that growth and maturation is supported by growth/differentiation factors including insulin-like growth factor I (IGF-1), transforming growth factor β_1 (TGF β_1), fibroblast growth factor 2 (FGF-2) [12,13] and bone morphogenetic proteins (BMP-2 and BMP-3) [14]. Biomaterial scaffolds may provide an opportunity for controlled local delivery of such agents.

Various approaches to combine growth factors and scaffolds have been investigated (see [15-17] for reviews). Absorption of growth factors into natural polymers (e.g. collagen [18], gelatin [19,20]) or adsorption to synthetic polymers (e.g. polylactides and copolymers thereof) [21] allows local delivery, but the opportunity to control release is often minimal [16].

Incorporation of proteins into polymers offers a tool to obtain well-controlled release of proteins over prolonged periods [10]. For instance, the advantage of slow delivery of growth factors over adsorption has been reported for bone formation using rhBMP-2 [22-24] and PDGF [25]. However, the methods used to prepare polymeric scaffolds are often not suitable for incorporation of labile proteins, due to the high temperatures used [10,26], exposure to organic solvents [26] or interaction with pressured gas [27]. Alternatively, suitable methods like porogen-leaching [10] often cause a premature lost of the protein during the leaching process [28].

Whereas the development of controlled delivery systems from scaffolds has always focussed on entrapping the molecule inside the scaffold polymer matrix itself [29-31], the aim of this study was to evaluate a new approach. Instead of entrapping the protein inside the scaffold matrix during fabrication of the scaffold, we propose coating of the inner pores of a prefabricated scaffold with a protein-loaded polymeric film.

Poly (ether-ester) multiblock copolymers, based on poly(butylene terephthalate) and poly(ethylene glycol) (PEGT/PBT) have been chosen as polymer matrix for both scaffolds and coating. Their properties, like hydrophilicity, elasticity, permeability and biodegradation, can be easily tailored to meet specific requirements. Porous PEGT/PBT scaffolds, prepared

by a compression moulding/salt leaching process, are under evaluation for tissue engineering of bone [32] and cartilage [33]. Furthermore, this material has been shown to be a successful release system for proteins [34]. In this paper, we evaluate various parameters of the coating process on coating characteristics, like homogeneity and thickness of the coated film and scaffold structure. The effect on release rate and stability of incorporated proteins was assessed using Lysozyme (Mw=14.5 kD) as a model protein.

Materials and Methods

Materials

Poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) multiblock copolymers (PEGT/PBT) were obtained from IsoTis NV, Bilthoven, The Netherlands, and were used as received. Polymers are indicated as aPEGTbPBTc, in which a is the PEG molecular weight, b the wt% PEG-terephthalate and c (=100-b) the wt% PBT. Vitamin B_{12}, lysozyme from chicken egg white (3x crystallized, dialyzed and lyophilized), and *Micrococcus Lysodeikticus* were purchased from Sigma Chem. corp. (St. Louis, USA). Eosin Y solution alcoholic was obtained from Sigma diagnostics (St. Louis, USA). Phosphate Buffered Saline (PBS), pH 7.4 was obtained from Life Technologies Ltd (Paisley, Scotland). Chloroform, purchased from Fluka chemica (Buchs, Switzerland), was of analytical grade.

Preparation of polymeric scaffolds.

Compression molded/salt leached scaffolds were obtained by applying pressure (10000 PSI during 10 minutes) and heat (240 °C) to a homogeneous mix of NaCl salt crystals and copolymer powder in a mold. The volume fraction of salt in the mixture was adjusted to 75 %. After cooling of the resultant dense block, the salt was extracted by successive immersions in RX-water. To ensure a complete salt removal, the water conductivity was controlled to be less than 25 μS. Subsequently, the porous blocks were dried in ambient air for at least 24 hours, and then placed in a vacuum oven (50 °C) for a minimum of 12 hours.
The PEGT/PBT copolymer used to prepare the scaffold had a PEGT content of 70 weight % and a PEG molecular weight of 1000 g/mol. The copolymer powder was produced by milling polymer granules under liquid nitrogen atmosphere and sieving. The fraction of the copolymer particles smaller than 600 μm was used. The salt crystals were sieved between 400 and 600 μm.

Preparation of coated scaffolds

Coated scaffolds were prepared using a water-in-oil (w/o) emulsion method. An aqueous

solution of a lysozyme in PBS was emulsified with a PEGT/PBT copolymer solution in chloroform, using an Ultra-Turrax (T25 Janke & Kunkel, IKA-Labortechnik) for 30 s at 19 krpm. The lysozyme concentration of the aqueous solution was fixed at 50 mg/ml. The volumes of the aqueous phase were varied between 0.5 and 2.0 ml per gram of copolymer used (water/polymer ratio = 0.5, 1 and 2 ml/g). The viscosity of the copolymer solution was tailored by varying the volume of chloroform used to dissolve one gram of copolymer (from 4 ml to 8 ml). Three different PEGT/PBT copolymer compositions were used in which the PEGT content was varied from 60 to 80 weight %, with a fixed PEG molecular weight of 1000 g/mol.
The emulsion was forced through a porous scaffold by applying a vacuum, which was varied between 160 mBars and 600 mBars. This vacuum was applied for at least 5 minutes, in order to evaporate as much as possible chloroform from the emulsion, thereby creating a polymeric coating on the scaffold. The resulting coated scaffolds were dried under vacuum over night.

Scanning electron microscopy

A Philips XL 30 ESEM-FEG was used to evaluate the internal morphology of the scaffolds. The internal porous structure was observed by cutting the scaffolds in the longitudinal axis with a razor blade. All samples were gold sputter-coated using a Cressington 108 auto apparatus before analysis.

Coating characterization

The coating homogeneity was assessed by using vitamin B_{12} as a dye in the emulsion coating instead of lysozyme. The vitamin B_{12} was dissolved in the emulsion water phase at a concentration of 10 mg/ml. Scaffolds were prepared as described above. Four longitudinal and two lateral cross sections of the treated scaffolds were made with a razor blade and observed under a binocular.
The coating thickness was evaluated using a staining method. Cross-sections of the sample embedded in PMMA were made by using a Leyca saw microtome (sp 1600). Cross-sections were subsequently stained using eosin Y alcoholic solution. The eosin taints the PEGT/PBT scaffold and the deposited coating. As the coating is less dense than the scaffold structure, the staining appears more intense in the coating. Subsequently, pictures of the cross-sections were observed by light microscopy.

Characterization of scaffold porosity

The average porosity (%) of the scaffolds was evaluated from their dry weight, dry volume and density of the PEGT/PBT copolymer (density = 1.2 g/ml) according to the following

equation:

$$p = I - \frac{\text{sample weight}}{\text{sample volume} \times I.2} \qquad \text{(Equation I)}$$

The scaffold pore interconnection before and after coating treatment was quantified using a method that applies Darcy's law, as described elsewhere [35,36]. A fluid is forced through the porous samples by applying a constant pressure and the flow rate is measured, from which a value for the pore interconnectivity can be evaluated.

Cylindrical samples (10 mm long and 7 mm in diameter) were enfolded in parafilm before being placed in a polystyrene tube (30 mm long). The polystyrene tubes were connected to a reservoir of demineralized water by a rubber tube (inner diameter = 11 mm). The difference of water level between the reservoir and the sample was corresponding to 0.898 m. Assuming that the pressure at the bottom surface of the samples equals zero, the pressure generated by the water level was 8.81 kPa. To keep the pressure difference approximately constant during the experiment, the flow volume was restricted at 100 ml. A volume of 100 ml would have reduced the water level in the reservoir by 7.1 mm, corresponding to a negligible 0.5 % pressure drop. The induced flow was deducted from the volume of water collected in a 100 ml cylinder during a certain time interval.

From the induced flow the fluid conductance can be evaluated as follows [36]:

$$C = \frac{\Delta Q}{\Delta P} \qquad \text{(Equation 2)}$$

where ΔQ is the induced flow (m^3/s) and ΔP is the pressure drop across the sample (Pa).

Applying Darcy's law to the porous sample, we can obtain the conductance as:

$$C = \frac{A\kappa}{L\mu} \qquad \text{(Equation 3)}$$

Where A is the cross sectional area of the sample (m^2), L is the sample length (m), μ is the kinematic fluid viscosity (η/ρ, in which the viscosity of water is 0.001 Pa.s), and κ is the permeability (m^2).

Thus, the permeability can be deducted from equations (2) and (3) [35]:

$$\kappa = \frac{\Delta Q.L.\mu}{\Delta P.A} \qquad \text{(Equation 4)}$$

The permeability can be used to compare the pore interconnectivity of different scaffolds, provided that porosities are comparable, which was the case for the emulsion coated scaffolds.

In vitro protein release

Emulsion coated scaffolds containing lysozyme (50 mg of each type) were incubated in 1 ml PBS (pH 7.4) at 37 °C. All samples were kept under constant agitation (25 rpm). Samples of the release medium were taken at various time points and the medium was refreshed after

sampling. Concentrations were quantified using a spectrophotometer (El 312e, BioTek instruments) at 570 nm and a standard protein assay (Micro BCA assay, Pierce).
To determine the quantity of emulsion effectively coated on the porous scaffold and establish the amount of lysozyme present, scaffolds were coated using vitamin B_{12} as a model compound, following the same preparation parameters as the corresponding protein-loaded scaffolds. Vitamin B_{12} was selected, since this small molecule (Mw=1355 D) is completely released within a few hours from 100 μm sheets of the selected polymers. This allows a fast evaluation of the amount of emulsion coated on the scaffold surface. This estimation was then used to determine the amount of lysozyme present on the coated scaffolds. The release of the vitamin from the treated scaffolds was evaluated as described above for lysozyme. Concentrations of the aqueous phase were quantified using a spectrophotometer at 380 nm (El 312e, BioTek instruments).
The activity of released lysozyme was determined using a *Micrococcus Lysodeikticus* assay [27]. To 150 μl of the lysozyme release medium, a suspension of *M. Lysodeikticus* (100 μl, 2.3 mg/ml), was added in a 96-wells microplate. The decrease in turbidity at 37 °C was measured at 450 nm, during 4 minutes at 15 seconds intervals. The initial kinetic rate (OD slope at t=0) was measured for each samples and the protein effective concentration deducted from a fresh standard curve. The lysozyme activity was then obtained by comparison of the concentrations obtained using the protein assay and the M. Lysodeikticus assay.

Results and discussion

Coating homogeneity and thickness

Three-dimensional scaffolds for tissue engineering were prepared by a compression moulding/salt leaching process. The resulting PEGT/PBT scaffolds were strong, but pliable, with a porosity of 77 volume % and a pore size of approximately 500 mm. In view of the harsh processing conditions used during the fabrication of the scaffolds, a two-step method has been developed for loading the porous scaffolds with growth factors. The method consists of coating a protein-loaded film onto a prefabricated scaffold. The coating is formed from a water-in-oil emulsion, consisting of an aqueous protein solution and a polymer solution in chloroform. Evaporation of the chloroform results in a protein-containing polymeric film on the surface of the porous scaffold. Simple coating of a prefabricated scaffold with the emulsion by dipping or soaking appeared to be impossible due to rapid dissolution of the PEGT/PBT scaffold. Thus, the contact time between the emulsion and the scaffold had to be reduced as much as possible, and this could be achieved by forcing the emulsion through the scaffold by applying a vacuum.
A visual evaluation of the coating homogeneity indicated that the deposited emulsion film was evenly distributed over the porous scaffold (Figure 1). The different cross sections of the treated scaffolds did not show uncoated parts, and the coating colour was homogeneous. A closer observation of the coating showed that its thickness could be adjusted by varying the

viscosity of the emulsion used. A more viscous emulsion resulted in a thicker coating, as presented in Figure 2.

The viscosity of the emulsion was modified by varying the volume of chloroform used to dissolve a fixed amount of copolymer. At a constant vacuum applied, a more viscous emul-

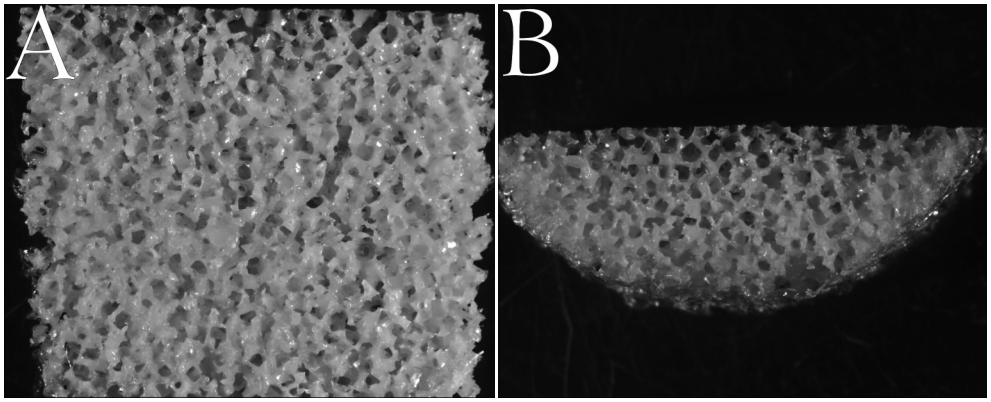

Figure 1: Coating homogeneity as assessed by using a vitamin B_{12}-containing coating. The emulsion was made of 1 ml vitamin B_{12} solution (10 mg/ml PBS) and 1 gram of 1000PEGT80PBT20 in 6 ml of chloroform; the vacuum applied was 300 mBar. The cross sections were made longitudinally (A) and laterally (B).

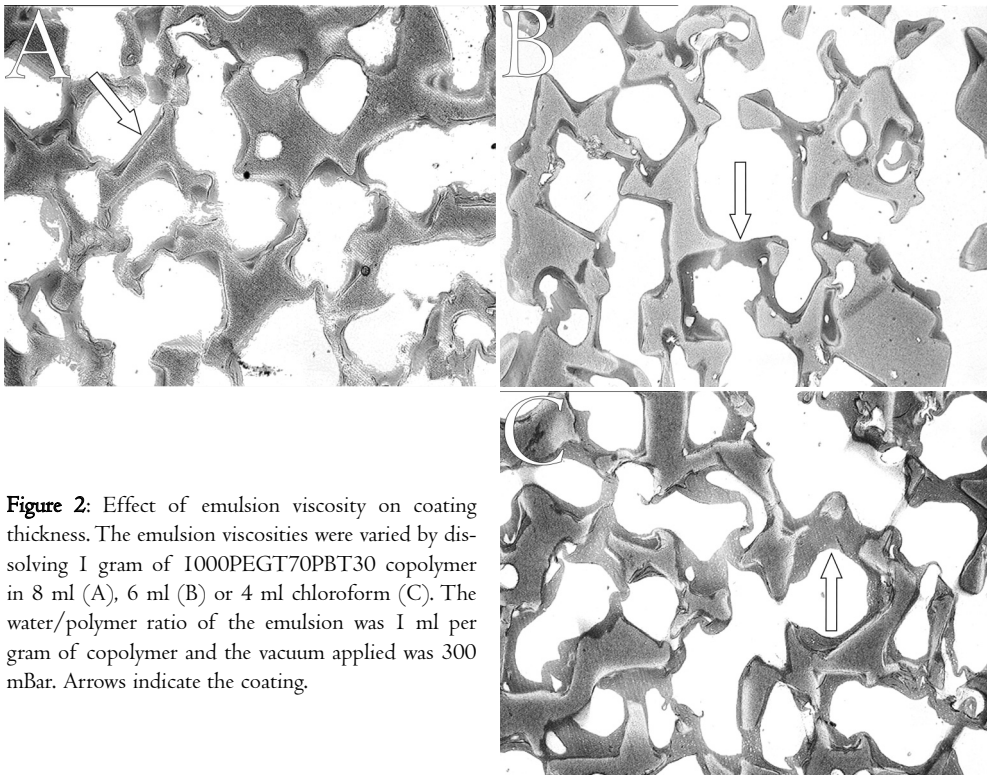

Figure 2: Effect of emulsion viscosity on coating thickness. The emulsion viscosities were varied by dissolving 1 gram of 1000PEGT70PBT30 copolymer in 8 ml (A), 6 ml (B) or 4 ml chloroform (C). The water/polymer ratio of the emulsion was 1 ml per gram of copolymer and the vacuum applied was 300 mBar. Arrows indicate the coating.

sion will move slower through the scaffold and will precipitate faster because of the lower amount of chloroform present. These two factors can explain the deposition of a thicker coating with increasing emulsion viscosity.

Effect of coating parameters on scaffold porosity

The emulsion-coating process clearly affected the scaffold structure. Visually, an improved interconnection and reduced porosity of the scaffolds was observed. In order to evaluate the effect of processing parameters on the scaffold properties more in detail, emulsion-coating process parameters were varied. The vacuum applied and the emulsion volume, viscosity, copolymer composition and water/polymer ratio were varied, and their effect on scaffold interconnection and porosity was measured.

The interconnection of the scaffolds was increased due to coating, regardless the different preparation parameters used. Variation of the vacuum applied from 600 mBar to 160 mBar resulted in a 2 to 4 fold increase in the permeability of the scaffold for water, respectively. As shown in Figure 3, a linear relationship between scaffold permeability and the emulsion volume or the amount of chloroform used in the emulsion could be established. Although the applied coating is likely to close some pores, it appears that a more important number is opened during the process, leading to the pore interconnection increase.

This may be explained by dissolution of the thin polymeric walls between pores by the chloroform present in the emulsion. This was confirmed by electron microscopy, as presented in Figure 4. A higher volume of the applied emulsion or the presence of more solvent in the emulsion results in a more extensive dissolution of the pore walls, and consequently a higher

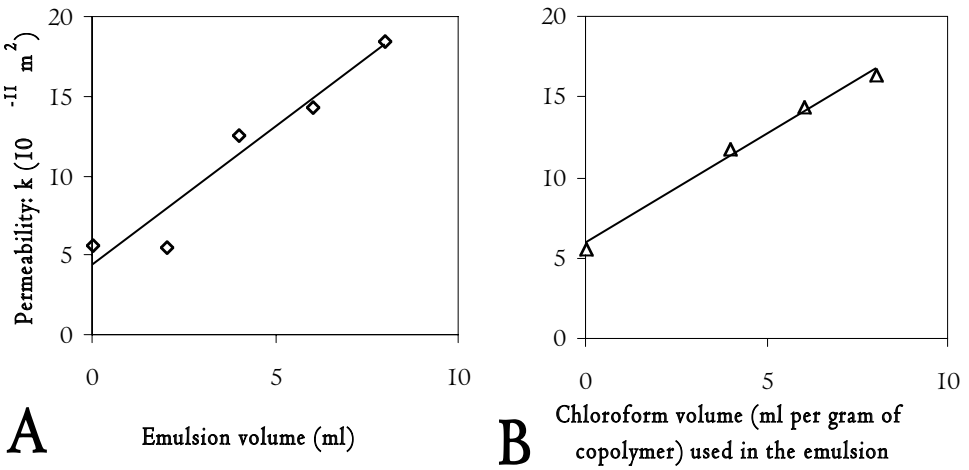

Figure 3: Water permeability of scaffolds as a function of emulsion volume (A) and chloroform volume used per gram of copolymer in the emulsion (B). A higher chloroform volume results in a lower emulsion viscosity. The water/polymer ratio of the emulsion was 1 ml per gram of copolymer (1000PEGT70PBT30) and the vacuum applied was 300 mBar.

interconnection. The observation that the coating process could improve the pore interconnection is of importance for scaffolds applied for tissue engineering. To allow nutrient diffusion, cell ingrowth and elimination of waste products, a good pore interconnection is considered as crucial [4,37].
Surprisingly, the porosity of the scaffolds decreased from 77 volume % before coating to 63-69 % after the treatment, irrespective the chosen coating parameters. Obviously, deposition of a coating in the scaffold will decrease its porosity. However, it was expected that a thicker coating would decrease the porosity to higher extent than a thinner coating. The observation that various coating parameters did not influence significantly the porosity decrease indicates that the deposition of a polymer coating is accompanied by a proportional dissolution of scaffold material. For example, coating of a more viscous emulsion would not only deposit a thicker coating, but also dissolve more of the scaffold matrix because of the longer contact-time.

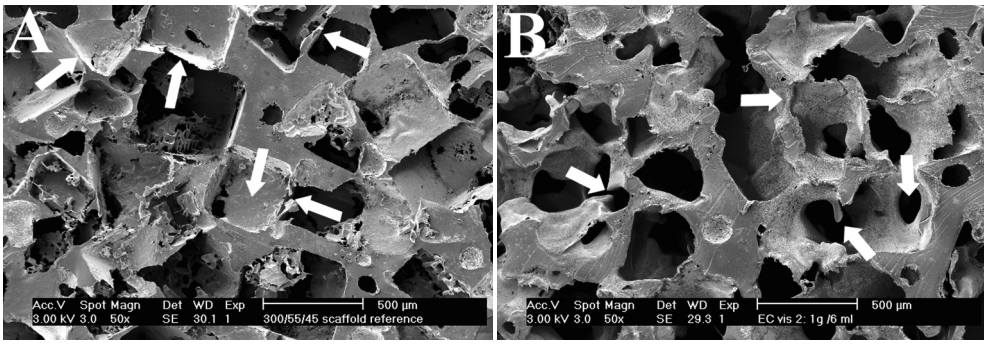

Figure 4: Effect of the emulsion coating treatment on the internal structure of porous scaffolds. Cross sections examined by scanning electron microscopy before (A) and after the coating process (B). Arrows indicate the change of the polymeric walls between pores due to the treatment. The water/polymer ratio of the emulsion (7 ml) was 1 ml per gram of copolymer (1000PEGT70PBT30) and the vacuum applied was 300 mBar.

Effect of emulsion coating on scaffold surface structure

The surface of the pores of the scaffold after coating displayed a homogeneous micro-porosity (Figure 5). The micro-pores, with diameters varying between 500 nm to 4µm, were well interconnected. Their structure was hardly influenced by the preparation parameters. In some cases, bigger micro-pores of diameters between 8 and 10 µm could be noticed, for instance when the emulsion viscosity was increased, or when higher water/polymer ratios were used (data not shown).
The micro-porous surface structure of the scaffolds is probably caused by the rapid evaporation of the chloroform due to the applied vacuum. This may result in a fast precipitation of the copolymer and consequently the formation of a micro-porous structure. In addition, the micro-pores may originate from the water droplets of the water-in-oil emulsion that was applied as coating. Therefore, the use of an emulsion with a higher water/polymer ratio is

expected to result in a more pronounced micro-porosity, which was indeed observed.
The surface structure of scaffolds is an important factor for cell attachment, proliferation and differentiation [38]. It has been reported that calcium phosphate micro-structures, similar to the structures presented here, play a key role in osteoinduction [39-41].

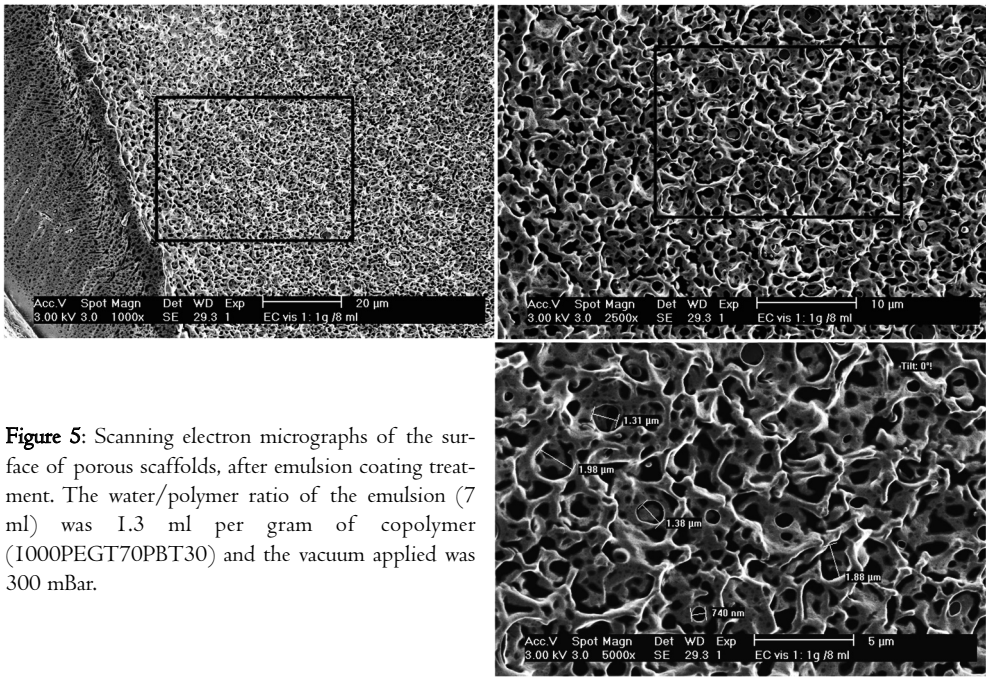

Figure 5: Scanning electron micrographs of the surface of porous scaffolds, after emulsion coating treatment. The water/polymer ratio of the emulsion (7 ml) was 1.3 ml per gram of copolymer (1000PEGT70PBT30) and the vacuum applied was 300 mBar.

Protein release from porous emulsion-coated scaffolds

A model protein (lysozyme) was effectively associated with the porous scaffolds by the emulsion-coating process. Using vitamin B_{12} the amount of emulsion effectively coated onto the porous scaffolds was evaluated. Depending on the processing parameters, up to 24 mg of protein was coated per g of scaffold. Lysozyme release could be tailored efficiently by varying the emulsion water/polymer ratio, or the coating copolymer composition, as presented in Figures 6 and 7. At a constant coating copolymer composition (1000PEGT70PBT30), a complete release of the protein was achieved within 3 days when the emulsion water/polymer ratio was set at 2 ml per gram of copolymer, whereas a release over more than 2 months was observed for a water/polymer ratio of 0.5 ml per gram. Furthermore, when the PEGT content of the coating copolymer was decreased from 80 to 60 weight %, the release rate was decreased, from a complete release within 20 days to a release period of more than 1 month. The effect of the water-polymer ratio of the emulsion on the lysozyme release rate can be explained by the coating structures. As mentioned above, a higher water/polymer resulted in

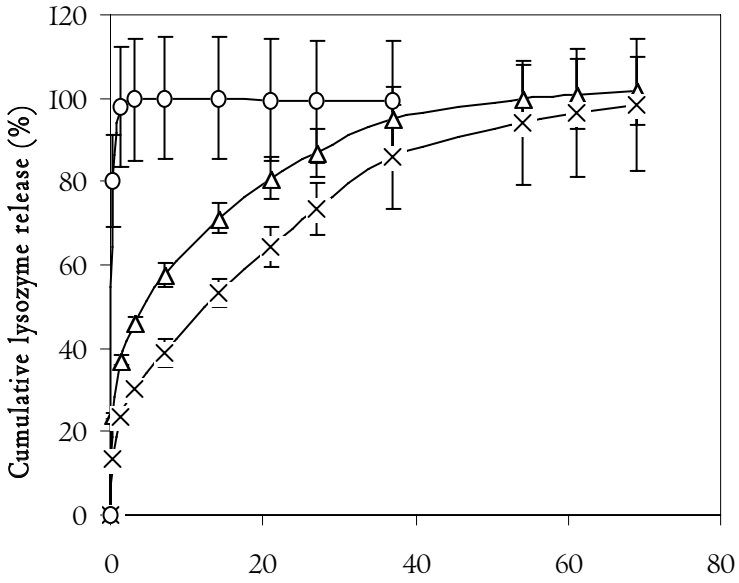

Figure 6: Cumulative lysozyme release from emulsion coated scaffolds. The emulsions (6 ml) used for the coating had a chloroform volume of 6 ml per gram of copolymer (1000PEGT70PBT30) and varied in water/polymer ratio: water/polymer ratio = 2 ml/g (○), water/polymer ratio = 1 ml/g (△), and water/polymer ratio = 0.5 ml/g (×). The vacuum applied was 300 mBar. (n=3; ± s.d.)

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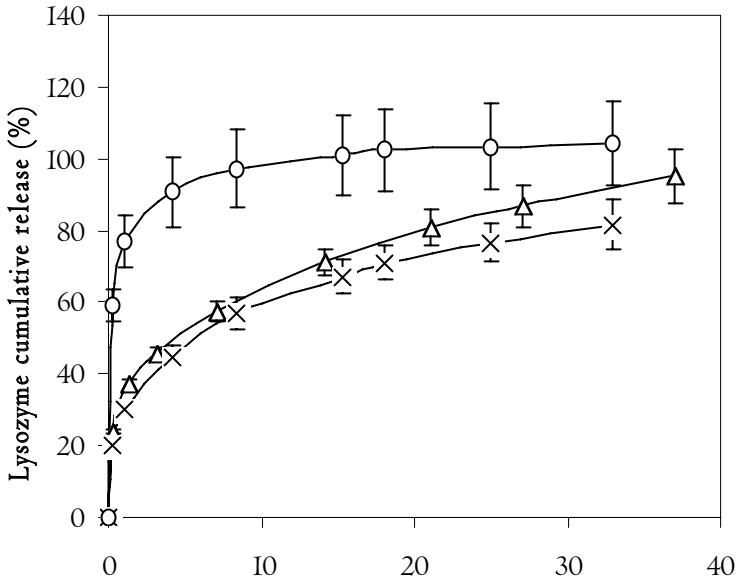

Figure 7: Cumulative lysozyme release from emulsion coated scaffolds. The emulsions (6 ml) used for the coating had a water/polymer ratio and chloroform volume set respectively to 1 ml and 6 ml per gram of copolymer 1000PEGT80PBT20 (○), 1000PEGT70PBT30 (△), and 1000PEGT60PBT40 (×). The vacuum applied was 300 mBar. (n=3; ± s.d.)

formation of a more pronounced micro-porosity of the coating. The increase in porosity may account for the faster release. Besides, modulation of release rates by varying the emulsion water/polymer ratio of PEGT/PBT copolymers has been described before [42] for lysozyme and BSA. It was then suggested that protein-loaded matrices prepared by water-in-oil emulsion methods are heterogeneous systems, composed of aqueous protein-rich droplets, dispersed in the polymeric matrix. At low volume fraction of water phase (low water/polymer ratio), these droplets are isolated from each other. The release of the protein is then mainly dependent on the permeability of the polymer matrix. However, at a critical volume fraction ("percolation threshold"), the aqueous domains connect to each other, allowing the protein to be released in a faster way by direct transport through the water-filled domains.

The trend in the lysozyme release rates obtained for the different coating copolymer compositions was in agreement with our previous results with PEGT/PBT films and microspheres [43]. The increasing release rates with increasing PEGT content in the copolymer coating can be attributed to the effect of the copolymer composition on the swelling and permeability. The (volume) swelling of the copolymers increases with increasing PEGT content. As discussed in detail elsewhere [43], the permeability of the PEG/PBT block copolymers for lysozyme is strongly dependent on the degree of swelling of the copolymers: an two-fold increase in swelling, caused an almost 50,000-fold increase in the lysozyme diffusion coefficient.

Surprisingly, the release profiles obtained from the coated scaffolds were different compared to the ones reported from films. Films provided an almost zero order release of lysozyme without an initial burst. The constant release rate was explained by the combined effect of protein diffusion and matrix degradation. In contrast, the release from coated scaffolds showed an initial burst, followed by a decreasing release rate over time. The occurrence of a burst can be explained by the micro-porous nature of the coating, as described above. The difference in release profile (constant vs. decreasing release rate) is most likely related to geometry and dimensions of the matrices. Whereas the films were only 100 μm in thickness, the scaffolds used for the release studies were approximately 5 mm in thickness. Although the scaffolds are macro-porous (the pore size is approximately 500 μm), apparently diffusion through the tortuous pore channels of the scaffold into the release medium is one of the rate limiting steps. In addition, it has to be noted that the protein-containing coatings on the scaffolds have only one side in contact with the release medium, which will reduce the release rates compared to a film with two sides in contact with the release medium. The observation that quantitative lysozyme release from a scaffold coated with 1000PEGT70PBT30 takes more than five weeks, whereas release form the corresponding films takes less than two weeks, confirms the above presented hypotheses.

Finally, the activity of the released lysozyme was investigated. It is well known that the emulsification procedure used to prepare the protein-loaded coatings may cause protein aggregation and incomplete release, as well as a decreased enzymatic activity [44,45]. As shown in Figure 8, the protein stability appeared to be constant and close to 100 % after the coating process and during the release from the coated scaffolds.

This indicates that the protein was not damaged by the emulsification procedure or during

the release period. As reported before, this may be attributed to the amphiphilic nature of the polymer. Possibly, this prevents very large aggregates to be formed, or alternatively, it serves as a template for refolding [46].

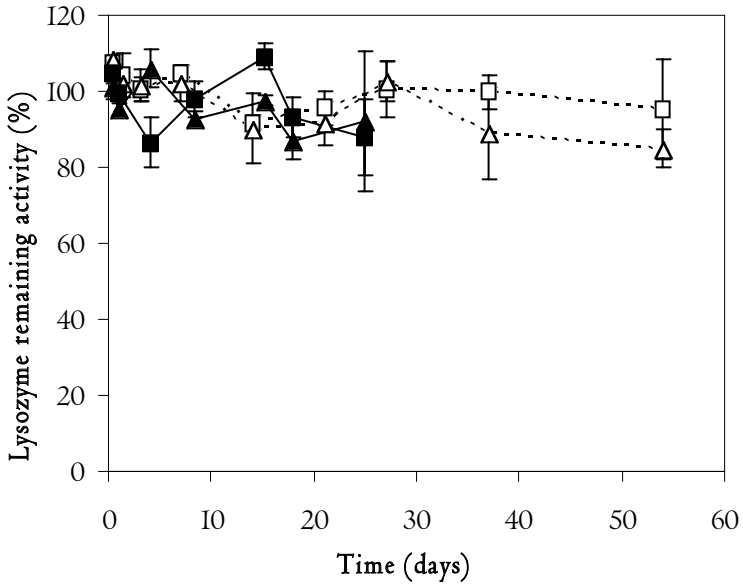

Figure 8: Activity of lysozyme released from coated porous scaffolds of different coating copolymer composition: 1000PEGT60PBT40 (▲), 1000PEGT80PBT20 (■) (water/polymer ratio = 1 ml/g); and of different water/polymer ratios: 1 (△) and 0.5 ml/g (□) (copolymer composition 1000PEGT70PBT30). (n=3; ± s.d.)

Conclusions

In order to create porous scaffolds that release growth factors in a controlled fashion, a novel method to associate proteins with porous polymeric scaffolds has been developed, based on an emulsion-coating technique. This new approach resulted in an effective, homogeneous and adjustable coating of the scaffolds. The structure of the porous scaffold was modified by the coating process. The scaffold interconnection was increased and interconnected micro-porosity appeared on the pore surface. The latter could be of value for cell attachment and differentiation. The release of a model protein (lysozyme) from the coated scaffolds could effectively be tailored from 3 days to more than 2 months, by varying process parameters such as emulsion copolymer composition or water/polymer ratio. Furthermore, the method did not denature the applied protein, as lysozyme activity remained close to 100% over the release period. Future experiments will focus on the loading of growth factors and in vivo evaluation of the scaffolds.

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Appendix to chapter 3

Emulsion-coated scaffolds loaded with bovine serum albumin

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Introduction

In order to further investigate the suitability of the emulsion-coating method regarding controlled release applications, a new protein was tested. In contrast to lysozyme, which is a relatively stable and small protein (14.3 kD), a large protein was selected (bovine serum albumin, BSA, 67 kD) and loaded scaffolds were prepared. Three different coating copolymer compositions were evaluated, in which the PEGT/PBT ratio and PEG molecular weight were varied (1000PEGT70PBT30, 1000PEGT80PBT20, 2000PEGT80PBT20). In addition, the release was investigated when varying the water to polymer ratio (w/p) of the coated water-in-oil emulsions between 0.5 and 4 ml/g. The w/p ratio proved to be a successful tool to control the release rate of lysozyme from emulsion-coated scaffolds [1]. Finally, a copolymer of poly(ethylene glycol) succinate and poly(butylene succinate) (PEGS/PBS) was used, with a fixed w/p ratio of 1 ml/g (1000PEGS67PBS33).

Materials and methods

Materials

Poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) (PEGT/PBT) and Poly(ethylene glycol)-succinate)/poly(butylene succinate) (PEGS/PBS) multiblock copolymers were obtained from Chienna BV, Bilthoven, The Netherlands, and were used as received. Polymers are indicated as aPEGTbPBTc or aPEGSbPBSc in which a is the PEG molecular weight, b the weight percentage (weight %) of Poly(ethylene glycol)-terephthalate or Poly(ethylene glycol)-succinate, and c (=100-b) the weight % of PBT and PBS. Bovine Serum Albumine (BSA) and vitamin B12 were purchased from Sigma Chem. corp. (St. Louis, USA). Phosphate Buffered Saline (PBS), pH 7.4 was obtained from Life Technologies Ltd (Paisley, Scotland). Paraffin was purchased from Fischer chemicals (Loughborough, UK). Poly (Vinyl Alcohol) (PVA, Mw=22 kg/mole) was obtained from Aldrich Chemicals (Milwaukee, USA). Chloroform and hexane, obtained respectively from Fluka chemica (Buchs, Switzerland) and Merck (Schuchardt, Germany), were of analytical grade.

Preparation of protein-loaded polymeric scaffolds.

Emulsion

The scaffolds were prepared using a water-in-oil (w/o) emulsion method. An aqueous solution BSA in PBS was emulsified with a PEGT/PBT copolymer solution in chloroform, using an Ultra-Turrax (T25 Janke & Kunkel, IKA-Labortechnik) for 30 s at 19 krpm. The protein concentration of the aqueous solution was varied between 50 and 12.5 mg/ml. The volume of the aqueous phase was varied between 0.5 and 4.0 ml (water/polymer ratio = 0.5, 1, 2, 3 and 4 ml/g). The copolymer solution was obtained by dissolving one gram of copolymer in 6 ml of chloroform. Four different PEGT/PBT copolymer compositions were used in which the PEGT content was varied from 70 to 80 weight %, with a PEG molecular weight of 1000 and 2000 g/mol.

Emulsion-coating method.

The emulsion-coated scaffolds were obtained as described elsewhere [1]. Briefly, compression molded/salt leached scaffolds (CMSL) were obtained by applying pressure (10000 PSI during 10 minutes) and heat (240 °C) to a homogeneous mix of NaCl salt crystals and copolymer powder in a mold. The volume fraction of salt in the mixture was adjusted to 75 %. After cooling of the resultant dense block, the salt was extracted by successive immersions in RX-water (until water conductivity was less than 25 μS). Subsequently, the porous blocks were

dried in ambient air for at least 24 hours, and then placed in a vacuum oven (50 °C) for a minimum of 12 hours. The PEGT/PBT copolymer used to prepare the scaffold had a PEGT content of 55 weight % and a PEG molecular weight of 300 g/mol. The salt crystals were sieved between 400 and 600 μm.
Coated scaffolds were prepared by forcing BSA-containing emulsion through a prefabricated porous scaffold with the use of vacuum (300 mBars). This vacuum was applied for at least 5 minutes, in order to evaporate as much as possible chloroform from the emulsion, thereby creating a polymeric coating on the scaffold. The resulting coated scaffolds were frozen in liquid nitrogen, and freeze-dried at room temperature for 24 hours.

Swelling of coated scaffolds

The swelling behavior of the coated scaffolds was determined by immersing dry scaffolds (small cubes of maximum 5x5x5 mm) of known weight in PBS at room temperature. After 4 days, a Pasteur pipette connected to a vacuum pump was applied softly over each of the faces of the cubes to remove the water present in the pores. The process was done in less than 20 seconds to prevent any drying of the scaffolds. The weight of the scaffolds was then measured. The water uptake (in ml per gram of polymer) was calculated from the weight increase. The equilibrium swelling ratio Q was determined from the weight of the swollen scaffolds using a density of 1.2 g/ml for all PEGT/PBT copolymers.

Quantification of coating applied on emulsion coated scaffolds

To estimate the weight ratio of copolymers effectively coated on the prefabricated scaffolds (*a*) and establish the amount of protein present, scaffolds were digested under vigorous agitation in a 1 N NaOH solution, for 40 hours at 37 °C. Bovine serum Albumin (BSA) standards in PBS and unloaded scaffolds were submitted to the same treatment. After neutralization of the samples with MOPS buffer and HCl (1M), the remaining protein content was quantified using a spectrophotometer (El 312e, BioTek instruments) at 570 nm and a standard protein assay (Micro BCA assay, Pierce). As the ratio of protein to polymer is known, the amount of protein in the scaffold allows to calculate the amount of coating present.
For scaffolds prepared without protein and water phase, similar coated scaffolds were prepared using a polymer solution containing 10 mg of vitamin B_{12} (fine powder) per gram of polymer. The vitamin containing polymer solution was finely homogenized prior to coating using a vortex. The small molecule was completely released within three days from the scaffold when immerged in 10 ml of PBS (completely refreshed three times over three days). The amount of vitamin released was calculated using a standard curve of vitamin B_{12} in PBS and a spectrophotometer (El 312e, BioTek instruments) at 380 nm. The weight ratio of coated copolymers was calculated as mentioned above, from the known ratio of vitamin to polymer (10 mg per gram of polymer).

In vitro protein release

Protein loaded scaffolds (50 mg) were incubated in 1 ml PBS (pH 7.4) at 37 °C. All samples were kept under constant agitation (25 rpm). Samples of the release medium were taken at various time points and the medium was refreshed after sampling. Concentrations were quantified using a standard protein assay and a spectrophotometer, as described above.
The integrity of released BSA was assessed by sodium dodecyl sulfate poly(acrylamide) gel electrophoresis (SDS page) under denaturating conditions.

Results and discussion

As presented in Figure 1A, no slow delivery of BSA could be obtained when using a 1000PEGT70PBT30 copolymer composition as coating matrix. A burst release comprised between 5 and 100 % was measured during the first day for the various w/p ratio (from 1 to 4 ml/g), followed by a very slow release that was still on going after 30 days. Only the highest w/p ratio (4 ml/g) of the coated emulsion showed a complete release of BSA. The total amount of BSA released over 30 days was increasing with increasing w/p ratios. The use of more hydrophilic copolymer compositions (1000PEGT80PBT20 and 2000PEGT80PBT20) in the coating showed also an incomplete release of maximum 14 % characterized by a burst within the first day, with no influence of the w/p ratio (data not shown). On the contrary, the use of 1000PEGS67PBS33 provided a nearly constant release of 80 % of the protein over 40 days after a lag time of 4 days (Figure 1B).

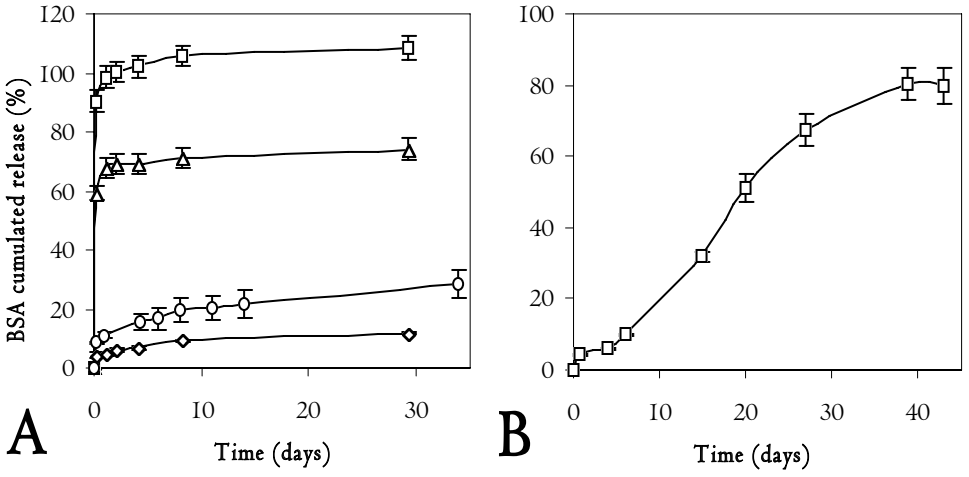

Figure 1: Cumulative BSA release from emulsion-coated scaffolds. A: the water to polymer ratio of the emulsions used for the coating was varied for a constant copolymer composition (1000PEGT70PBT30): 1 ml/g (◇), 2 ml/g (○), 3 ml/g (△), and 4 ml/g (□). B: a copolymer of poly(ethylene glycol) succinate and poly(butylene succinate) (1000PEGS67PBS33) was tested, with a fixed water to polymer ratio of 1. All polymers were dissolved in a chloroform volume of 6 ml per gram and the vacuum applied was 300 mBar (n=3; ± s.d.)

The integrity of the BSA released from a coating composed of 1000PEGS67PBS33 copolymer was evaluated by electrophoresis (SDS-Page) and did not reveal any sign of aggregation or denaturation in the release medium (data not shown). Considering that the protein was fully released from these scaffolds (100 % release), it can be assumed that the emulsion-coating method or the succinated copolymer do not hamper the protein structure.

To better understand the low and incomplete release profiles observed from the various PEGT/PBT copolymer compositions used as coating, we evaluated the different copolymer network structures. An empirical relation between equilibrium swelling ratio of PEGT/PBT copolymers and polymer network mesh size has been established for polymeric films [2]:

$$\xi = 21.8Q + 15.9 \qquad \text{(Equation 1)}$$

where ξ is the copolymer network mesh size (Å) and Q the equilibrium swelling ratio of the copolymer. This relation can be applied in the case of coated scaffolds, as the coated emulsion can be considered as a film distributed on the pores of a prefabricated scaffold. The determination of the water uptake of the coated emulsion, necessary to calculate the equilibrium swelling ratio, can be obtained from the water uptake of coated and prefabricated (uncoated) scaffold:

$$WU_{\text{coated emulsion}} = \frac{WU_{\text{coated scaffold}} - WU_{\text{prefabricated scaffold}} \times (1-a)}{a} \qquad \text{(Equation 2)}$$

where WU is the water uptake of the different copolymers (ml/g) and a is the weight ratio of coated emulsion present in each scaffold. The water uptake of the prefabricated scaffold was measured using uncoated scaffolds. Considering the low swelling of the prefabricated scaffold copolymer composition in comparison to the coated emulsions, the water uptake of the prefabricated scaffold is assumed to be constant and independent of the coated emulsion. The resultant network mesh sizes for the different copolymers used in the coating are presented in Table 1. All PEGT /PBT copolymers used in the coated emulsions in this study presented a network mesh size smaller than the hydrodynamic diameter of BSA (72 Å [3,4]). Therefore, the protein cannot diffuse through the polymeric matrix. As the release of pro-

Table 1: Network mesh size of the different coated copolymers (Å) (n=3; ± s.d..).

Copolymer composition of the coated emulsion	Mass ratio of coated emulsion (a)	Equilibrium swelling ratio of coated emulsion (Q)	Network mesh size (ξ, Å)
CMSL scaffolds [1]	n.a.	1.3 ± 0.02 [2]	44.9 ± 0.4 [2]
1000PEGT70PBT30	0.52	1.72 ± 0.02	53.3 ± 0.3
1000PEGT80PBT20	0.54	2.04 ± 0.05	60 ± 1
2000PEGT80PBT20	0.46	2.28 ± 0.05	65 ± 1

[1] CMSL scaffolds: compression molded/salt leached scaffolds prior coating
[2] Equilibrium swelling ratio and network mesh size of CMSL scaffolds prior coating.
n.a. : not applicable

teins from PEGT/PBT matrices is primarily diffusion driven during the first weeks of release (with little influence of matrix degradation) [5], the incomplete BSA release observed is due to the network structure of the copolymers used.

Contradictorily, emulsion coatings made from 1000PEGT70PBT30 copolymer showed that BSA could be released when increasing the w/p ratio of the emulsion. This has also been previously reported for 1000PEGT70PBT30 films and microspheres (prepared by the use of a water-in-oil emulsion method) [6]. It was there hypothesized that the emulsification process introduces water-rich domains in the PEGT/PBT matrices. Whereas a part of the water used in the emulsion is effectively absorbed by the copolymer during swelling, the other part remains dispersed in the matrix. Above a critical w/p ratio, the dispersed aqueous domains form an interconnected network through the copolymer matrix, through which the BSA can diffuse. To assess the veracity of this theory for coated scaffolds, the volume fraction of the dispersed aqueous domains in swollen coated emulsions was calculated as follow:

$$\varepsilon = 1 - \frac{Q_{\text{coated emulsion, w/p=0}}}{Q_{\text{coated emulsion}}} \qquad \text{(Equation 3)}$$

where ε is the volume fraction of dispersed aqueous domains and $Q_{\text{coated emulsion, w/p=0}}$ and $Q_{\text{coated emulsion}}$ are the equilibrium swelling ratio of coated emulsions prepared without and with protein solutions of different volume, respectively. Figure 2 presents the different ε obtained for the various copolymer compositions and w/p used in the coated emulsion, in relation with the percentage of BSA released after 10 days.

Above an ε of about 0.3, the amount of BSA released was increased up to completeness. This tends to confirm the formation of an interconnected aqueous network as mentioned above. Once the aqueous domains interconnect to each other, the protein present in these domains can freely diffuse in the water, out of the polymeric coating. This could explain the burst release noticed. By increasing the volume fraction of dispersed aqueous domains, the number of interconnected aqueous domains increases and a higher amount of protein can be released. Interestingly, values of higher than 0.3 were obtained only for 1000PEGT70PBT30 coated emulsions whereas 1000PEGT80PBT20 and 2000PEGT80PBT20 ones were lower. This can be linked to the higher equilibrium swelling ratios of 1000PEGT80PBT20 and 2000PEGT80PBT20 unloaded coatings that were measured. A higher swelling probably hampers the formation of an interconnected aqueous network by reducing the volume fraction of the dispersed aqueous domains and consequently prevents the release of BSA.

The linear release of BSA over 40 days observed when using a 1000PEGS67PBS33 copolymer with a w/p of 1 is probably due to another mechanism. It has already been reported that the substitution of butylene terephthalate (BT) sequences (aromatic groups) by butylene succinate (BS) (aliphatic) increased the degradation rate of poly(ether-ester) copolymers [7]. Consequently, the release of large proteins like BSA could be obtained from films, based mainly on matrix degradation for succinate-substituted copolymers as the one used in this study. The lag time observed in BSA delivery from the 1000PEGS67PBS33-coated scaffold confirms this degradation driven release mechanism. Initially no protein was released from the matrix, indicating an initial mesh size smaller than the protein size. After 4 days the BSA

release started, meaning that the size of the polymer meshes had increased up to a critical mesh size, due to the decrease of molecular weight of the matrix.

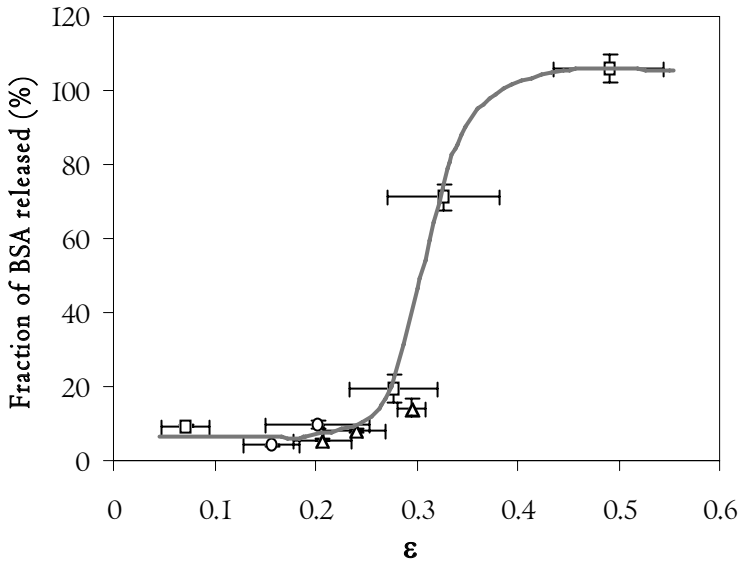

Figure 2: Fraction of the amount of incorporated BSA released after 10 days as a function of the volume fraction of aqueous domains in coated emulsions (ε). Different copolymer compositions were used to obtain the emulsions: 1000PEGT70PBT30 (□), 1000PEGT80PBT20 (△), and 2000PEGT80PBT20 (○). The water/polymer ratio of the emulsions was varied between 0.5 and 3 ml/g. (n=3; ± s.d.).

Conclusion

The controlled release of large protein such as BSA from scaffolds coated with PEGT/PBT emulsion could not be achieved due to the size of the protein that hampers its diffusion from the polymeric matrix. A known way to tailor the release of proteins from PEGT/PBT copolymers (increasing amounts of water during the preparation of the emulsion) induced increasing amounts of the protein released in a burst fashion. This phenomenon was linked to the formation of a permeation threshold within the polymer matrix resulting from increasing amounts of water in the emulsion. However, the emulsion-coating method proved to be suitable for BSA release by the use of succinate substituted copolymers. The applied protein was not denaturated by the emulsion-coating preparation method.

These results underline the potency of the emulsion-coating method regarding release of active protein, from different type of coated polymers. The different unsuccessful and successful release profiles of BSA from the various coated emulsions used in this study underline the flexibility of the emulsion-coating method regarding different polymers and formulations

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Chapter 4

Water-in-oil emulsions in the design of protein-releasing scaffolds: an evaluation

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Abstract

To obtain well defined macro-porous polymeric scaffolds suitable for cartilage tissue engineering and releasing proteins, water-in-oil emulsions as starting material were evaluated. Two novel methods based on the precipitation of emulsion around paraffin templates were used in view of controlling the scaffold structure, and preventing any protein loss. The resulting scaffolds were evaluated in the light of known requirements for cartilage tissue engineering, with regard to their external and internal structure, mechanical properties, and protein release abilities. A previously described method, based on coating of water-in-oil emulsions on prefabricated scaffolds, was used as comparison. Poly(ether-ester) multiblock copolymers were chosen as polymer matrix for the different scaffolds for their release abilities. The methods produced successfully protein-containing scaffolds of different structures. The organization of the paraffin template was of importance to control the scaffold porosity and interconnection. The scaffold stiffness, measured dynamically, could be adjusted by varying their porosity. All scaffolds obtained from paraffin leaching showed an internal micro-porosity whereas the ones obtained from emulsion-coating were internally dense. A model protein (lysozyme) could effectively be associated with the scaffolds without any loss, and be released without denaturation. Nevertheless, possibly due to the internal micro-porosity of the scaffolds, no long-term release of the protein could be obtained.

Introduction

Tissue engineering aims to repair or replace damaged or worn-out tissues in the body by combining different approaches such as biotechnology, biology and material science [1]. It has been demonstrated that new tissues could be created from living cells and three-dimensional scaffolds [2,3]. The properties of the supporting scaffolds are crucial for the success of these approaches. For instance, the scaffold should possess a high porosity and a good inter-pore connection to allow nutrient diffusion, cell ingrowth and elimination of waste [4]. Its mechanical properties should approach the one of the targeted tissue, and optimally, it should be degraded and replaced by the newly formed tissue. In addition, the scaffolds should support and enhance growth and differentiation of cells.

Although some applications are under clinical evaluation [5], many difficulties remain in obtaining adequate porous structures with all the properties required, especially with respect to enhancement of tissue growth and differentiation. Therefore, recently, novel approaches combining porous supportive structure with bioactive molecules such as growth or differentiation factors have been pursued. As an example, potential candidates could be insulin-like growth factor 1 and 2 (IGF-1, 2), basic fibroblast growth factor (bFGF), transforming growth factors (TGFs), and bone morphogenetic proteins (BMPs) for cartilage and bone applications [6,7]. The relevance of local release of various growth factors from scaffolds has been shown for bone [8-10], cartilage [11,12], and angiogenesis [13-15].

The success of this approach, however, depends on the well-timed delivery of the bioactive compounds from the scaffold, as shown for rhBMP-2 [16,17], bFGF [18], TGF-β_1 [19], and platelet-derived growth factor (PDGF) [20]. Therefore, methods have to be developed to carefully control the delivery rate and kinetics of selected compounds from porous scaffolds. The incorporation of proteins into suitable biodegradable polymer matrices (films, rods or microspheres) offers the possibility to tailor their release over various time periods. However, the methods commonly used to prepare porous scaffolds are not suitable for the incorporation of labile proteins as the use of heat [21], organic solvents [22], or pressure [23] might cause denaturation.

Surprisingly, water-in-oil emulsions (w/o), which is the most common method to associate proteins with polymers and release them in a controlled fashion, has rarely been used to produce porous scaffolds. Poly(L-lactic-co-glycolic acid) polymers (PLGA) w/o emulsions were used in conjunction with freeze-drying or super critical CO_2 techniques to produce scaffolds releasing bFGF, BSA or BMP-2 [9,24,25]. Although protein release was achieved, these methods were not optimal regarding scaffold architecture and protein stability. The pore size of scaffolds obtained from freeze-drying are difficult to control and pore interconnection is poor, whereas super critical CO_2 can induce protein denaturation.

A more promising approach was recently presented, based on the coating of a prefabricated scaffold with a layer of w/o emulsion (emulsion-coating [26]). This method resulted in scaffolds of well-defined structure, allowing a real control on protein release rates. Nevertheless, a considerable part of the w/o emulsion is lost during the process, resulting in a protein loss. This might be problematic when expensive proteins are used.

To overcome the loss of protein during scaffold production, we designed a novel method to create protein-releasing porous scaffolds from w/o emulsions, based on application of paraffin spheres as porogen. Paraffin-based methods have been reported before [27,28], however, never in combination with a w/o emulsion. The combination of paraffin templating and w/o emulsions, could result in well-defined porous polymeric scaffolds releasing proteins in a controlled way, without any protein loss. Hence, the potential use of polymeric w/o emulsions was investigated based on these two novel methods.
As emulsification procedures of Poly lactic acids polymers (PLA) and copolymers are known to cause protein aggregation and activity decrease [29,30], a poly(ether-ester) multiblock hydrogel copolymer was used instead. This biodegradable copolymer, based on poly(butylene terephtalate) and poly(ethylene glycol) (PEGT/PBT), can be successfully used as a release system for proteins [31]. In addition, PEGT/PBT copolymers are known to prevent formation of aggregates when used in w/o emulsions [32]. Its properties (hydrophilicity, elasticity, permeability and biodegradability) can readily be tailored to meet specific requirements. Porous scaffolds based on these polymers have successfully been evaluated for bone [33], cartilage [34] or skin applications [35].
The resulting scaffolds were evaluated with regard to porosity, pore interconnection, internal structure, and mechanical properties, and compared to emulsion coated scaffolds. The ability of the scaffolds to release non-denaturated proteins in a controlled way was evaluated using lysozyme as model protein. A critical examination of the scaffolds was done based on the known requirements for cartilage tissue engineering applications.

Materials and Methods

Materials

Poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) (PEGT/PBT) and Poly(ethylene glycol)-succinate)/poly(butylene succinate) (PEGS/PBS) multiblock copolymers were obtained from Chienna BV, Bilthoven, The Netherlands, and were used as received. Polymers are indicated as aPEGTbPBTc or aPEGSbPBSc in which a is the PEG molecular weight, b the weight percentage (weight %) of Poly(ethylene glycol)-terephthalate or Poly(ethylene glycol)-succinate, and c (=100-b) the weight % of PBT and PBS. Vitamin B_{12}, lysozyme from chicken egg white (3x crystallized, dialyzed and lyophilised), bovine serum albumine (BSA), rhodamine B and *micrococcus lysodeikticus* were purchased from Sigma Chem. corp. (St. Louis, USA). Phosphate buffered saline (PBS), pH 7.4 was obtained from Life Technologies Ltd (Paisley, Scotland). Paraffin was purchased from Fischer chemicals (Loughborough, UK). Poly (vinyl alcohol) (PVA, Mw=22 kg/mole) was obtained from Aldrich Chemicals (Milwaukee, USA). Glycol methacrylate embedding solutions (GMA) were purchased from Technovit (Heraeus Kulzer, Germany). Chloroform and hexane, obtained respectively from Fluka chemica (Buchs, Switzerland) and Merck (Schuchardt, Germany), were of analytical grade.

Preparation of protein-loaded polymeric scaffolds.

Emulsion

The three different types of scaffolds were prepared using a water-in-oil (w/o) emulsion method. An aqueous solution of lysozyme in PBS was emulsified with a PEGT/PBT copolymer solution in chloroform, using an Ultra-Turrax (T25 Janke & Kunkel, IKA-Labortechnik) for 30 s at 19 krpm. The protein concentration of the aqueous solution was fixed at 50 mg/ml for lysozyme. The volume ratio of the aqueous phase was set to 1 ml per gram of copolymer used for protein-loaded scaffolds (water/polymer ratio = 1 ml/g). The copolymer solution was obtained by dissolving one gram of copolymer in 6 ml of chloroform. Three different PEGT/PBT copolymer compositions were used in which the PEGT content was varied from 60 to 80 weight %, with a PEG molecular weight of 1000 g/mol.
To better illustrate the different preparation methods described hereafter, a schematic representation is given in figure 1.

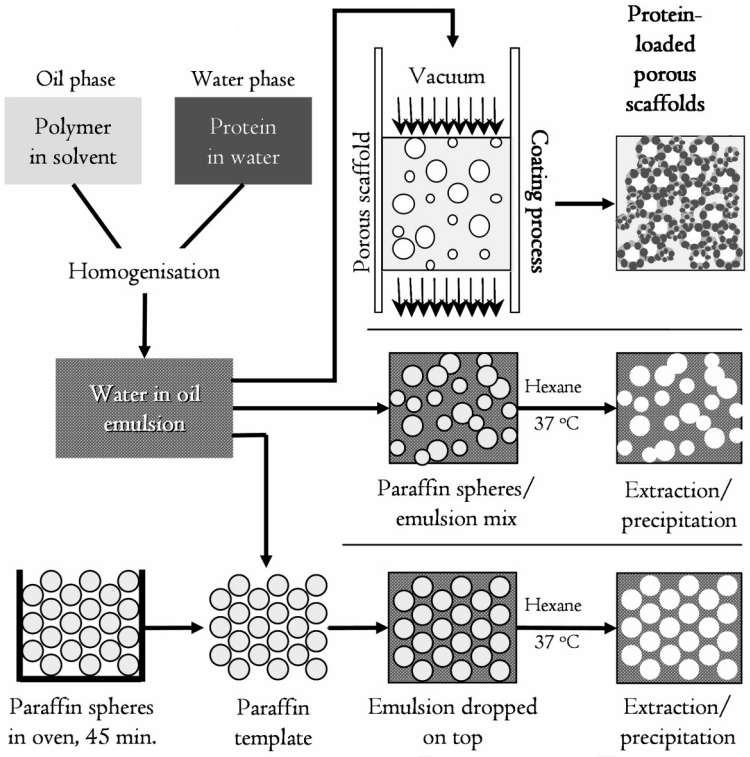


Figure 1: Different emulsion-based methods used to prepare protein containing and releasing scaffolds.

Paraffin microspheres

In order to obtain paraffin spheres of defined diameters, a 0.5% (g/ml) PVA solution was prepared in water for injection and heated to 70°C in a 200 ml beaker. Five grams of paraffin were heated to melt in a glass vial and were added to 150 ml of the PVA solution. The mixture was stirred energetically with a magnetic stirrer for a few seconds. Subsequently, cold water was poured in the suspension to harden the paraffin. The spheres were then collected and sieved in different fractions. Washing of the different fractions to remove the remaining PVA was done during the sieving with double distilled water.

Paraffin-template method

The method described by Ma *et al.* [28] was modified to be used with PEGT/PBT w/o emulsions as follows. The porous scaffolds were prepared by adding 0.75 g of 400 to 600 mm paraffin spheres in a Teflon mold (diameter 1 cm, length 2 cm). Once the spheres were packed together (by tapping gently the mold on a flat surface), the mold was heated at 50°C during 45 minutes in an oven, and cooled back at room temperature. A lysozyme-containing emulsion (obtained as described above) was then slowly added onto the paraffin structure, until the mold was filled, and placed under low vacuum to remove the air trapped inside the paraffin construct. When no bubbles were noticed at the surface, the mold containing the paraffin spheres and the copolymer emulsion was immersed in 200 ml of hexane (thermo-stated at 37 °C). After 30 minutes the scaffold was removed from the mold and placed in a fresh hexane solution to fully precipitate the copolymer matrix and extract the paraffin. The scaffolds were kept in hexane between 45 minutes and 1.5 hour (depending on the copolymer composition used in the emulsion). Finally, they were frozen in liquid nitrogen, and freeze-dried at room temperature for 24 hours.

paraffin-mixing method

The method described by Shastri *et al.* [27] was modified as described hereafter. A lysozyme-containing emulsion was prepared and stirred manually with a spatula till reaching the viscosity of a paste. Then 200 mg, 1.2 g and 1 g of respectively 600 to 1 mm, 300 to 600 μm and 100 to 300 μm paraffin spheres were added and homogenously mixed with the thickened emulsion. Subsequently, the mix was compacted in a cubic Teflon mold (1 cm^3) opened on every face with tiny holes. The mold was immersed in 200 ml of hexane (thermo-stated at 37 °C). After 45 minutes to one hour, the scaffold was removed from the mold and placed in a fresh hexane solution to fully precipitate the copolymer matrix and extract the paraffin. The scaffolds were kept in hexane between 45 minutes and 2 hour (depending on the copolymer composition used in the emulsion). Finally, they were frozen in liquid nitrogen, and freeze-dried at room temperature for 24 hours.To decrease the scaffolds porosity, the copoly-

mer to paraffin ratio was increased.

Emulsion-coating method.

The emulsion-coated scaffolds were obtained as described elsewhere [26]. Briefly, compression molded/salt leached scaffolds (CMSL) were obtained by applying pressure (10000 PSI during 10 minutes) and heat (240 °C) to a homogeneous mix of NaCl salt crystals and copolymer powder in a mold (75 volume %). After cooling of the resultant dense block, the salt was extracted by successive immersions in RX-water (until water conductivity was less than 25 μS). Subsequently, the porous blocks were dried in ambient air and placed in a vacuum oven (50 °C) for a minimum of 12 hours. The PEGT/PBT copolymer used to prepare the scaffold had a PEGT content of 55 weight % and a PEG molecular weight of 300 g/mol. The salt crystals were sieved between 400 and 600 μm.
Coated scaffolds were prepared by forcing a lysozyme containing emulsion through a prefabricated porous scaffold with the use of vacuum (300 mBars). This vacuum was applied for at least 5 minutes, in order to evaporate as much as possible chloroform from the emulsion, thereby creating a polymeric coating on the scaffold. The resulting coated scaffolds were frozen in liquid nitrogen, and freeze-dried at room temperature for 24 hours.
Scaffolds of lower porosity were obtained by increasing the concentration of the polymer phase of the emulsion.

Scanning Electron Microscopy

A Philips XL 30 ESEM-FEG was used to evaluate the internal morphology of the scaffolds. The internal porous structure was observed by cutting the scaffolds in the longitudinal axis with a razor blade. All samples were gold sputter-coated using a Cressington 108 auto apparatus before analysis.

Characterization of scaffold porosity

The average porosity (%) of the scaffolds was evaluated from their dry weight, dry volume and density of the PEGT/PBT copolymer (density = 1.2 g/ml) according to the following equation:

$$p = 1 - \frac{\text{sample weight}}{\text{sample volume} \times 1.2} \qquad \text{(Equation 1)}$$

The scaffold pore interconnection before and after coating treatment was quantified using a method that applies Darcy's law, as described elsewhere [36-38,26]. In brief, a fluid is forced through the porous samples by applying a constant pressure and the flow rate is measured, from which the sample permeability (κ, [39]) can be calculated. This value reflects the sam-

ple porosity and pores interconnectiok; therefore, it can be used to compare different scaffolds.

Coating characterization of emulsion coated scaffolds

The coating was evaluated using fluoroisothiocyanate labelled bovine serum albumin (FITC-BSA) as incorporated protein (12.5 mg/ml of PBS). Samples were embedded in GMA and 10 μm cross-sections made by using a Microm microtome (HM 355 S). Subsequently, the cross-sections were observed by fluorescence microscopy (FITC-Texas red multi-band dual filter, Nikon, Tokyo, Japan). 2 μl of a 1 weight % rhodamine B solution in water were added to 5 ml of GMA-A solution to be able to distinguish the embedding matrix from the prefabricated scaffold under fluorescent light.

Mechanical evaluation

The stiffness of the different scaffolds was evaluated using a tension/compression machine (Z050, Zwick, Germany). Cylindrical scaffolds of diameter 4.7 millimeter and 6.7 mm of length were cyclically loaded between 5 and 20 % strain at a frequency of 1 Hertz. Force amplitude equilibrium was attained within 30 cycles. The dynamic stiffness (Mpa) was calculated by taking the ratio of average force amplitude for the last 10 cycles to cross sectional area, and dividing by the applied strain.

Quantification of protein present in emulsion coated scaffolds

To estimate the amount of protein effectively coated on the prefabricated scaffold, similar coated scaffolds were prepared using a polymer emulsion containing 10 mg of vitamin B_{12} per gram of polymer. The small molecule was completely released within three days from the scaffold when immerged in 10 ml of PBS (completely refreshed three times over three days). The amount of vitamin released was calculated using a standard curve of vitamin B_{12} in PBS and a spectrophotometer (El 312e, BioTek instruments) at 380 nm.

In vitro protein release

Protein loaded scaffolds (50 mg of emulsion coated scaffolds, and 10 mg of paraffin-template ones) were incubated in 1 ml PBS (pH 7.4) at 37 °C. All samples were kept under constant agitation (25 rpm). Samples of the release medium were taken at various time points and the medium was refreshed after sampling. Concentrations were quantified using a standard protein assay (micro bicinchoninic acid (μBCA)) and a spectrophotometer, as described above. The cumulated release of emulsion-coated scaffolds was corrected for the amount of

protein effectively associated to the scaffold.
The activity of released lysozyme was determined using a *Micrococcus Lysodeikticus* assay [40]. To 150 µl of the lysozyme release medium, a suspension of *M. Lysodeikticus* (100 µl, 2.3 mg/ml), was added in a 96-wells microplate. The decrease in turbidity at 37 °C was measured at 450 nm, during 4 minutes at 15 seconds intervals. The initial kinetic rate (OD slope at t=0) was measured for each samples and the protein effective concentration deducted from a fresh standard curve. The lysozyme activity was then obtained by comparison of the concentrations obtained using the protein assay and the *M. Lysodeikticus* assay.

Results

Scaffolds structure

An overview of the scaffolds morphology, as evaluated by Scanning Electron Microscopy (SEM), is presented in Figure 2. The paraffin-template method resulted in scaffolds with a highly open structure (Figure 2A) whereas the scaffolds obtained from the paraffin-mixing and emulsion-coating methods were visually less porous and homogeneous (Figure 2B and Figure 2C), especially for copolymer compositions of higher PBT weight percentage (30 and 40 %).

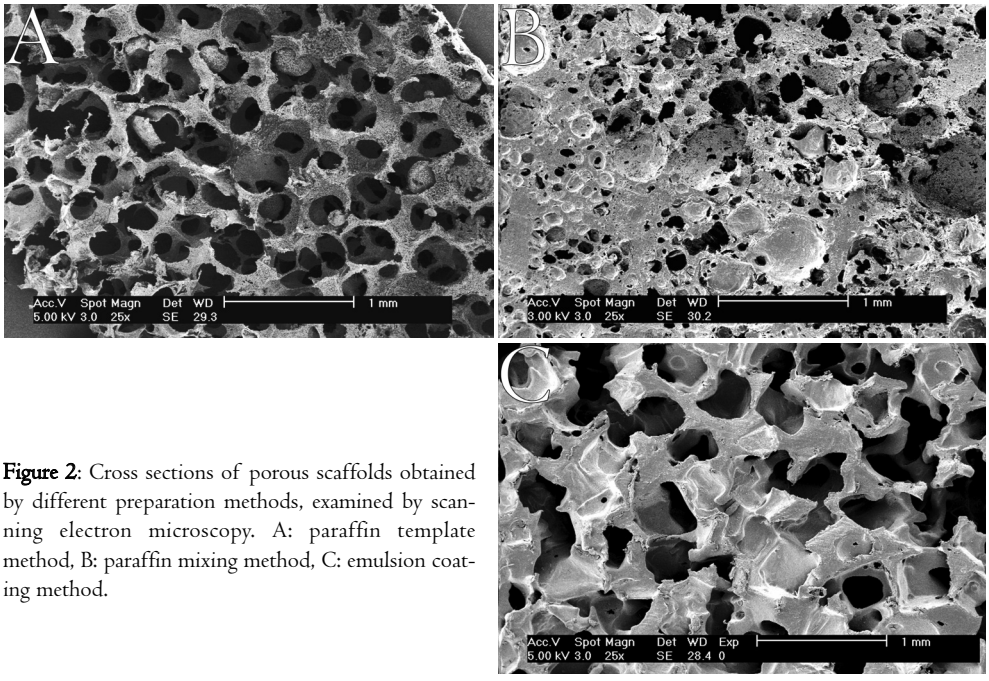


Figure 2: Cross sections of porous scaffolds obtained by different preparation methods, examined by scanning electron microscopy. A: paraffin template method, B: paraffin mixing method, C: emulsion coating method.

These observations were confirmed by the porosity of the scaffolds, as presented in Table I. The paraffin-templated scaffolds showed the highest porosity values (comprised between 89 and 94 %), whereas the emulsion-coated and the paraffin-mixed scaffolds had lower values (respectively comprised between 55-68% and 58-76%). The copolymer composition used in the emulsion influenced the porosity of the scaffolds obtained from the emulsion-coating and paraffin-mixing methods. An increasing PEGT/PBT ratio resulted in higher porosities. A quantification of the permeability of the scaffolds towards water was done to be able to compare the scaffolds with respect to their pores interconnection. Unfortunately, it was not possible to measure the water permeability of the paraffin-template scaffolds because of their foamy and weak structure, but it can be expected that they show the highest interconnection between pores. As shown in Table I, the paraffin-mixed scaffolds were not permeable to water, indicating a low interconnection between pores, with exception of the scaffold with the highest porosity value. The emulsion-coated scaffolds were more permeable than the paraffin mixed and compression molded/salt leached scaffolds prior to coating.

Table I: Porosity (%) and water permeability of the scaffolds (k (10^{-11} m^2)), (n=2; ± s.d.)

	Scaffolds porosity (%)				Water permeability, κ (10^{-11} m^2)			
PEGT/PBT ratio (%) / Method	CMSL[1]	60/40	70/30	80/20	CMSL[1]	60/40	70/30	80/20
Paraffin template[2]	n.a.	92 ± 2	90 ± 4	90 ± 1	n.a.	n.d.	n.d.	n.d.
Paraffin mixing	n.a.	58 ± 2	61 ± 3	76 ± 1	n.a.	0	0	6 ± 1
Emulsion coating	75 ± 1	55 ± 2	64 ± 2	68 ± 1	2 ± 1	6 ± 1	12 ± 2	11 ± 1

[1] CMSL scaffolds: compression molded/salt leached scaffolds prior coating.

[2] The permeability of the paraffin template scaffolds could not be measured due to their weak structure.

n.a.: not applicable; n.d. : not determined

Scaffolds surface and internal structure

Cryo-fracture of the scaffolds showed a clear difference between paraffin-based and emulsion-coated scaffolds regarding pores surface and internal organization at high magnification (Figure 3). Regardless of copolymer composition, the pores surface of paraffin-templated and mixed scaffolds was dense with irregular roughness whereas emulsion coated scaffolds presented a strong micro-porosity (diameter of the micro-pores ranging from 0.2 to 4 μm). The opposite structure was seen when considering the internal organization of the scaffolds. paraffin-templated and mixed scaffolds were fully microporous whereas only the surface of the emulsion-coated scaffolds was porous (depth not higher than 3 μm). The thickness of the emulsion-coating applied on the scaffold was evaluated by preparing scaffolds containing fluoroisothiocyanate labelled bovine serum albumin (FITC-BSA) as fluorescent marker. Cross-sections revealed that the thickness of the coating applied was in average comprised

between 4 and 346 μm (Figure 4).

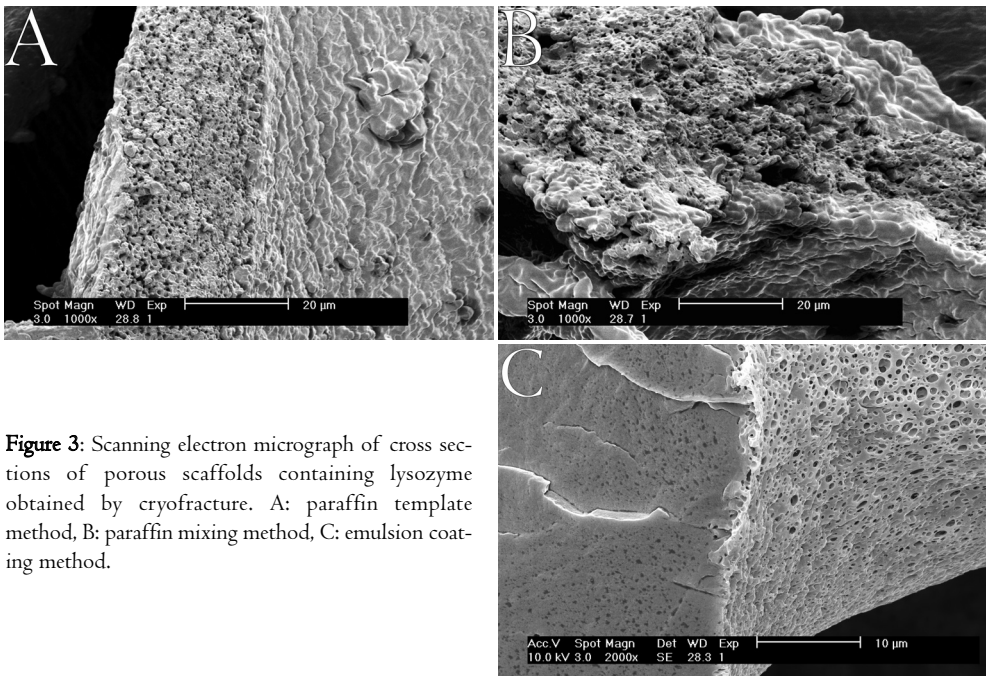


Figure 3: Scanning electron micrograph of cross sections of porous scaffolds containing lysozyme obtained by cryofracture. A: paraffin template method, B: paraffin mixing method, C: emulsion coating method.

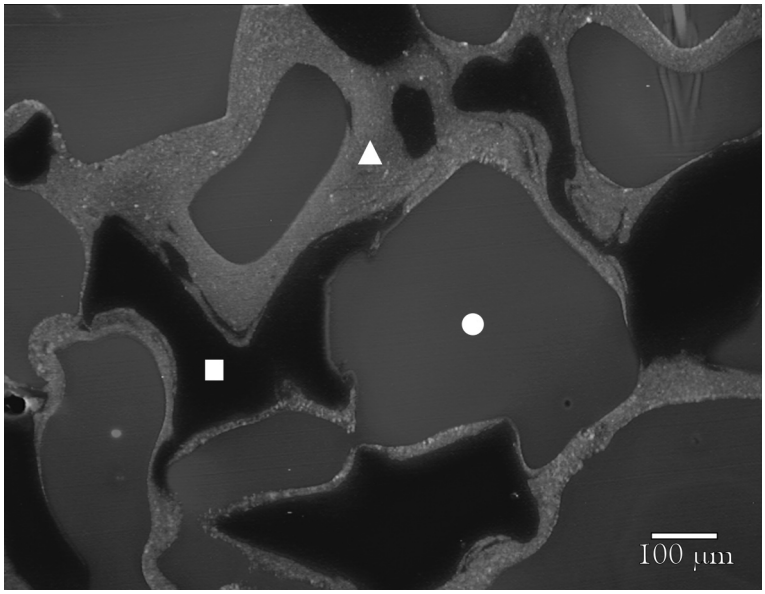
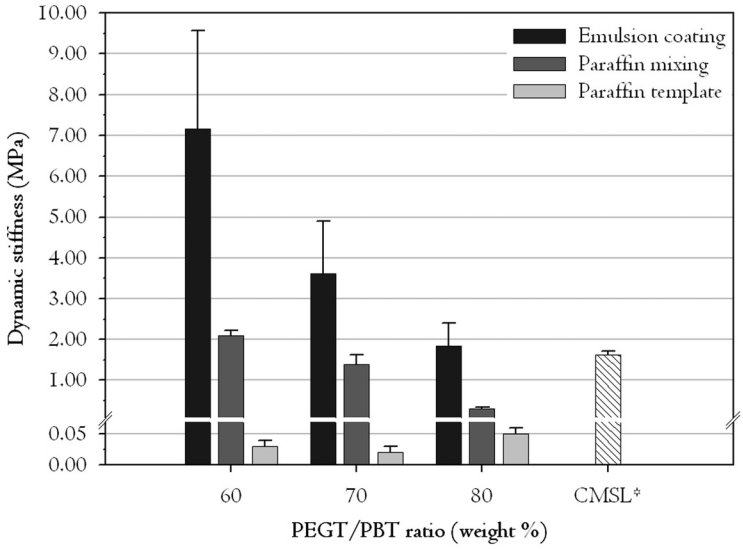


Figure 4: Optical fluorescent micrograph of cross sections of emulsion-coated scaffolds. The emulsion coating contained FITC-BSA (▲) and the GMA embedding rhodamine (●). The polymer appears as black (■).

Scaffolds mechanical properties

The stiffness of the different scaffolds was measured under dynamic conditions. With respect to scaffold preparation methods, the paraffin template showed the lowest dynamic stiffness (comprised between 30 and 50 Kpa) as compared to the two other methods (Figure 5). Emulsion coating resulted in the stiffest scaffolds (dynamic stiffness between 1.9 and 7.2 Mpa) and the paraffin-mixed scaffolds had intermediate mechanical properties (dynamic stiffness between 0.3 and 2.1 Mpa). An effect of the scaffolds copolymer composition was noticed on the paraffin-mixed and emulsion-coated scaffolds, as an increasing amount of PBT weight % resulted in higher stiffness. On the contrary, the paraffin-templated scaffolds did not show the same behavior. It has to be mentioned that the sensitivity of the apparatus used was probably not sufficient to establish differences within the low values measured for the paraffin-templated scaffolds.



*CMSL: compression molded/salt leached scaffolds

Figure 5: Dynamic stiffness of porous scaffolds obtained by different preparation methods and by varying the matrix copolymer compositions (MPa) (n=4 ± s.d.).

Controlled release of protein

To evaluate the ability of the paraffin-leached scaffolds to release proteins in a controlled fashion, different templated and mixed paraffin scaffolds were prepared in which the PEGT weight % of the copolymer was varied. The release of the protein was quantified and the enzymatic activity measured. In addition, the protein yield (expressed as the percentage of protein used during preparation that was incorporated in the scaffold) of the different methods was measured. The paraffin-leached method had a protein yield of 100 %, whereas the

emulsion-coating scaffolds showed a loss of 87% of the protein applied. As presented in Figure 6, a complete lysozyme release was obtained from the paraffin-templated scaffolds within 3 days

The three different copolymer compositions used as scaffold matrix showed little differences in release rates, which were characterized by an important burst during the first 24 hours of release (up to 100 %). The same type of release profiles were obtained from the paraffin-mixed scaffolds. The lyzosyme release was completed within 3 to 6 days for the different matrix copolymer compositions, with a burst release during the first day. This contrasts with the emulsion-coated scaffolds, for which lysozyme release was completed within 20 days for a PEGT weight % of 80 %, and still ongoing after one month for a PEGT weight % of 60 %.

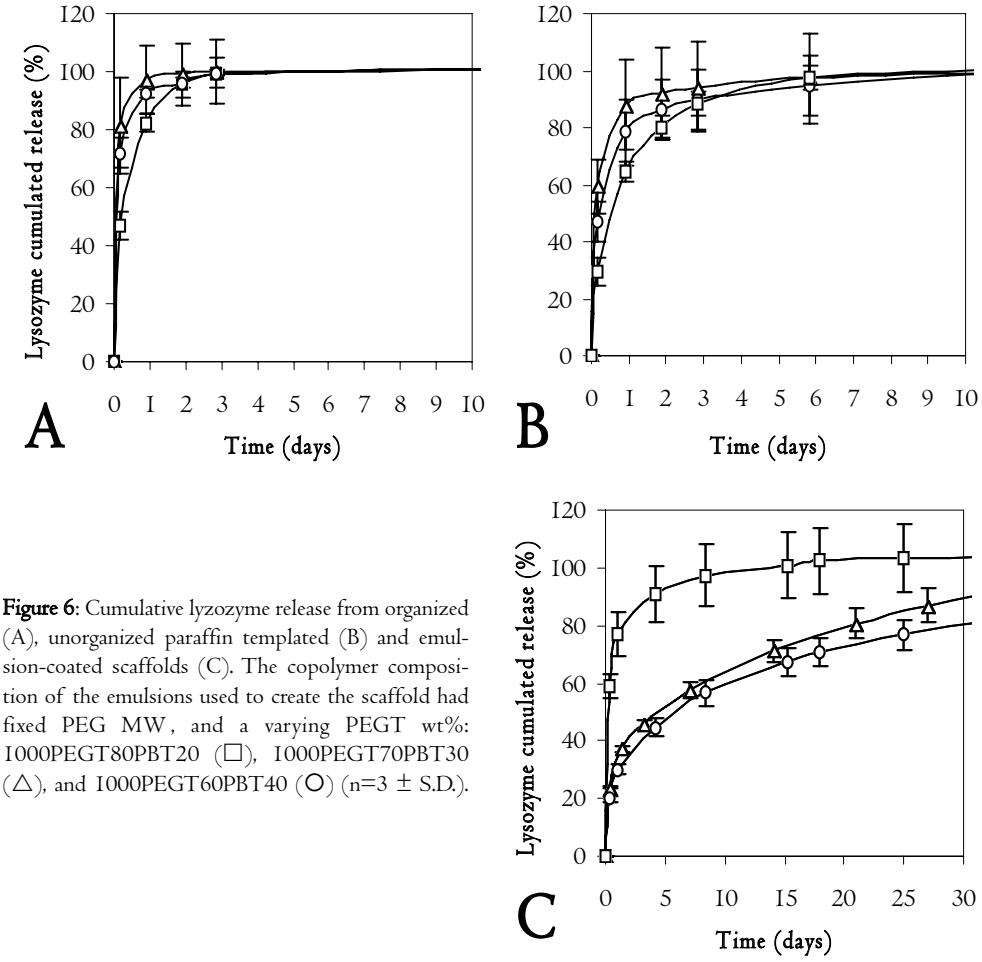


Figure 6: Cumulative lyzozyme release from organized (A), unorganized paraffin templated (B) and emulsion-coated scaffolds (C). The copolymer composition of the emulsions used to create the scaffold had fixed PEG MW, and a varying PEGT wt%: 1000PEGT80PBT20 (□), 1000PEGT70PBT30 (△), and 1000PEGT60PBT40 (○) (n=3 ± S.D.).

Subsequently, the activity of the released lysozyme was investigated. As shown in Figure 7, the protein activity was constant and close to 100 % during the release from the paraffin-templated scaffolds.

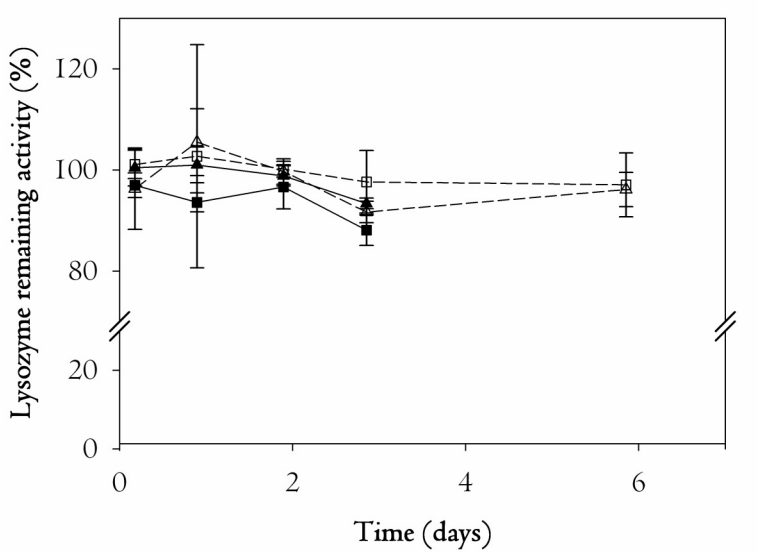


Figure 7: Enzymatic activity of lysozyme released from organized and unorganized paraffin templated scaffolds (respectivel full and empty symbols), of different copolymer compositions: 1000PEGT60PBT40 (□, ■) and 1000PEGT80PBT20 (▲, △).

Discussion

The aim of this study was to prepare protein-releasing scaffolds suitable for cartilage regeneration. Such scaffolds should meet specific requirements [41,42]. The porosity and pore interconnection should be sufficient to allow cell growth, maximize nutrient/waste exchange and limit oxygen gradients. The surface structure should be suitable for cell attachment, proliferation and cartilage differentiation. The mechanical properties should be able to match the surrounding native tissue and mediate mechanical stimulus to cells. Optimally, bioactive factors should be delivered at determined rates to further maintain or induce cartilage phenotype.

Whereas the preparation of well-defined porous structures and of controlled release systems is feasible, the combination of the two is difficult with conventional methods. Therefore, we developed novel methods to design such scaffolds based on the use of water-in-oil emulsions. The use of paraffin particles as pore forming agent was compared with coating of prefabricated porous scaffolds. The structures obtained were evaluated with respect to morphology, mechanical properties and release behavior.

The use of an emulsion in combination with paraffin did not hamper the formation of

porous scaffolds, as can be seen in Figure 2-A and Figure 2-B, indicating that this biphasic system reacted to the precipitation treatment in a similar way as a plain polymer solution. The size of the pores correlated with the size of the paraffin particles, however, each of the methods resulted in a different scaffold structure, regarding porosity and interconnection. Concerning the paraffin-template method, the scaffolds porosity was in direct agreement with the one obtained by Ma *et al.* with plain PLLA solutions [28]. The proportion between the volumes of paraffin and emulsion explains the high porosity observed. The structure of the fused paraffin is clearly reflected in the excellent pore interconnection of the resulting polymer scaffolds (Figure 2-A). On the contrary, the paraffin-mixed scaffolds showed in most cases low pore interconnection (Figure 1-B). The porosities observed were lower than the one obtained from plain polymer solutions, as presented by Shastri *et al.* using the same conditions [27]. The viscosity of the PEGT/PBT emulsions may have prevented adequate mixing of the paraffin spheres with the emulsion. This underlines the limitation of this method when used with polymeric emulsions, as high pore interconnection is of utmost importance for tissue engineering applications, to allow nutrient diffusion, cell ingrowth and waste elimination [4,43].

The emulsion-coating method is not based on the use of a porogen to create porous scaffolds and is related to the properties of the compression molded-salt leached scaffold on which the coating is applied. The porosity decrease observed in all cases after treatment is due to the coating of the emulsion layer on top of the pores. The increase of pore interconnection noticed is linked to the process of emulsion-coating which opens the pores by dissolving the thin polymeric membranes in between [26].

The cryo-fracture of the scaffolds revealed a consistent internal micro- porosity (Figure 3). This is possibly caused by the precipitation of the copolymer matrix (in hexane). It is known that the phase inversion of a polymer solution by immersion in a non solvent (here hexane) results in a micro-porous matrix [44,45,27,28]. Although the emulsion used here is a biphasic system (polymer and aqueous phase), the polymer phase is continuous. Therefore, the same phenomenon might occur, resulting in microporosity throughout the all scaffold structure. On the contrary, for the emulsion-coated scaffolds, the vacuum applied on the scaffolds during the treatment might induce a different polymer precipitation pattern. As the surface of the emulsion is directly subjected to the surrounding vacuum, a fast solvent evaporation and accordingly phase separation of the copolymer will be induced, ensuing in a microporous surface. The chloroform present deeper in the coating will be extracted in a more slowly fashion because not directly in contact with the vacuum, therefore creating a denser and more homogeneous matrix. Cross sections of FITC-BSA loaded scaffolds revealed that the majority of the coating was dense (Figure 4). The presence of an irregular and rough surface could be beneficial to sustain chondrocyte phenotype [46].

To efficiently regenerate cartilage tissue, the stability of the newly formed tissue has to be assured by the scaffold. In addition, mechanical inputs should be transmitted to the cells for a good tissue organization [47-49]. Therefore, the stiffness of the different scaffolds was measured under dynamic conditions and the dynamic stiffness of human articular femoral cartilage (measured at 1 Hz by Treppo *et al.* [50]) was considered as bench mark (4.5 MPa).

In this context, the low dynamic moduli of the paraffin-templated and paraffin-mixed scaffolds may prevent their applications in cartilaginous sites. On the other hand, emulsion coated scaffolds showed values similar or higher than 4.5 MPa. In addition to the inherent mechanical properties of the copolymer used as matrix, a way to modulate the compressive strength of porous scaffolds consists of varying the porosity, which is linked to the compressive strength by a power-law relation [51,52,34]. To assess this with our structures, we prepared scaffolds of different copolymer compositions and porosity values, using the paraffin-mixing and emulsion-coating methods. As presented in Figure 8, a power-law relation was indeed found between porosity and dynamic stiffness for the different copolymer compositions tested. Nevertheless low porosity values (lower than 50 %) were necessary for paraffin-mixed scaffolds to reach 4.5 MPa, thus reducing their potential use for cartilage tissue engineering.

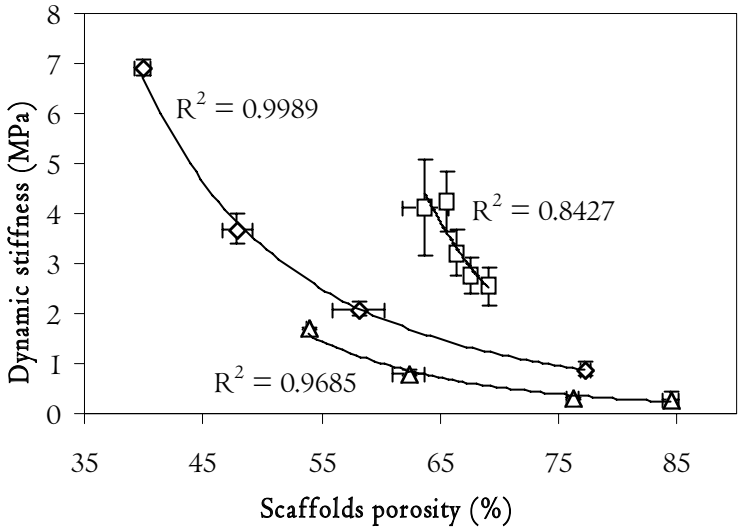


Figure 8: Relation of porosity and dynamic stiffness of porous scaffolds obtained by paraffin mixing method from different copolymer compositions: 1000PEGT80PBT20 (△) and 1000PEGT60PBT40 (◇), and by emulsion coated scaffolds from 1000PEGT70PBT30 copolymers of different viscosities (□) (n=2 ± s.d. for porosity values and n=4 ± s.d. for dynamic stiffness values).

As the main purpose of using water-in-oil emulsion to prepare porous scaffolds is the incorporation and release of proteins, the ability of the emulsion-based scaffolds to release a model protein (lysozyme) in a controlled fashion was investigated. For emulsion-coated scaffolds, although the method results in a loss of the applied protein, a successful release can be obtained [26]. It was shown that the release of the protein could be tailored by varying the PEGT weight % of the coating copolymer composition. As discussed elsewhere, the release of proteins from PEGT/PBT copolymers is due to a combination of primarily diffusion and polymer degradation [31]. The diffusion coefficient of lysozyme is linked to the swelling behavior of the copolymer [53]. Therefore, an increase of copolymer swelling (by increasing

the PEGT weight %) results in higher protein diffusion coefficient through the matrix and faster release rates. Surprisingly, the paraffin-leached scaffolds did not follow this pattern, as presented in Figure 6. The variation of the copolymer composition did not permit to control significantly the release rate of lysozyme. The similar release profiles of both type of scaffolds clearly indicates that another mechanism plays a role, preventing to control the protein release rate by varying the copolymer composition. The fast release observed is most probably correlated to the internal microporosity of the scaffolds matrices as described above. The microporous structure caused by the precipitation of the emulsion in hexane shortens the diffusion length within the copolymer matrix. As the release of a protein from PEGT/PBT matrices is mainly diffusion driven during the first days of release [31], a decrease of diffusion length will reduce the influence of the copolymer compositions on the protein release profile. This result in the similar fast lysozyme release observed for the different copolymer composition used. The lack of control on the release rate hampers the potential use of these scaffolds for applications requiring a well defined and long term release of proteins. Alternatively, the paraffin-leached methods do not induce any protein loss, and could be of interest for applications requiring a burst-like or fast release.

Although the emulsification procedure to prepare the various scaffolds is known to cause protein aggregation and decrease of activity with other polymers [29,30], the intact activity of the released protein indicated that lysozyme was not irreversibly aggregated or denaturated during the scaffold preparation or the release period. This is in agreement with previous studies that showed the stability of proteins when entrapped in PEGT/PBT copolymers [31,32]. In addition, the use of hexane was not found detrimental for the protein.

With a view to evaluating the scaffolds for cartilage applications, their characteristics are summarized in Table 2. Both paraffin based methods resulted in porous scaffolds lacking important features for cartilage tissue engineering. While paraffin-templated scaffolds were highly porous and interconnected, their mechanical properties prevent them to be used for load-bearing applications. On the contrary, paraffin-mixed scaffolds could attain the required mechanical strength but, as a result, their porosity and pore interconnection was low.

Table 2: Overview of scaffolds properties.

Properties / Scaffold type	Pores surface	Porosity	Pore inter - connection	Dynamic Stiffness	Release of proteins
Paraffin templated	Rough	High (90 %)	High	Low Not suitable for load - bearing applications	Burst like Not controllable
Paraffin mixed	Rough	Broad range (40 to 85 %)	Low to null	Broad range Inversely related to porosity	Burst like Not controllable
Emulsion coated	Micro-porous	Intermediate (60 to 70 %)	High	High In the range of native cartilage	Controllable over one month

Furthermore, the release rate of proteins could not be controlled and only a burst release was achieved. In view of these drawbacks, the emulsion coated scaffolds appear to be the most suitable for cartilage tissue engineering applications, provided that the protein loss during preparation is reduced. Future studies will focus on the use of these scaffolds with relevant growth factor for cartilage tissue engineering such as TGF-β_1.

Conclusions

This study showed that poly(butylene terephtalate)/poly(ethylene glycol) water-in-oil emulsions could be used in conjunction with paraffin templating methods to design defined porous scaffolds containing proteins. The two methods used, based on the precipitation of the emulsions in hexane around an organized or unorganized paraffin template, resulted in scaffolds of different morphology, porosity and pore interconnection. The structures obtained from organized paraffin templates showed well defined and controlled porosity and pore interconnection. On the contrary unorganized templated scaffolds were less controllable, especially regarding pore interconnection, which was low. The mechanical properties of the scaffolds were correlated to the porosity by a power law. As a result, the paraffin-templated scaffolds showed low dynamic stiffness values, preventing their use for load-bearing sites. Possibly due to the precipitation of the emulsions in hexane, the surface of the paraffin-templated scaffolds was dense whereas their internal structure was microporous. The opposite was seen for the emulsion-coated scaffolds. This discrepancy of internal structure had consequences on the release rate of lysozyme from the different scaffolds. Burst release were obtained from the paraffin-templated scaffolds (microporous internal structure), whereas the emulsion-coated ones allowed a slow delivery comprised between 20 days to more than one month by varying the copolymer composition of the coating (dense internal structure). On the other hand, while the emulsion-coating method induced a protein loss, the paraffin based scaffolds showed a 100% entrapment efficiency. The lack of control on the release rate of proteins from the paraffin based scaffolds hamper their use for long-term release applications. The applied protein was not denaturated by any of the preparation methods used, demonstrating the interest of w/o PEGT/PBT emulsions.

As to cartilage tissue engineering, paraffin based scaffolds obtained from w/o emulsions showed important deficiencies, especially regarding mechanical properties, pore interconnection or ability to control protein release rate. In this context, emulsion coated scaffolds were selected for further experiments with relevant growth factors for cartilage applications.

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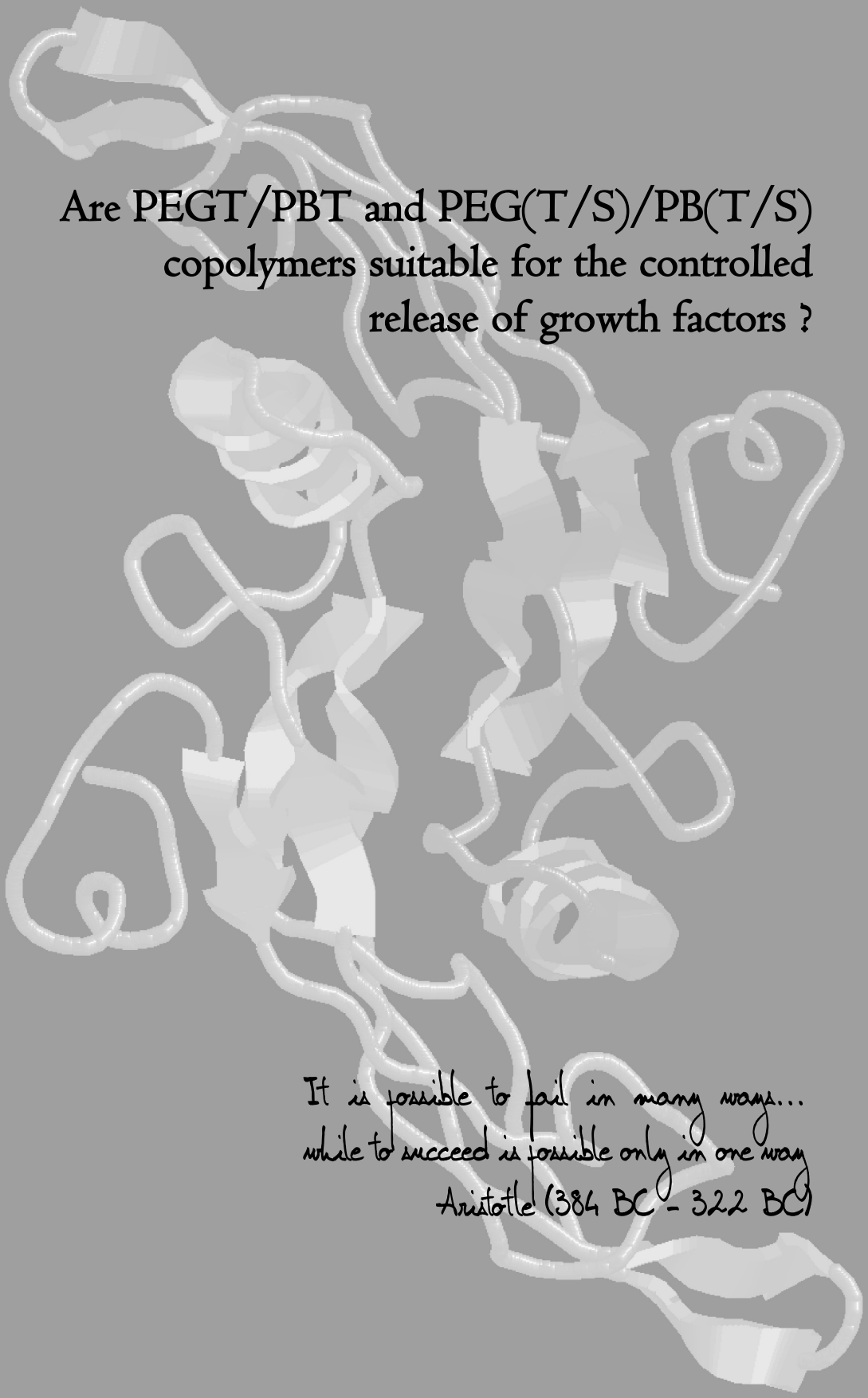
Are PEGT/PBT and PEG(T/S)/PB(T/S) copolymers suitable for the controlled release of growth factors ?



It is possible to fail in many ways...
while to succeed is possible only in one way
Aristotle (384 BC - 322 BC)

Chapter 5

Tailored release of TGF-β1 from porous scaffolds and potential for cartilage tissue engineering

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Abstract

In view of cartilage tissue engineering, the possibility to prepare porous scaffolds releasing transforming growth factor-β1 (TGF-β1) in a well controlled fashion was investigated by means of an emulsion-coating method. Poly(ether–ester) multiblock copolymers were used to prepare emulsions containing TGF-β1 which were subsequently applied onto prefabricated scaffolds. This approach resulted in defined porous structures (66 %) with interconnected porosity, which were suitable to allow tissue ingrowth and migration of progenitor cells in osteochondral defects. The scaffolds were effectively associated with TGF-β1 and allowed to tailor precisely the release of the growth factor from 12 to more than 50 days by varying the copolymer composition of the coating. The increase of copolymer hydrophilicity and degradation rate resulted in faster release rates of the protein. The growth factor retained its biological activity as was assessed by a cell proliferation assay and by the ability of the released protein to induce cartilage differentiation of bone marrow-derived

mesenchymal stem cells. Therefore, these scaffolds appear promising candidates for cartilage tissue engineering applications requiring precise release rates of TGF-β_1.

Introduction

In tissue engineering approaches, the possibility to create new tissues or functional organs usually requires the use of three-dimensional scaffolds as guide and support structures [1]. In addition to the classical requirement such as high porosity and inter-pore connection, specific mechanical properties and degradation rates [2,3], the scaffolds should have the potency to support, enhance or even induce the growth and differentiation of cells or tissue towards the desired lineage. To do so, porous scaffolds could act as a release matrix for bioactive molecules such as growth and differentiation factors or cytokines. Different molecules can be considered that showed their interest for cartilage and bone applications (insulin-like growth factor I and 2, basic fibroblast growth factor, transforming growth factors, and bone morphogenetic proteins) [4,5].
Promising data were previously reported showing the relevance of local release of various growth factors from scaffolds for bone, cartilage, and angiogenesis [6-9]. However, the well-timed delivery and suitable dosing of the compounds appears to be of high importance to achieve an optimal tissue induction while avoiding adversary or inhibitory effects [10-15]. Therefore, methods must be investigated to achieve a precise control of the release kinetics of selected compounds from porous scaffolds.
Transforming growth factor-β_1 (TGF-β_1) is a pleiotropic growth factor which has regulatory effects on many different cell types. For instance, it plays an important role in cell proliferation and differentiation, bone formation [16-18], angiogenesis [19,20], neuroprotection [21] and wound repair [22-24]. It controls the production of extracellular matrices by stimulating the synthesis of collagens, fibronectin and proteoglycans [25,26]. It also appeared to have positive effects on cartilage differentiation and repair [27-30]. Nevertheless, this multipotency induces drawbacks linked to the dependency of the tissue responses towards its dose and length of exposure. For instance, a long exposure of high doses of TGF-β_1 results in fibrosis and hypertrophic scars [31], while a too high dosage in cartilaginous sites results in osteophytes formation [30]. Therefore, the ability to release TGF-β_1 in a controlled fashion is of high importance to use this protein in the most optimal way for cartilage applications. Hence, the opportunity to create scaffolds allowing a wide range of TGF-β_1 release periods (from days to months) was here investigated.
A potential approach to create TGF-β_1 releasing scaffolds is based on the coating of prefabricated porous polymeric scaffolds with protein-containing emulsions. This method has been successfully applied to control the release of a model protein (lysozyme) [32]. Nevertheless, lysozyme is a relatively stable molecule while TGF-β_1 is extremely labile. Therefore, in addition to the ability of the method to produce scaffolds with broad release rates, the activity of the released TGF-β_1 was investigated. Poly(ether-ester) multiblock hydrogel copolymers were selected as matrix for prefabricated scaffolds and emulsions. These biodegradable hydrogels, based on poly(ethylene glycol)-terephtalate and poly(butylene terephtalate) (PEGT/PBT),

and poly(ethylene glycol)-succinate and poly(butylene succinate) (PEG(T/S)PB(T/S)), are successfully used as protein release system as they allow to tailor release rates easily by varying the copolymer composition [33,34]. It was demonstrated that the protein release was controlled by a combination of diffusion and degradation of the polymeric matrix [35,36]. The resulting scaffolds were evaluated with regard to their structure, TGF-β1 release capacities, stability of the released protein, and their potential interest for cartilage tissue engineering.

Materials and Methods

Materials

Poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) (PEGT/PBT) and poly(ethylene glycol)-succinate)/poly(butylene succinate) (PEGS/PBS) multiblock copolymers were obtained from OctoPlus, Leiden, The Netherlands, and were used as received. Polymers are indicated as aPEGTbPBTc or aPEG(T/S)bPB(T/S)c (dT/eS) in which a is the PEG molecular weight, b the weight percentage (weight %) of PEGT or the combined weight % of PEGT and PEGS, and c (=100-b) the weight % of PBT or the combined weight % of PBT and PBS. d/e is the molar T/S ratio in the copolymer. Vitamin B_{12}, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), L-ascorbic acid-2-phosphate, proline, insulin-transferrin-selenium (ITS+1), and dexamethasone were purchased from Sigma Chem. corp. (St. Louis, USA). Recombinant human transforming growth factor β-1 (rhTGF-β1 later referred as TGF-β1) and enzyme-linked immunosorbent Assay (ELISA) kit were purchased from R&D Systems Inc. (Minneapolis, USA). Dulbecco and alpha modified eagle medium (DMEM and α-MEM), pyruvate, L-glutamine, penicillin and streptomycin were obtained from Gibco (Invitrogen, Carlsbad, USA). Roswell park memorial institute medium (RPMI 1640) and foetal bovine serum (FBS) were purchased from Cambrex (East Rutherford, USA). Glycol methacrylate embedding solutions (GMA) were purchased from Technovit (Heraeus Kulzer, Germany). Basic-fibroblast growth factor (bFGF) was obtained from VWR international (Roden, The Netherlands). Chloroform, obtained from Fluka chemica (Buchs, Switzerland), was of analytical grade.

Preparation of TGF-β1-loaded polymeric scaffolds.

Emulsion

The protein was associated to the porous scaffolds by means of a water-in-oil (w/o) emulsion method. An aqueous solution of TGF-β1 in a 4 mM HCL solution (with 1 mg/ml BSA, according to the supplier's protocol) was emulsified with a PEGT/PBT or

PEG(T/S)PB(T/S) copolymer solution in chloroform, using an Ultra-Turrax (T25 Janke & Kunkel, IKA-Labortechnik) for 30 s at 19 krpm. The TGF-β_1 concentration of the aqueous solution was set at 1 μg/ml for release and bioactivity experiments, and 20 μg/ml for cell culture and in vivo experiments. The volume of the aqueous phase was set to 1 ml per gram of copolymer (water/polymer ratio = 1 ml/g). The copolymer solution was obtained by dissolving 0.5 gram of copolymer in 3 ml of chloroform. Three PEGT/PBT and two PEG(T/S)PB(T/S) copolymer compositions were used in which the PEGT content was of 70 or 80 weight %, the PEG MW of 600, 1000 or 2000 g/mol, and the T/S molar ratio varied between 0 and 100 %.

Emulsion-coating method.

The emulsion-coated scaffolds were obtained as described elsewhere [32]. Briefly, compression molded/salt leached scaffolds were obtained by applying pressure (10000 PSI during 10 minutes) and heat (240 °C) to a homogeneous mix of NaCl salt crystals and copolymer powder in a mold. The volume fraction of salt in the mixture was adjusted to 75 %. After cooling of the resultant dense block, the salt was extracted by successive immersions in RX-water (water conductivity less than 25 μS). Subsequently, the porous blocks were dried in ambient air for at least 24 hours, and then placed in a vacuum oven (50 °C) for a minimum of 12 hours. The PEGT/PBT copolymer used to prepare the scaffold had a PEGT content of 55 weight % and a PEG molecular weight of 300 g/mol. The salt crystals were sieved between 400 and 600 μm.

Coated scaffolds were prepared by forcing a TGF-β_1-containing emulsion through a prefabricated porous scaffold with the use of vacuum (300 mBars) [32]. This vacuum was applied for at least 5 minutes, in order to evaporate chloroform as much as possible from the emulsion, thereby creating a polymeric coating on the scaffold. The resulting coated scaffolds were frozen in liquid nitrogen and freeze-dried at room temperature for 24 hours.

Blank scaffolds were prepared by using a TGF-β_1-free 4 mM HCL solution (with 1 mg/ml BSA) in the same conditions as TGF-β_1 containing scaffolds.

Characterization of scaffold porosity

The average porosity (%) of the scaffolds was evaluated from their dry weight, dry volume and density of the PEGT/PBT copolymer (density = 1.2 g/ml) according to the following equation:

$$p = 1 - \frac{\text{sample weight}}{\text{sample volume} \times 1.2} \qquad \text{(Equation 1)}$$

Three scaffolds pieces (8x4x4 mm) were used to determine the porosity of a scaffold.

The scaffold pore interconnection before and after coating treatment was quantified using a method that applies Darcy's law, as described elsewhere [37-39,32]. In brief, water is forced

through the porous samples by applying a constant pressure and the flow rate is measured, from which the sample permeability (κ, μm^2) can be calculated. This parameter reflects the sample porosity and pore interconnection and can therefore be used to compare different scaffolds.

Scanning Electron Microscopy

A Philips XL 30 ESEM-FEG was used to evaluate the internal morphology of the scaffolds. The internal porous structure was observed by cutting the scaffolds in the longitudinal axis with a razor blade. All samples were gold sputter-coated using a Cressington 108 auto apparatus before analysis.

In vitro protein release

TGF-β_1 loaded scaffolds (around 100 mg) were incubated in 1 ml RPMI 1640 medium at 37 °C in polypropylene tubes. All samples were kept under constant agitation (25 rpm). The release medium was entirely refreshed at various time points, immediately frozen in liquid nitrogen and conserved at -20 °C until quantification. TGF-β_1 concentrations were quantified using an ELISA kit obtained from R&D Systems (Quantiquine human TGF-β_1 immunoassay). The TGF-β_1 used for the standards and the preparation of the releasing scaffolds originated from the same batch. Aliquots of different volumes were frozen in liquid nitrogen immediately after reconstituting the protein solution and stored at -20°C. They were thawed immediately prior to use for scaffold preparation or as standards.
To determine the quantity of emulsion effectively coated on the porous scaffold and establish the amount of protein present, coated scaffolds were prepared in the same conditions using polymer emulsions containing 10 mg of vitamin B_{12} per gram of polymer, for each copolymer composition used. The size of this molecule allows a complete release within three days when entrapped in the copolymers used in this study. The quantity of vitamin released is correlated to the amount of polymer coated onto a given scaffold, as the vitamin is homogeneously distributed through the emulsion. The amount of polymer coated can then be related to the amount of protein associated with the scaffold. This indirect detection method was proven to be accurate for other proteins [32]. The amount of vitamin released was calculated using a standard curve of vitamin B_{12} in phosphate buffered saline and a spectrophotometer (El 312e, BioTek instruments) at 380 nm.

TGF-β_1 stability in solution

The stability of TGF-β_1 in the release or culture medium was assessed by measuring the protein concentration with ELISA as a function of time (from 20 minutes to 6 days). For absolute concentration decrease, fresh TGF-β_1 was added at a concentration of 5 and 10

ng/ml to the release or culture medium (1 ml) containing unloaded scaffolds (1000PEG(T/S)70PB(T/S)30 (0T/100S)) and 2000PEGT80PBT20. At each time interval, the medium was collected in triplicate and assayed for concentration. The unloaded scaffolds were then discarded.

TGF-β_1 bio-activity assay

The activity of released TGF-β_1 was determined using a modified cell growth inhibition assay based on Mv 1 Lu mink lung fibroblast (ATCC# CCL64) [40]. CCL64 cells were cultured in DMEM supplemented with 10 volume % FBS, 100 UI/ml penicillin and 100 µg/ml streptomycin. The cells were always kept sub-confluent. For the growth inhibition assay, CCL64 cells were seeded at a density of 1 x 10^4 cells/well in 48-well plates and subsequently let to attach for 3 hours at 37°C in a 5% CO_2 humidified atmosphere. The cell culture was performed with 1 ml of RPMI 1640, supplemented with 10% v/v FBS, 100 UI/ml penicillin and 100 µg/ml streptomycin. After 3 hours, releasing scaffolds (100 mg) or standards of known TGF-β_1 concentration were added in single or multiple boluses to the wells containing cells, in duplicate on the same plate. A schematic drawing of the different conditions assayed is presented in Figure 1. Each culture well contained porous scaffolds (100 mg) of similar coating composition for a given plate, either loaded with TGF-β_1 (releasing) or unloaded. The plates were then incubated for 40 hours and the relative amount of cells was evaluated using an alamar blue assay (200 µl added per well and incubated for 4 hours prior reading).

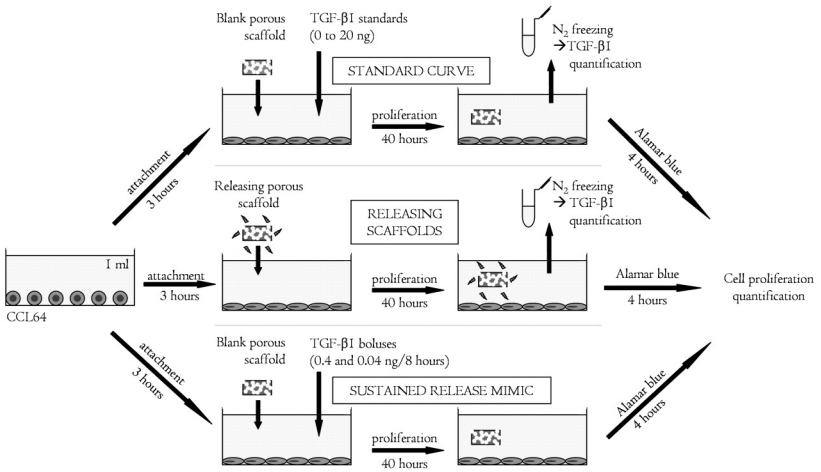

Figure 1: Schematic representation of the different conditions used to evaluate the bioactivity of the released TGF-β_1 in a CCL64 growth inhibition assay.

The linearity of the alamar blue response towards cell number was assessed for the range of the bioassay, by incubating cells dilutions from 0 to 8 x 10^5 cells/well over 40 hours. Each assay was performed with a standard curve of fresh TGF-β_1 comprised between 0 and 20 ng/ml. The activity of the protein was defined as the ratio between concentrations obtained from the standards and concentration released from the scaffolds. The same batch of TGF-β_1 was used for the standards and the preparation of the releasing scaffolds. Aliquots of different volumes were frozen in liquid nitrogen immediately after reconstituting the protein solution and stored at -20°C. They were thawed immediately prior to use for scaffold preparation or as standards.

Goat mesenchymal stem cells pellet culture

GMSC were harvested from the iliac crest of 4 years old female dutch milk goats, under general inhalation anaesthesia. The bone marrow aspirate was collected in heparin tubes. The nucleated cells were plated at a density of 5 x 10^5 cells/cm^2 in α-MEM supplemented with 12 % FBS, 100 UI/ml penicillin, 100 µg/ml streptomycin, 0.1 mM L-ascorbic acid-2-phosphate, 1 ng/ml bFGF and 2mM L-glutamine. The medium was refreshed first after 3 days and then twice a week until confluency (8 to 10 days). Cells were passaged with 0.05 % trypsin-EDTA to obtain the primary cells, and replated at 5000 cells/cm^2. Passage 1 cells were cryo-preserved in 50 % supplemented α-MEM, 40 % FBS, 10 % DMSO. When needed, cells were thawed, plated and grown until confluency in α-MEM supplemented with 15 % FBS, 100 UI/ml penicillin, 100 µg/ml streptomycin, 0.1 mM L-ascorbic acid-2-phosphate and 2mM L-glutamine (expansion medium).
Passage 3 cells were used to prepare the pellets. After trypsinization, 5 x 10^5 cells were spun down at 500 g for 2 minutes in 10 ml polystyrene conical tubes. The expansion medium was then replaced by serum free medium consisting of 1 ml of DMEM with 100 UI/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml pyruvate, 40 µg/ml proline, 50 µg/ml L-ascorbic acid-2-phosphate, 1 % ITS+1 and 100 nM dexamethasone.
One TGF-β_1 loaded or blank scaffold (100 mg) was added to the culture tube, directly in the medium. The culture tubes containing pelleted cells were incubated at 37°C, in a 5% CO_2 humidified atmosphere. Three pellets were cultured for each scaffold condition. After 24 hours of incubation, the cells formed round aggregate, not adhering to the tube walls. Medium changes were carried out every 3 days.
Pellets (n=3) were harvested after 15 and 21 days and fixed overnight in 0.14 M cacodylate buffer (pH=7.2–7.4) containing 0.25% glutaraldehyde (Merck, Darmstadt, Germany). They were subsequently dehydrated in a graded ethanol series and embedded in GMA. 5 µm thick cross-sections were made by using a Microm microtome (HM 355 S). The sections were stained with hematoxylin (Sigma) and fast green (Merck) for cells and with safranin-O (Sigma) for glycosaminoglycans (GAG).

Results and discussion

Scaffold characterization

In view of cartilage tissue engineering applications, the possibility to prepare porous polymeric scaffolds containing and releasing TGF-β_1 was here investigated. An emulsion-coating method [32] was used for this purpose with the objective to obtain a wide range of release profiles (from days to months) without TGF-β_1 loss of activity. The morphology of the emulsion-coated scaffolds, as evaluated by Scanning Electron Microscopy (SEM), is presented in Figure 2. The pores size was ranging from 100 to 650 μm while the pores appeared visually interconnected. The porosity of the scaffolds was decreased by the coating application from 77 % to 66 ± 3 %. As was previously reported for emulsion coated scaffolds [32], the coated layers partly filled the pores and consequently decreased porosity. In parallel, the permeability of the scaffolds toward water was modified by the coatings. κ increased from 18 to 82 μm^2 after coating application. Increasing κ values indicate a higher inter-pore connection of the scaffolds [39]. The increase of permeability is due to the dissolution of the thin polymeric membranes present between pores of the prefabricated compression molded-salt leached scaffolds by the applied emulsion.

The scaffolds porosity and pore interconnection are suitable to allow tissue ingrowth and integration as was shown by a preliminary in vivo study, performed with similar scaffolds implanted in rabbit knee osteochondral defects [41]. After three weeks of implantation, the scaffolds were filled with a tissue consisting of undifferentiated mesenchymal cells, histiocytotic cells and new bone. The pore interconnection was sufficient to allow progenitor cells present in the bone marrow to reach the cartilage zone.

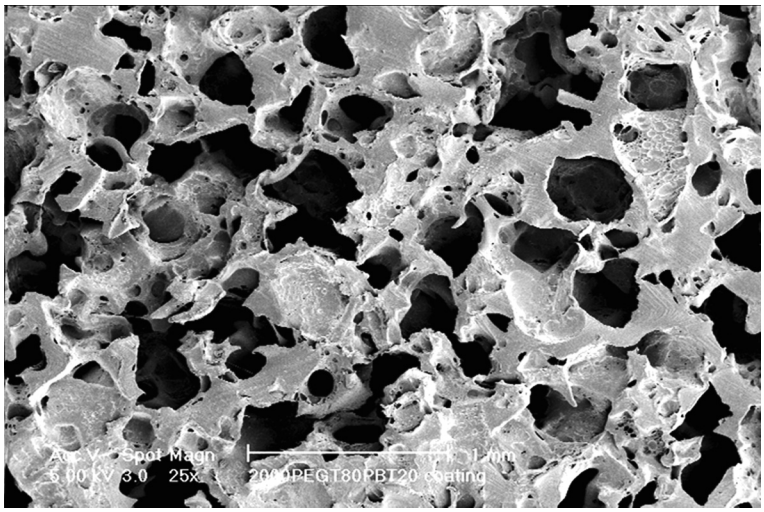

Figure 2: Cross sections of a porous scaffold obtained after application of a TGF-β_1 containing emulsion, examined by scanning electron microscopy.

The effectiveness of the coating process during the application of the emulsion was evaluated. About half of the emulsion prepared was effectively coated on the porous scaffolds (49 ± 1 %).

Protein release kinetics

The ability of the coated porous scaffolds was first determined for copolymers of fixed PEGT weight percentage (80 wt-%) and of varying PEG segment length. As presented in Figure 3, the PEG molecular weight (MW) of the coated copolymer appeared of high influence on the growth factor release rate.
A MW of 600 g/mol resulted in a very slow release after a small burst while a MW of 1000 showed a first order release completed within 10 to 20 days. Interestingly, further increase of the MW from 1000 to 2000 resulted only in a slightly faster release. The modulation of proteins release rates by varying the PEGT/PBT copolymers composition is a well described phenomenon [35,33,42-44]. Increasing values of PEG MW are related to an increase of matrix degradation rate, higher swelling and subsequent larger hydrogel mesh size, resulting in a faster diffusion of the incorporated proteins through the polymeric matrix [45]. The important difference in release rate obtained by a small variation of the PEG MW (from 600 to 1000) suggests that a threshold of hydrogel mesh size has been reached for the 600 PEG MW composition, below which the protein cannot diffuse through the coated copolymer.

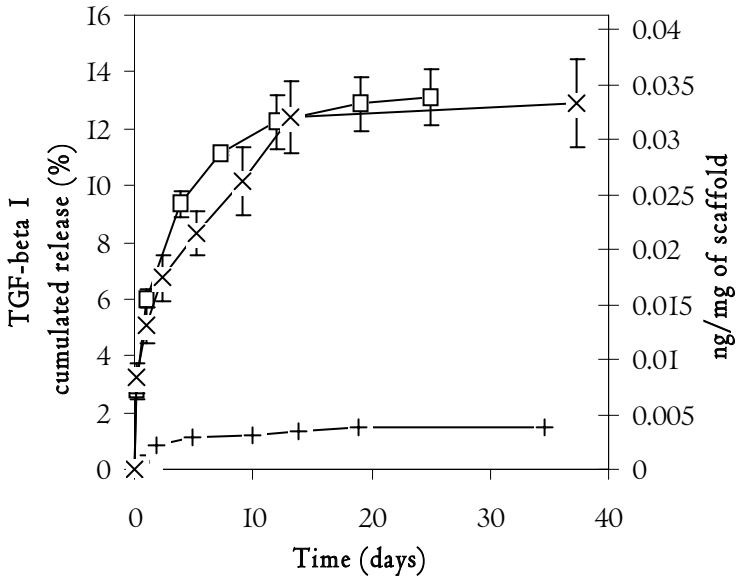

Figure 3: Cumulated release of TGF-β1 from porous polymeric scaffolds coated with water-in-oil emulsions of different copolymeric compositions: 2000PEGT80PBT20 (□), 1000PEGT80PBT20 (×), 1000PEGT70PBT30 (○) and 600PEGT80PBT20 (+). (n=3 ± s.d.).

To fine-tune the release, the copolymer composition could be further adjusted with regard to the PEGT weight percentage (wt-%), which has a similar effect on the hydrogel mesh size as the PEG MW [45]. To investigate this possibility, scaffolds were prepared with a 1000PEGT70PBT30 coating. In addition, varying the degradation behavior of the copolymers could allow further fine-tuning of the release. Therefore, coated scaffolds were prepared using 1000PEG(T/S)PB(T/S) copolymers. The release of proteins from succinated copolymers is based on the same degradation and diffusion mechanism [36]. The substitution of aromatic groups (terephtalate) by aliphatic moieties (succinate) results in higher swelling of the copolymer and higher degradation rates of the copolymer due to the higher accessibility of the ester bond for hydrolysis [36]. As a consequence, the protein diffusion coefficients are increased by the degree of substitution [34]. As presented in Figure 4, the use of 1000PEGT70PBT30 or PEG(T/S)PB(T/S) copolymers resulted indeed in intermediate release profiles.

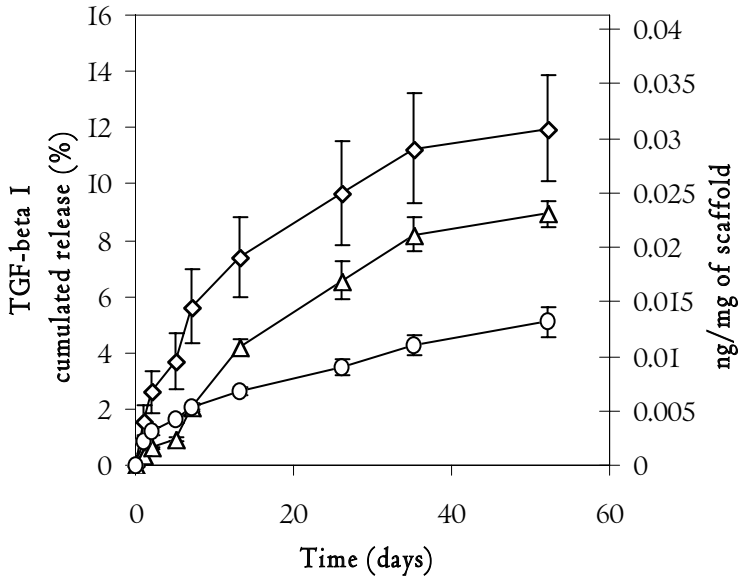

Figure 4: Cumulated release of TGF-β_1 from porous polymeric scaffolds coated with water-in-oil emulsions of different copolymeric compositions: 1000PEG(T/S)70PB(T/S) (0T/100S) (◇), 1000PEG(T/S)70PB(T/S) (50T/50S) (△) and 1000PEGT70PBT30 (○). (n=3 ± s.d.).

A 1000PEGT70PBT30 coated copolymer showed a zero order release still on going after 50 days, while 1000PEG(T/S)70PB(T/S)30 (50T/50S) and 1000PEG(T/S)70PB(T/S)30 (0T/100S) copolymers presented a release completed within 40 days. The effect of the PEGT wt-% on the release rate was clear as a 10 wt-% decrease of PEGT (from 1000PEGT80PBT20 to 1000PEGT70PBT30) resulted in an important decrease of the protein release rate. The substitution of terephtalate groups by succinates groups increased the release rate of TGF-β_1 from the coated scaffolds, as expected.

Noticeably, the total amount of TGF-β1 released (as measured by ELISA) never exceeded 14 % of the total amount entrapped in the scaffolds (24 ng). This low recovery during release is surprising and could be due to an extensive denaturation of the protein by the coating process or during the release period. Nevertheless, previous release experiments using the emulsion-coating method and a model protein (lysozyme) indicated no degradation of the protein during either scaffolds preparation or release [32]. This discrepancy could be linked to the intrinsic stability of TGF-β1 in solution. The half-life of TGF-β1 in vivo is less than 30 minutes [20,31] when in its active form. In addition, due to its high hydrophobicity, TGF-β1 tends to adsorb quickly to plastic surface, reducing so the concentration of the protein in solution [40]. To assess the effective degradation of the protein in our release experiment condition, the concentration decrease of two TGF-β1 standards was measured over time. As can be seen in Figure 5, the amount of TGF-β1 left in the release medium was decreasing rapidly to reach a stable value close to 2.5 % after 12 hours. Within 20 minutes, 60 % of the protein amount could not be measured anymore in the solution. This fast decrease of concentration contributes to the low recovery obtained from the releasing scaffolds, as the amount of protein measured by ELISA at each medium refreshment corresponds to a small fraction of the amount effectively released. The depletion therefore hampers the determination of the release completeness.

To determine if the growth factor depletion is linked to adsorption phenomena (as the release medium does not contain proteins), the release of TGF-β1 from 1000PEGT80PBT20 coated scaffolds was measured in a release medium supplemented with BSA (1 mg/ml). The resulting release profiles were not significantly different in the presence

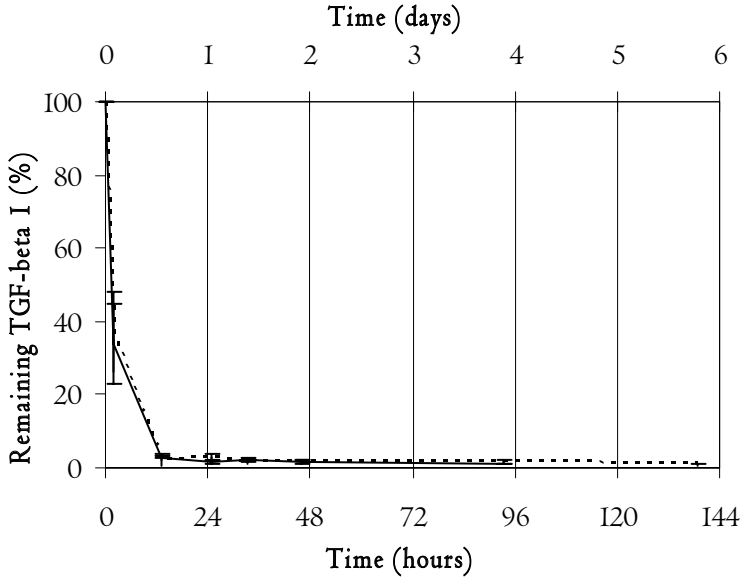

Figure 5: Concentration decrease of TGF-β1 in release medium. Two different concentrations were supplemented as a bolus: 5 ng/ml (full line) and 10 ng/ml (dotted line). (n=3 ± s.d.).

of BSA (data not shown) suggesting that the protein concentration decrease cannot be entirely related to adsorption. Additionally, the freezing and thawing of the samples prior to quantification could have played a role in the protein depletion. However, the TGF-β_1 concentrations of release samples measured after one and two cycles of freezing and thawing were similar.

In summary, the release rate and profile of TGF-β_1 from porous scaffolds could be effectively tailored by the copolymer composition of the coating. The release of the growth factor was varied from 10 to more than 50 days. The incomplete release detected for all coating composition is most likely caused by the intrinsic instability of the protein in solution. Besides the completeness of the release, the activity of the released protein is an important factor in view of cartilage application. Therefore, the activity of the released protein was evaluated.

Activity of released TGF-β_1

To confirm that the released protein is not denaturated by the emulsion-coating method, the activity of the released protein was measured in a cell growth inhibition assay based on CCL64 cells. A schematic drawing of the different conditions assayed is presented in Figure 1. The activity of TGF-β_1 directly released from emulsion-coated scaffolds was assessed during the length of the growth inhibition assay (40 hours). Releasing scaffolds (coated with 2000PEGT80PBT20 and 1000PEG(T/S)70PB(T/S)30 (50T/50S) copolymer) were placed in the culture medium and the amount of TGF-β_1 released was measured by ELISA. The resulting cell growth inhibition of the released TGF-β_1 appeared higher than the one obtained from similar TGF-β_1 concentrations used as standards (Figure 6). The protein activity, calculated by comparing the concentrations deduced from the TGF-β_1 standard curve and the ELISA, was of respectively 472 ± 140 % and 1500 ± 63 % for 2000PEGT80PBT20 and 1000PEG(T/S)70PB(T/S)30 (50T/50S) coatings. This apparent high activity is surprising and might be linked to the sensitivity of the CCL64 cells towards the sustained delivery of the protein. It is possible that the continuous presence of TGF-β_1 in the culture medium, when released from the scaffolds, induce a higher inhibition of the cell growth compare to a single supplementation (standards). To assess if the growth of the cells was reduced by a sustained delivery of the protein, two different TGF-β_1 concentrations (0.04 and 0.4 ng/ml) were added sequentially to the medium every 8 hours (5 times). As can be seen in Figure 6-A, the total cumulated amount of TGF-β_1 (0.2 and 2 ng/ml) resulted in a cell inhibition similar to the one obtained with the standards. This indicates that the delivery rate had no effect on the cell growth.

Another potential cause of the high activity can be found in the detection of the released protein in the culture medium. As stated above, the protein concentration measured by ELISA most likely only reflects a part of the amount effectively released. Therefore, the level of decrease of the protein in the cell culture medium was measured for each type of releasing coated scaffold assayed over 40 hours. Subsequently, the total amount of released protein measured by ELISA was corrected for the protein depletion. A 2000PEGT80PBT20 and 1000PEG(T/S)70PB(T/S)30 (50T/50S) coating respectively showed a protein loss of 86

and 87 %. As a result, the corrected activity of the released protein was 85 ± 25 % for scaffolds coated with a 2000PEGT80PBT20 copolymer and 200 ± 8 % for 1000PEG(T/S)70PB(T/S)30 (50T/50S) coated scaffolds. Although these activity values can only be considered as indicative, due to the growth factor depletion which prohibited accurate concentration measurements, they tend to indicate that the bioactivity of the TGF-β_1 was preserved during preparation of the scaffolds.

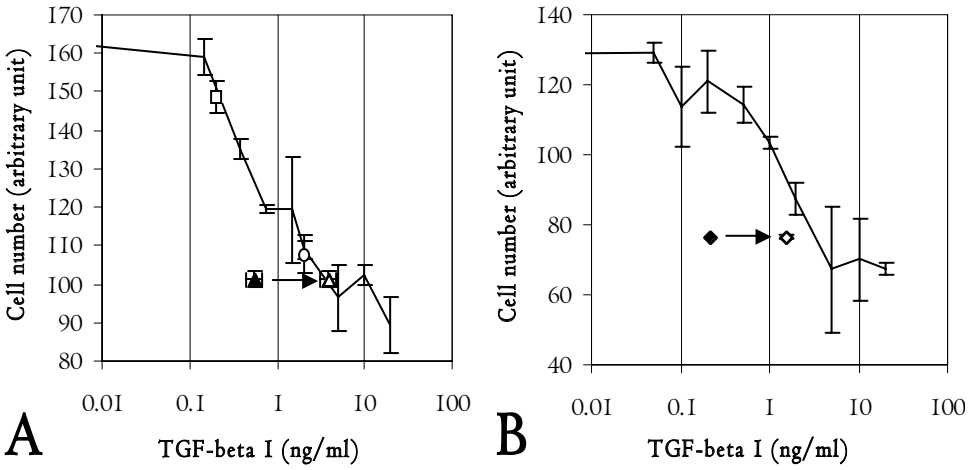

Figure 6: Activity of TGF-β_1 releasing scaffolds and samples mimicking a controlled release system, measured in a cell growth inhibition assay, based on the growth of CCL64 cells. Increasing values of TGF-β_1 result in a lower number of cells after 40 hours of culture. The releasing scaffold tested were coated with a 2000PEGT80PBT20 (A) or 1000PEG(T/S)70PB(T/S)30 (50T/50S) (B) copolymer. The cumulated released concentrations mentioned were obtained by elisa, before (▲, ◆) and after correction for the protein depletion in the medium (△, ◇). The samples mimicking a sustained delivery of TGF-β_1 were of cumulated concentration of 0.2 (□) and 2 (○) ng/ml and were supplemented in the culture medium in 5 regular time intervals (8 hours).

Effect of control released TGF-β_1 on cartilage formation in vitro

To confirm the activity of the released protein and the potential benefit of the controlled release of TGF-β_1 from porous scaffolds, the ability of the releasing scaffolds to induce cartilage formation in cell pellets was investigated. Bone marrow-derived mesenchymal stem cells were selected as they are able to differentiate into the cartilage lineage when exposed to appropriate signals. For instance, goat and rabbit bone marrow cell pellets successfully produced cartilage-like matrix when subjected to TGF-β_1 [46,47]. The releasing scaffolds (80 mg) were placed directly in the pellet culture medium, but not directly in contact with the cells. A pellet culture was preferred to avoid any potential effects related to seeding efficiency on the scaffold or cellular differentiation due to the cell contact with the copolymer used as coating. Scaffolds coated with a 1000PEG(T/S)70PB(T/S)30 (0T/100S) copolymer that showed

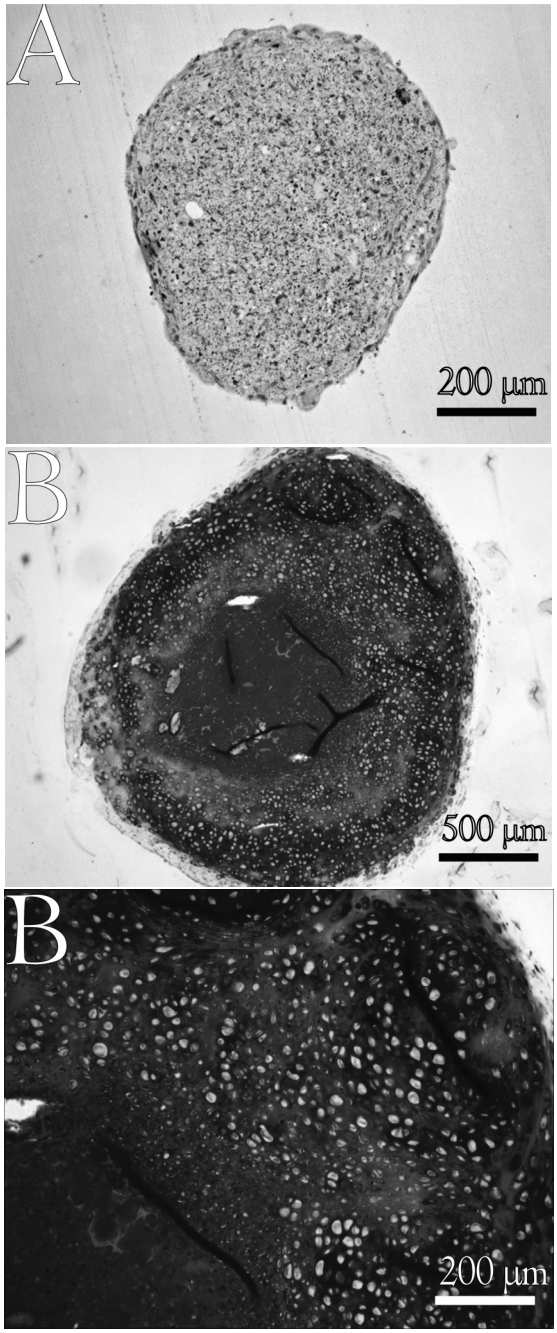

Figure 7: Histological sections of the bone marrow-derived mesenchymal stem cells pellets cultures during 21 days, in the presence of emulsion-coated porous scaffolds. The cross sections were stained with safranin O/fast green. A: pellets cultured in the presence of an unloaded emulsion-coated scaffold, B: pellets cultured in the presence of a releasing TGF-β_1 loaded scaffold.

a slow delivery over 40 days were selected. Considering the fast degradation of TGF-β1 in vitro, scaffolds of higher protein content (360 ng/scaffold) were prepared in order to obtain a growth factor release potentially inducing the chondrogenic differentiation of the cells. As negative control, unloaded coated scaffolds were included in the study as well.
The effect of the released TGF-β1 on cell pellets differentiation after 15 and 21 days was assessed histologically. Figure 7 depicts histological sections of the pellets after 21 days, stained with fast green/safranin O, which stains cytoplasm green and negatively charged glycosaminoglycans (GAG) red.
The positive effect of the releasing scaffolds on cartilage formation was clearly visible after 15 days and was further demonstrated after 21 days. While the negative control, not subjected to TGF-β1, presented no sign of GAG formation, the group containing releasing scaffolds showed an intense safranin O positive staining. This staining was more intense at the periphery of the pellets which also contained more cells. The cell morphology displayed similarities with hyaline cartilage, including round cells surrounded by large lacunae, creating chondron-like structures. The pellet core was characterized by a low number of cells and the presence of cell debris. Nevertheless, it was positively stained, indicating a strong formation of GAG. The lower cell density and cellular debris might have been caused by a limitation of nutrient diffusion to the pellet core.
The ability of the TGF-β1 releasing scaffolds to induce cartilage in this cell pellet model confirms qualitatively the activity of the released protein.

Conclusions

To associate TGF-β1 to porous polymeric scaffolds and release it in a controlled fashion, an emulsion-coating method was investigated. This approach resulted in scaffolds of defined porosity and pore interconnection which were shown suitable for tissue ingrowth. The growth factor was effectively released from the scaffolds. By varying the copolymer composition used as coating, the release rate of TGF-β1 could be precisely tailored from 12 to more than 50 days. The released protein was not denaturated by the emulsion-coating process and retained its bioactivity, indicating the safety of the emulsion-coating method and poly(ether–ester) multiblock copolymers regarding sensitive proteins. This was further confirmed by the ability of the released protein to induce chondrogenic differentiation of bone marrow-derived mesenchymal stem cells. Therefore, emulsion coated scaffolds appear as potential candidates for cartilage tissue engineering as they release TGF-β1 in a biologically active form and allow a broad control on the growth factor release rates. This last property would be useful to investigate the relative effect of TGF-β1 release rate on cartilage formation and determine the most optimal release profile in vivo.

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What is the effect of growth factor controlled release on cartilage formation ?

Ni la contradiction n'est marque de fausseté,

ni l'incontradiction n'est marque de vérité

Blaise Pascal (1623 - 1662)

Chapter 6

TGF-β1 released from porous scaffolds: influence of delivery rates on cartilage formation

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Abstract

In the perspective of repairing deficient cartilage, an increasing interest is given to the controlled release of TGF-β1 from porous supportive structures to enhance the differentiation of cells. Although the sustained release of growth factors is generally considered beneficial due to their native instability and high potency, the assumption that sustained exposure of stem cells to TGF-β1 results in optimal cartilage formation was not addressed. In other words, for a given amount of TGF-β1, the most ideal delivery profile is not known. Therefore, the present study investigates the effect of different TGF-β1 release profiles from copolymeric porous scaffolds and different supplementation rates on the chondrogenic differentiation of goat mesenchymal stem cell pellets. A similar cumulated dose of growth factor was either released over 12 and 40 days from scaffolds, or supplemented in the culture medium at once (bolus) or every 3 days (positive control). After 21 days, the pellets were evaluated by histology, GAG/DNA quantification and gene expression of cartilage markers (collagen type 1, 2 and aggrecan). In contrast to general belief, not the sustained delivery but rather the bolus supplementation of the growth factor was the most effective approach to induce the cells towards the chondrogenic phenotype. The amount and quality of the cartilage formed was increased with an increasing delivery rate of the growth factor. Surprisingly,

the scaffold copolymer composition, although not in direct contact with the pellets, influenced the cartilage formation in parallel to the TGF-β_1 supplementation conditions. The beneficial effect of the TGF-β_1 instantaneous delivery and the effect of scaffold copolymer composition were related to the amount of growth factor present at the early culture times and could be explained by the physiological mode of action of TGF-β_1.
The demonstration that mesenchymal stem cells are induced in a better way to the chondrogenic pathway by instantaneous or fast supplementations or releases has important implications for the use of these cells in cartilage tissue engineering applications and for the use of TGF-β_1 releasing scaffolds in clinical applications.

Introduction

Among the potential targets of tissue engineering, cartilage is of special importance as this avascularized tissue has poor regeneration capabilities [1]. Concomitantly, an increasing interest is given to the controlled release of growth factors from porous polymeric scaffolds to achieve a better tissue formation [2-6]. Consequently, the current research to improve cartilage regeneration or formation in engineered constructs tends to focus on the controlled released of TGF-β_1 [7-10]. TGF-β_1 is a pleiotropic growth factor which has regulatory effects on many different cell types. For instance, it plays an important role in cell proliferation and differentiation, bone formation [11-13], angiogenesis [14,15], neuroprotection [16] and wound repair [17-19]. More importantly, it controls the production of extracellular matrices by stimulating the synthesis of collagens, fibronectin and proteoglycans [20,21] and has positive effects on cartilage differentiation and repair [22]. Additionally, TGF-β_1 seems the ideal candidate to benefit from a sustained delivery due to its rapid denaturation and high potency.
Controlled release of TGF-β_1 from porous scaffolds has been shown to enhance cartilage formation [23,24]. However, the underlying assumption that sustained release is the optimum way to induce or enhance cartilage formation is seldom questioned and has not been verified. In addition, considering the cost of commercially available growth factors, it is of interest to determine the most effective way to employ a given dose. Therefore, the aim of this study was to investigate the effect of TGF-β_1 release profiles from scaffolds on cartilage formation and to evaluate the potential beneficial effect of long term delivery over bolus supplementation.
To address this, a recently developed method based on the coating of porous scaffolds with poly(ether-ester) copolymeric emulsions was used [25]. This biodegradable multiblock copolymer, based on poly(ethylene glycol)-terephtalate and poly(butylene terephtalate) (PEGT/PBT) or poly(ethylene glycol)-succinate and poly(butylene succinate) (PEGS/PBS), is successfully used as protein release system [26,27] and as scaffold matrix for tissue engineering applications [28-32]. The emulsion-coating method allows the associate TGF-β_1 with porous scaffolds and to adjust its release kinetics, ranging from release within 12 days up to more than 50 days [33]. Control of the release rate can be obtained by varying the coating copolymer composition.

To investigate the effect of supplementation rates of TGF-β_1 on cartilage formation, three different delivery profiles (bolus supplementation, release completed in 12 days and completed in 40 days), a negative (without TGF-β_1) and a positive control (regular refreshment of TGF-β_1 every 3 days) were compared with respect to the cartilage formation observed in Goat Mesenchymal Stem Cell (GMSC) pellets over 21 days of culture in vitro. MSC were chosen as they undergo chondrogenic differentiation when exposed to TGF-β_1, which makes them attractive candidates for cartilage tissue engineering applications [34-37]. The use of pellets as a model system for chondrogenesis was preferred as it allows cell-cell interactions analogous to those occurring in precartilage condensation during embryonic development [34]. Moreover, this system avoids any effect of seeding efficiency on the scaffolds and minimizes the potential cellular differentiation due to the cell contact with the different copolymers used as coating.
Beside the effect of the supplementation rates, and as the copolymer compositions used as coating were varied to tailor the release rate of the growth factor, the effect of the coating copolymer compositions on cartilage formation was investigated as well.

Materials and methods

Materials

Poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) (PEGT/PBT) and poly(ethylene glycol)-succinate)/poly(butylene succinate) (PEGS/PBS) multiblock copolymers were obtained from OctoPlus, Leiden, The Netherlands, and were used as received. Polymers are indicated as aPEGTbPBTc or aPEGSbPBSc in which a is the PEG molecular weight, b the weight percentage (weight %) of poly(ethylene glycol)-terephthalate (PEGT) or poly(ethylene glycol)-succinate (PEGS), and c (=100-b) the weight % of PBT or PBS. Vitamin B_{12}, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), L-ascorbic acid-2-phosphate, proline, insulin-transferrin-selenium (ITS+1), dexamethasone, proteinase K, pepstatin-A and iodoacetamide were purchased from Sigma Chem. corp. (St. Louis, USA). Recombinant human transforming growth factor β_1 (rhTGF-β_1, later referred as TGF-β_1) and ELISA kit were purchased from R&D Systems Inc. (Minneapolis, USA). Dubbelco and alpha modified eagle medium ((DMEM and α-MEM), pyruvate, L-glutamine, penicillin and streptomycin were obtained from Gibco (Invitrogen, Carlsbad, USA). Foetal bovine serum (FBS) was purchased from Cambrex (East Rutherford, USA). Glycol methacrylate embedding solutions (GMA) were purchased from Technovit (Heraeus Kulzer, Germany). Basic-fibroblast growth factor (bFGF) was obtained from VWR international (Roden, The Netherlands). Chloroform, obtained from Fluka chemica (Buchs, Switzerland), was of analytical grade.

Preparation of TGF-β_1-loaded polymeric scaffolds

Emulsion

The protein was associated to the porous scaffolds by mean of a water-in-oil (w/o) emulsion method. An aqueous solution of TGF-β_1 in a 4 mM HCL solution (containing 1 mg/ml BSA, according to the supplier recommendation) was emulsified with a PEGT/PBT or PEGS/PBS copolymer solution in chloroform, using an Ultra-Turrax (T25 Janke & Kunkel, IKA-Labortechnik) for 30 s at 19 krpm. The TGF-β_1 concentration of the aqueous solution was set at 3.3 μg/ml. The volume of the aqueous phase was set to 1 ml per gram of copolymer (water/polymer ratio = 1 ml/g). The copolymer solution was obtained by dissolving 0.5 gram of copolymer in 3 ml of chloroform. Two different copolymers were used: 2000PEGT80PBT20 and 1000PEGS70PBS30.

Emulsion-coating method

The emulsion-coated scaffolds were obtained as described elsewhere [25]. Briefly, compression molded/salt leached scaffolds were prepared from 300PEGT55PBT45 granules and 400-600 μm salt crystals (75 volume %). Coated scaffolds were prepared by forcing a TGF-β_1-containing emulsion through a prefabricated porous scaffold with the use of vacuum (300 mBars). This vacuum was applied for at least 5 minutes in order to evaporate chloroform as much as possible from the emulsion, thereby creating a polymeric coating on the scaffold. The resulting coated scaffolds were frozen in liquid nitrogen and freeze-dried for 24 hours.
Blank scaffolds were prepared by using a TGF-β_1-free 4 mM HCL solution (with 1 mg/ml BSA) in the same conditions as TGF-β_1 containing scaffolds.
The quantity of emulsion effectively coated on the porous scaffold and the resulting amount of TGF-β_1 present was determined by an indirect method which was proven to be accurate for other proteins [25]. Briefly, emulsion-coated scaffolds containing vitamin B_{12} were prepared for each copolymer composition used and the fast release of the small molecule was measured. The quantity of vitamin released is correlated to the amount of polymer coated onto a given scaffold. In turn, the amount of polymer coated can be related to the amount of protein associated with the scaffold. The amount of TGF-β_1 incorporated in a scaffold piece of 100 mg was about 70 ng.
The copolymer compositions used in the coating were selected considering their ability to release TGF-β_1 from the scaffolds in a controlled way. A scaffold coated with a 2000PEGT80PBT20 copolymer results in a release of the protein completed within 12 days while a 1000PEGS70PBS30 coating shows a similar amount of protein gradually released over 40 days. In addition, the protein released from the scaffolds was not denaturated during the coating process and retained its bioactivity. A complete characterisation of the scaffolds has been reported previously [33].

TGF-β_1 concentration decrease in culture medium

TGF-β_1 concentrations in the culture medium were assessed by ELISA. To determine the absolute concentration decrease over 3 days, triplicates of the bolus and positive control culture medium (containing originally 70 ng/ml and 10 ng/ml) were collected and assayed for TGF-β_1 remaining concentration. To determine the concentration equilibrium over 21 days, the medium of three pellets from the positive control group (containing originally 10 ng/ml) was collected every 3 days for TGF-β_1 quantification. The different groups evaluated in this study are summarized in Figure 1.

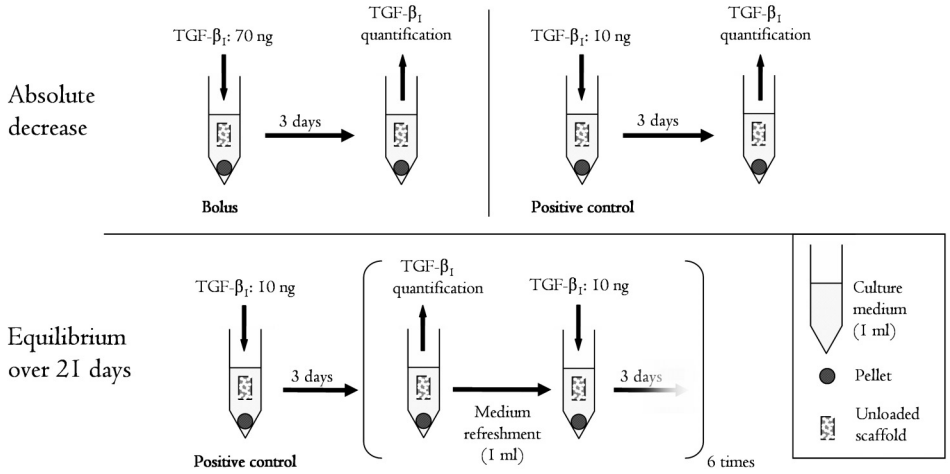

Figure 1: Different experimental conditions used to investigate the TGF-β_1 concentration decrease in the pellet culture medium. Each condition was performed twice, with two different unloaded scaffolds coated with two different copolymer compositions (2000PEGT80PBT20 and 1000PEGS70PBS30).

Goat mesenchymal stem cells pellet culture

GMSC were harvested from the iliac crest of 4 years old female dutch milk goats, under general inhalation anaesthesia. The bone marrow aspirate was collected in heparin tubes. The nucleated cells were plated at a density of 5 x 105 cells/cm^2 in α-MEM supplemented with 12 v/v% FBS, 100 UI/ml penicillin, 100 µg/ml streptomycin, 0.1 mM L-ascorbic acid-2-phosphate, 1 ng/ml bFGF and 2mM L-glutamine. The medium was refreshed after 3 days and then twice a week until confluency (8 to 10 days). Cells were passaged with 0.05 % trypsin-EDTA and replated at 5000 cells/cm^2. Passage 1 cells were cryo-preserved in 50 % supplemented α-MEM, 40 % FBS, 10 % DMSO. When needed, cells were thawed, plated and grown until confluency in α-MEM supplemented with 12 v/v% FBS, 100 UI/ml penicillin, 100 µg/ml streptomycin, 0.1 mM L-ascorbic acid-2-phosphate and 2mM L-glutamine (expansion medium).

Passage 3 cells were used to prepare the pellets. After trypsinization, 5 x 105 cells were cen-

trifuged at 500 g for 2 minutes in 10 ml polystyrene conical tubes. The expansion medium was then replaced by serum free medium, consisting of 1 ml of DMEM with 100 UI/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml pyruvate, 40 μg/ml proline, 50 μg/ml L-ascorbic acid-2-phosphate, 1 volume % ITS+1, and 100 nM dexamethasone.
To study the effect of the TGF-β_1 released from the scaffolds, one TGF-β_1 loaded scaffold (100 mg) was added to the culture tube containing one pellet, directly in the medium. Pellets exposed to TGF-β_1 freshly supplemented in the culture medium (70 ng/ml as a bolus or 10 ng/ml repeated every 3 days) were incubated with 1000PEGS70PBS30-coated blank scaffold unless indicated otherwise. The culture tubes were incubated at 37°C, in a 5% CO_2 humidified atmosphere. After 24 hours of incubation, the cells formed round aggregates, non-adhering to the tube walls. The medium was refreshed every 3 days, and pellets were harvested after 3, 12 and 21 days.
The different conditions evaluated in this study are summarized in Table 1.

Table 1: Overview of the different experimental groups evaluated. The delivery of TGF-β_1 to the pelleted cells was either obtained from releasing scaffolds of different release rate or by supplementing the medium with the growth factor in different pattern. Each pellet was cultured in the same conditions, in the presence of a similar emulsion-coated scaffold (100 mg), either containing and releasing TGF-β_1 or unloaded.

Experimental group	Coating copolymer composition	Delivery rate (days)	TGF-β_1 amount incorporated in scaffolds	TGF-β_1 amount supplemented in medium
Negative control	1000PEGS70PBS30	0	0	0
	2000PEGT80PBT20			
Slow release	1000PEGS70PBS30	40	70 ng	0
Fast release	2000PEGT80PBT20	12	70 ng	0
Bolus	1000PEGS70PBS30	Instantaneous	0	70 ng once
	2000PEGT80PBT20			
Positive control	1000PEGS70PBS30	21	0	7 x 10 ng (every 3 days)
	2000PEGT80PBT20			

Histology

Pellets (n=3) taken after 12 and 21 days were fixed overnight in 0.14 M cacodylate buffer (pH=7.2–7.4) containing 0.25% glutaraldehyde (Merck, Darmstadt, Germany) and subsequently dehydrated in a graded ethanol series and embedded in glycol methacrylate (Technovit, Heraeus Kulzer, Germany). 5 μm thick cross-sections were made by using a Microm microtome (HM 355 S, Waldorf, Germany) which were stained with hematoxylin (Sigma) and fast green (Merck) for cells and with safranin-O (Sigma) for glycosaminoglycans (GAG).

RNA isolation and quantitative PCR

The effect of the TGF-β_1 release profile on articular cartilage marker-genes expression was analyzed from the cultured pellets. Primers for all genes were designed using sequences published on the NCBI website. Because the caprine complete gene sequences are not available, we designed primers based on bovine and mouse cDNA information. The genes examined in this study were collagen type 1 and 2 (coll. I and II) and aggrecan (AGC), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as house keeping gene. The sequence, accession number and product size of each primer are listed in Table 2. The amplified product sizes were confirmed by gel electrophoresis and sequencing to eliminate the possibility of cross contamination by mouse or bovine sources.

Total RNA was isolated by crushing the pellets and using a RNeasy kit (Qiagen, Hilden, Germany) and on-column DNase treated with 10U RNase free DNase I (Gibco) at 37°C for 30 minutes. DNase was inactivated at 72°C for 15 minutes. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry. One µg of RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturer's protocol. 2 µl of 100x diluted cDNA was used for GAPDH amplification, 2 µl of 50x diluted cDNA for aggrecan, and 2 µl of undiluted cDNA for collagen type 1 and 2. PCR was performed on a Light Cycler real time PCR machine (Roche, Basel, Switzerland) using SYBR green I master mix (Invitrogen). Data were analyzed with the Light Cycler software version 3.5.3 using the fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. The relative expression of the genes was calculated by normalizing to the house keeping gene (GAPDH) and comparing to the negative control group (without TGF-β_1) by the comparative $2^{-\Delta\Delta CT}$ method [38]. For all groups, the sample size was 3, representing three different pellets. If data passed the normality and variance tests, multiple student's t-tests were performed to compare the different groups. If not, the non parametric Mann-Whitney test was used. A p-value$<$0.05 was considered significant for all tests.

Table 2: Primer sequences used with caprine cDNA for quantitative PCR reactions.

Primer name (abbreviation, accession number, product size)	Forward sequence (5' -3')	Reverse sequence (5' -3')
glyceraldehyde-3-phosphate dehydrogenase (GAPDH, M32599, 191 bp)	AACGACCCCTTCATT GAC	TCCACGACATACTCA GCAC
Collagen type 1 (coll. I, NM_007742, 82 b p)	GCATGGCCAAGAAG ACATCC	CCTCGGGTTTCCAC GTCTC
Collagen type 2 (coll. II, NM_031163, 216 bp)	CAAGGCCCCCGAGG TGACAAA	GGGGCCAGGATTCC ATTAGAG
Aggrecan (AGC, U76615, 473 bp)	AAGGGCGGGTGCGG GTCAACAG	CGCGAAGCAGTACAC GTCATAGG

Biochemical assays

Pellets (n=3) were collected after 21 days and digested overnight at 56°C in a solution containing proteinase K (1 mg/ml), pepstatin-A (10 μg/ml) and iodoacetamide (185 μg/ml). Quantification of total DNA was done by Cyquant dye kit (Molecular Probes, Invitrogen) using a spectrofluorometer (Perkin-Elmer, Boston, USA). The amount of GAG was determined spectrophotometrically after reaction with dimethylmethylene blue dye (DMMB, Sigma), using a spectrophotometer at 520 nm (EL 312e Bio-TEK Instruments). Chondroitin sulphate B (Sigma) was used as standard to calculate the amount of GAG. Statistically significant differences between groups were evaluated using student's t-test with a level of significance set at 0.05.

Results and discussion

Effect of TGF-β_1 supplementation rate on cartilage formation

Histological evaluation

To evaluate the effect of different TGF-β_1 treatment strategies on the chondrogenic differentiation of GMSC, we exposed pellets three different delivery profiles (bolus supplementation, release completed in 12 days and completed in 40 days), a negative (without TGF-β_1) and a positive control (regular refreshment of TGF-β_1 every 3 days). The releasing scaffolds were coated with 2 different copolymer compositions to allow different release rates.
The different delivery rates of TGF-β_1 to the culture medium resulted in different tissue organization of the pellets. Representative cross sections of the pellets cultured in various conditions and stained with Safranin O/fast green (stains negatively charged GlycosAminoGlycans (GAG) red and nuclei green) are presented in Figure 2.
Irrespective of the copolymer used as coating, the positive effect of TGF-β_1 on the differentiation of GMSC towards cartilage could clearly be observed. In the absence of TGF-β_1 (negative control) no GAG was found in the pellets, which were of smaller size than for the other conditions. The cells were small and a lot of debris was observed, suggesting a low cell survival during culture. In contrats, all the pellets cultured in the presence of TGF-β_1 (either supplemented or released from scaffolds) showed GAG formation at different intensities and localizations. The morphology of the cells present in the positively stained areas was similar regardless of the culturing conditions and resembled articular chondrocytes. The cells were rounded and located in lacunae surrounded by GAG positive extra cellular matrix. The chondrogenic differentiation of GMSC by TGF-β_1 is in line with previous studies conducted on goat [37], rabbit [34], or human [35,39] cells.
The rate at which TGF-β_1 was applied to the pellets influenced the chondrogenesis. For a 1000PEGS70PBS30 coating, a slow release of the protein (within 40 days) resulted in the

Figure 2. Gross evaluation and histological cross sections of GMSC pellets cultured in the presence of porous scaffolds of different TGF-β1 release profiles and different coating copolymers: A: 1000PEGS70PBS30 coating, B: 2000PEGS80PBS30 coating. The cross sections were stained with safranin O/fast green. The negative control pellets were not exposed to TGF-β1. The positive control consists of unloaded scaffold where constant amounts of TGF-β1 are added to the culture medium regularly (10 ng/3 days) while in the bolus groups 70 ng of growth factor was added at the beginning of the culture. (n=2)

formation of GAG in discontinuous areas, mainly at the pellets rim and in the smaller structures present around it. The pellet core was of similar structure to the negative control, without apparent GAG formation, indicating a partial differentiation of the pellets. The bolus supplementation of the TGF-β_1 at the beginning of the culture resulted in an intermediary GAG staining at the pellet rim and a small and less intensively stained core. The core was composed of a mix of distressed-looking and round cells surrounded by a faint positive staining. Surprisingly, the addition of TGF-β_1 in the culture medium at regular time interval (10 ng/ml every 3 days, positive control for long term release) resulted in a structure more comparable to the bolus than to the slow release. Unexpectedly, 2000PEGT80PBT20 coatings resulted in pellets of more homogeneous GAG distribution, with a lower amount of small and undifferentiated cells in the pellet core. In addition, the pellets were larger for similar culture conditions and some cells were stacked in chondron-like structures of two to five cells. The differences in GAG formation were less obvious between the delivery conditions (either released or supplemented) as compared to the 1000PEGS70PBS30 coating. The bolus supplementation resulted in a cartilage formation as effective as the fast release from the scaffolds or the repeated supplementation.

Quantitative characterization of the TGF-β_1 supplementation rate effect

The differences in GAG formation due to the TGF-β_1 supplementation rate was evaluated more accurately by a quantitative characterisation of the pellets GAG and DNA content. The pellet DNA content indicated that the number of cells was influenced by the culture conditions, as presented in Figure 3-A. A slow delivery and a lack of TGF-β_1 (negative control for both coating copolymers) resulted in a significantly lower DNA content ($p<0.05$) than the other culture conditions. This indicates that the pellets cultured with slow releasing scaffolds or without TGF-β_1 had a similarly low cell number. Apparently, the slow growth factor release resulted in a cell survival or proliferation rate similar to the one observed without TGF-β_1. Nevertheless, the size of the pellets obtained for a slow TGF-β_1 release (Figure 1) confirmed the extra cellular matrix (ECM) production, as previously observed for human MSC [35,40,36]. All the other culture conditions resulted in higher and variable amount of DNA per pellet, without statistical difference.

The differentiation state of the cells was quantified by measuring the amount of GAG produced per DNA (Figure 3-B). As expected from the histological sections of the pellets, the lack of TGF-β_1 (negative control) resulted in a minimal GAG formation ($p<0.05$) for both coating copolymers. For the 1000PEGS70PBS30 coating, the bolus, repeated supplementation (positive control) and slow release of TGF-β_1 showed intermediate GAG levels which seemed to increase from slow release to bolus, but not significantly ($p \approx 0.1$). The low amount of cartilage formation and low cell number observed for the slow release group is probably linked to the lower amount of TGF-β1 released over the 21 days of culture. As the release from the scaffold was completed in 40 days, only 75 % of the protein was delivered to the culture medium at the end of the study. The localized and sporadic chondrogenic dif-

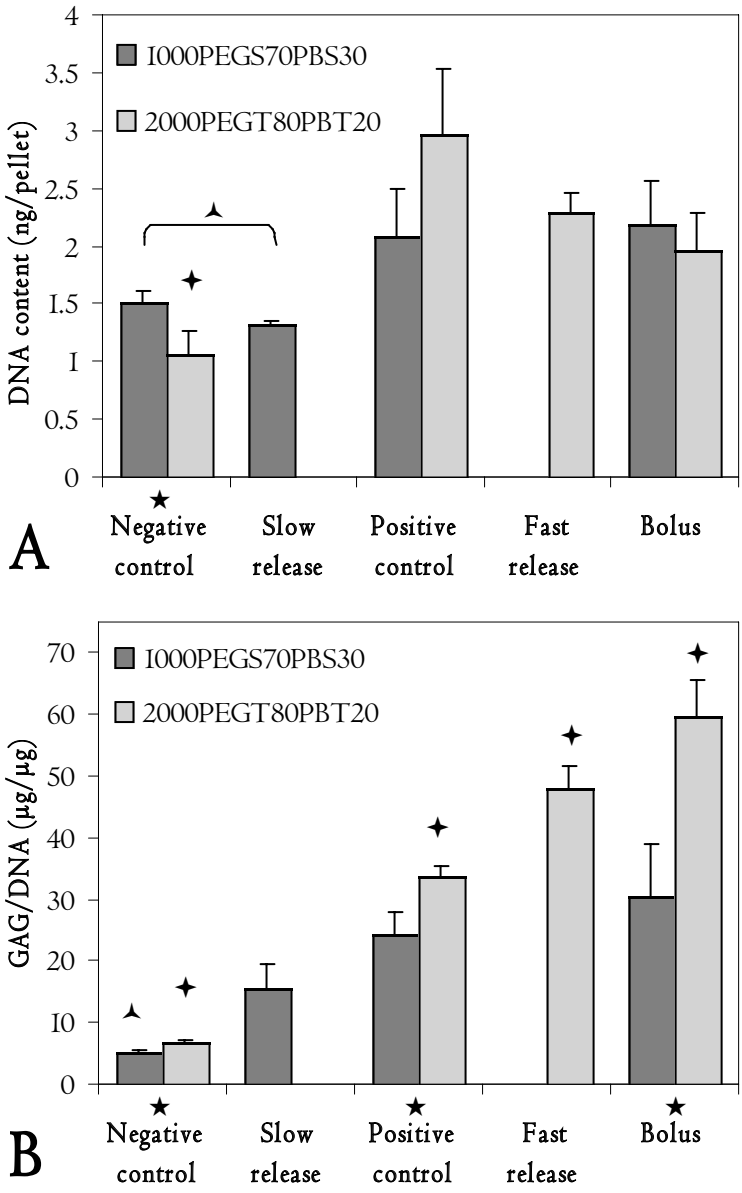

Figure 3: Effect of the TGF-β_1 supplementation rate and coating copolymer composition on GMSC pellets DNA (A) and GAG/DNA (B) content. TGF-β_1-unloaded scaffolds were coated with I000PEGS70PBS30 (dotted bars) or 2000PEGT80PBT20 (stripped bars) and were cultured without supplementation of TGF-β_1 (negative control), with regular supplementation every 3 days (positive control), or with a bolus supplementation of TGF-β_1 at the beginning of the culture. TGF-β_1-loaded scaffolds released the growth factor over 12 days (fast release) or 40 days (slow release). ➹ denotes a significant difference ($p<0.05$) between culture conditions for a I000PEGS70PBS30 coating copolymer, ✦ denotes a significant difference ($p<0.05$) between the different culture conditions for a 2000PEGT80PBT20 coating copolymer and ★ denotes a significant difference ($p<0.05$) between coated copolymer for a similar culture condition. ($n=3 \pm$ s.d.)

ferentiation could therefore be linked to the TGF-β_1 dose. This was confirmed in an independent experiment which demonstrated that lower doses of TGF-β_1 (3 ng/ml every 3 days) result in very few small conglomerates of cartilage-like cells at the periphery of the pellet (data not shown).
The scaffolds coated with 2000PEGT80PBT20 copolymer induced as well an evolution of the GAG synthesis linked to the rate of TGF-β_1 supplementation. The amount of GAG/DNA regularly and significantly increased from a slow supplementation of TGF-β_1 in the medium over 21 days (positive control) to an instantaneous and unique supplementation (bolus) ($p<0.05$). In agreement with the histological observations, a 2000PEGT80PBT20 coating (unloaded or releasing TGF-β_1) always showed a higher GAG formation in comparison to scaffolds coated with a 1000PEGS70PBS30 copolymer. As a result, the bolus group induced the highest GAG formation of all delivery conditions investigated. This suggests that a sustained delivery of the growth factor, either release from scaffolds or supplemented in the culture medium is not as effective as a bolus delivery.

Temporal pattern of chondrogenic gene expression

To better understand the relative effects of the different supplementation and release rates on the chondrogenic differentiation, the expressions of collagen type I (coll. I), collagen type 2 (coll. II) and aggrecan (AGC) genes were quantified as a function of time. Three different time points were evaluated (after 3, 12 and 21 days of culture). The pellets cultured with a 1000PEGS70PBS30 coating were used as the differences in GAG formation appeared more acute by histology when using this coating copolymer. As depicted in Figure 4, the coll. I expression remained unchanged after 12 days of culture while after 21 days all the culture conditions showed a higher gene expression. The increase of coll. I expression varied between 1.7 and 6.6 folds as compared to the negative control (without TGF-β_1). The coll. II expression appeared highly up-regulated after 12 days of culture. The bolus supplementation resulted in an up-regulation close to 200 times, followed by the slow release scaffolds and the repeated supplementation (positive control, 50 times). The expression of coll. II appeared to decrease for some culture conditions after 21 days (slow release and its control), although no statistically significant differences could be found in comparison with 12 days. The opposite trend was seen for the bolus condition, without significant difference. The expression of the AGC gene was highly up-regulated by a bolus delivery after 12 days of culture (by 70 fold). Similarly to coll. II, a slow TGF-β_1 release or the positive control resulted in the smallest up-regulation of the gene (between 25 and 35 fold). Although the AGC expression was increasing slightly for all groups after 21 days, no statistically significant difference could be found between the gene expression at day 12 and day 21.
In all culture conditions containing TGF-β_1, a high and variable ratio of coll. II gene expression versus coll. I was seen after 12 and 21 days, corroborating the chondrocyte differentiation of the GMSC noticed by histology. Several publications outlined that a high ratio was representative of hyaline articular chondrocytes of different mammal species including goat [41-46].

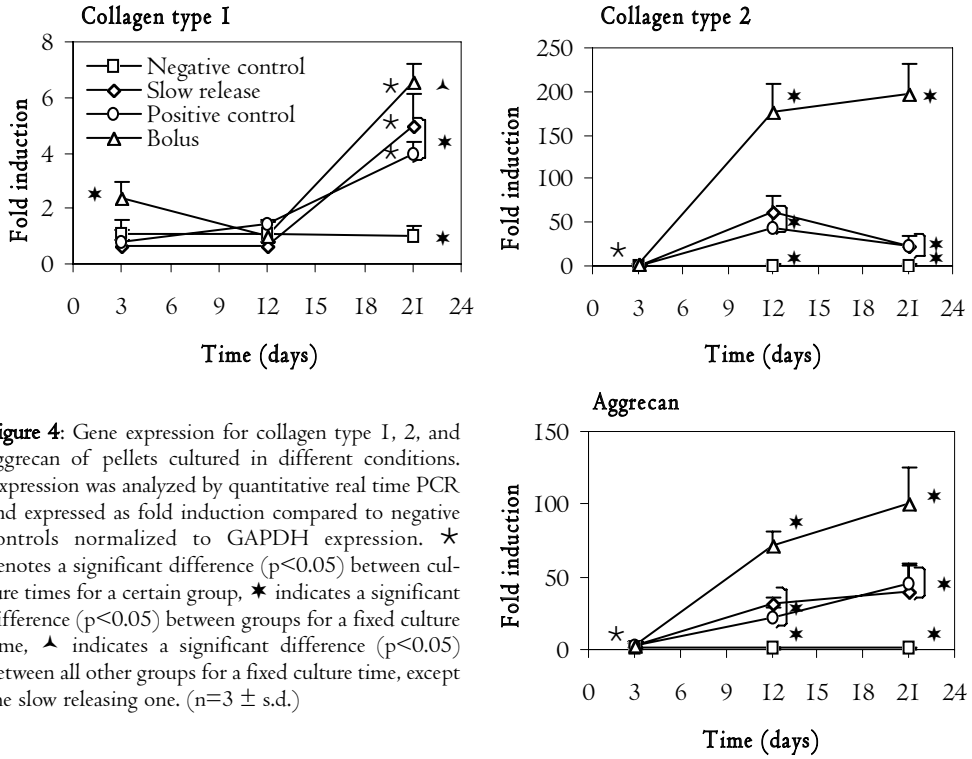

Figure 4: Gene expression for collagen type 1, 2, and aggrecan of pellets cultured in different conditions. Expression was analyzed by quantitative real time PCR and expressed as fold induction compared to negative controls normalized to GAPDH expression. ✶ denotes a significant difference ($p<0.05$) between culture times for a certain group, ✶ indicates a significant difference ($p<0.05$) between groups for a fixed culture time, ✶ indicates a significant difference ($p<0.05$) between all other groups for a fixed culture time, except the slow releasing one. (n=3 ± s.d.)

The coll. II and AGC gene expression of the pellets clearly confirms the beneficial effect of the bolus supplementation on cartilage formation, in comparison to slower deliveries (positive control and slow release from scaffolds). Interestingly, the different supplementation rates up-regulated the coll. II and AGC expression mainly over the first 12 days of culture. At later culture time, the gene expression was not significantly varied. This indicates that the up-regulation of these cartilage-related genes is mainly triggered over the first 12 days of culture. Moreover, it can even be assumed that the first 3 days are the most important as TGF-β_1 is only available to the pellets over the first 3 days for the bolus group, which resulted in the highest up-regulation.

Effect of coating copolymer

Although the rate of TGF-β_1 supplementation from slow (positive control) to instantaneous (bolus) resulted in an increasing beneficial effect on cartilage formation (evident from histological evaluation, GAG quantification and cartilage genes expression), the coating copolymer composition showed a large effect as well. The cause of the beneficial effect of the 2000PEGT80PBT20 copolymer observed by histology (Figure 2) and GAG quantification (Figure 3) is unclear. A direct induction of the cells to the cartilage lineage by the copolymer

can be excluded as the culture of pellets without TGF-β_1 did not show any cartilage formation.

Alternatively, the presence of smaller cells and cellular debris in the pellets core when cultured with a 1000PEGS70PBS30 coating suggests a cytotoxic effect on the cells. Nevertheless, this copolymer did not induce statistically significant differences in the pellets cell number when compared to the 2000PEGT80PBT20 copolymer, and succinated copolymers were not found cytotoxic in previous evaluations [47]. In addition, change in the culture medium pH due to polymer degradation occurred in the same fashion for both copolymers used (from 8.3 ± 0.1 to 7.8 ± 0.2 after three days).

A third possibility may reside in the effect of the copolymers on the amount of TGF-β_1 available in the culture medium. TGF-β_1 is known to be unstable and rapidly degraded [48]. In vivo, the half life time of TGF-β_1 is shorter than 30 minutes [15,2] when in its active form. In addition, due to its high hydrophobicity, TGF-β_1 tends to rapidly adsorb to plastic

Table 3: TGF-β_1 decrease in the culture medium over three days in the presence of unloaded scaffolds coated with 2000PEGT80PBT20 or 1000PEGS70PBS30, after supplementation of different amount of TGF-β_1. The difference between the two coated scaffolds was significant for both culture conditions ($p<0.05$). (n=3 ± s.d.)

Coating copolymer		1000PEGS70PBS30	2000PEGT80PBT20
Remaining TGF-β_1 in the culture medium after the first three days of culture (bolus supplementation in ng)	70	12.4 ± 1.2 % (8.7 ± 0.9 ng)	58.7 ± 10.2 % (41.1 ± 7.1 ng)
	10	14.4 ± 6.4 % (1.8 ± 0.2 ng)	40.6 ± 1.5 % (4.1 ± 0.2 ng)

surfaces, reducing so the biologically active concentration of the protein in solution. Due to the difference in hydrophilicity of the two coating copolymers used, a different TGF-β_1 adsorption behavior can be expected. To assess the effect of the copolymers on TGF-β_1 availability in the culture medium, the protein concentration was measured at different time points and under different conditions schematically summarized in Figure 1. Table 3 shows that indeed the amount of TGF-β_1 was decreased over the first three days of culture, in a different fashion for each coating copolymer.

While only 12 to 14 % of the TGF-β_1 was still present when using an unloaded 1000PEGS70PBS30 coating, between 40 and 59 % remained when using a 2000PEGT80PBT20 copolymer. Over longer culture times, the trend in the growth factor disappearance was similar, with always a more acute clearance when using a 1000PEGS70PBS30 copolymer (Figure 5). The complete refreshment of the TGF-β_1 containing medium at regular time intervals (3-4 days) showed that two different concentration equilibria were reached for each coating copolymer. Around 20 % of the protein was found after each refreshment of the 1000PEGS70PBS30 coating while 40 to 60 % remained in the 2000PEGT80PBT20-containing medium. The presence of different equilibria supports the hypothesis of different adsorption affinities of the protein for the two copolymers. However, the differential concentration decrease could be due to other events. For instance the copolymer degradation products could interact or bind with the protein in a different fashion.

Therefore, similarly to the lower cartilage formation seen from the slow releasing scaffolds (which released less TGF-β_1 than the other conditions), the beneficial effect of the 2000PEGT80PBT20 coating copolymer could be linked to the amount of TGF-β_1 available in the culture medium. The pellets cultured in the presence of this type of coated scaffold will always be exposed to a higher concentration of TGF-β_1, regardless of the supplementation or release rate used. As a result the cartilage formation observed was always higher when 2000PEGT80PBT20 coatings were used. This is well reflected in the more homogeneous GAG formation observed by histology (Figure 2) for all culture conditions and confirmed in previous studies on rabbit MSC which reported the apparition of an undifferentiated core in pellets cultured with concentrations of TGF-β_1 lower than 10 ng/ml in the culture medium [34]. The fact that polymeric scaffolds, not in direct contact with MSC, have an indirect influence on their chondrogenic differentiation via TGF-β_1 availability is important to consider as they can diminish the effect of the growth factor.

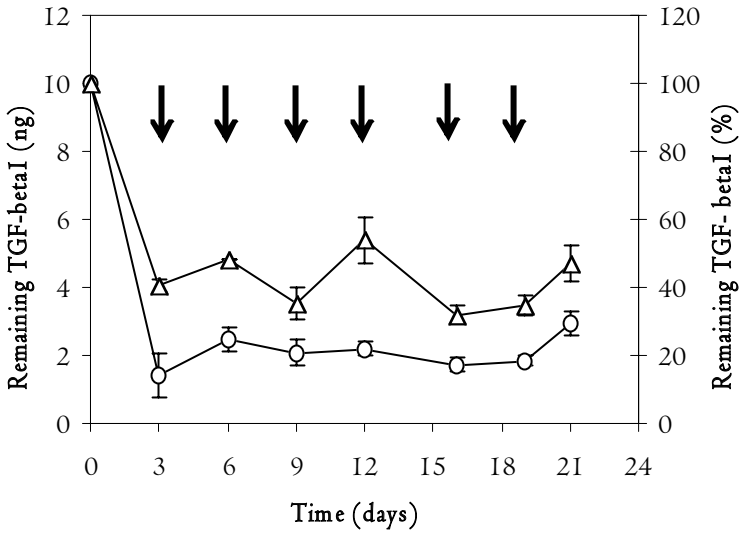

Figure 5: TGF-β_1 decrease in the culture medium after repeated refreshments, over a culture period of 21 days, in the presence of unloaded 2000PEGT80PBT20 (△) or 1000PEGS70PBS30 (○) coatings. Arrows indicate a complete refreshment of the medium, with a fresh solution containing 10 ng/ml of TGF-β_1 (1 ml). For each time point, the difference between the two groups was significant ($p<0.05$). No statistically significant difference was found between the different boluses of TGF-β_1, for a similar coating copolymer. (n=3 ± s.d.)

Overall discussion and conclusion

In the perspective of repairing deficient cartilage, an increasing interest is given to the controlled release of TGF-β_1 from porous supportive structure to enhance the differentiation of cells [7,9,10]. In general, it is assumed that a sustained release of TGF-β_1 results in optimal cartilage formation. On the contrary, the present study indicates that the sustained exposition of TGF-β_1 is not the most effective approach for a given amount of growth factor.

Irrespective of the copolymer used as coating, a bolus exposure to TGF-β_1 resulted in the most successful cartilage differentiation of GMSC pellets. In general, the cartilage formation was increasing with an increasing delivery rate of growth factor to the cells, either released from a porous scaffold or supplemented in the culture medium. Even the repeated supplementation of TGF-β_1 over 21 days (10 ng/ml every 3 days), which is the way commonly used to induce cartilage differentiation of dedifferentiated chondrocytes or MSC [35,49,36,50,37,51], resulted in a lower chondrogenic differentiation as compared to a fast release (within 12 days) or a bolus supplementation.

These differences can be related to the TGF-β_1 concentration readily available in the medium at a given point. This was suggested by the low cartilage formation observed from slow releasing scaffolds, which released less TGF-β_1 than the other conditions. Besides, 2000PEGT80PBT20 coatings always resulted in a higher TGF-β_1 concentration available for the cells and consequently showed a better and more homogeneous cartilage differentiation for all delivery conditions (release or supplementation). The effect of TGF-β_1 concentration was as well indicated in previous studies on rabbit MSC which reported the apparition of an undifferentiated core in pellets cultured with concentrations of TGF-β_1 lower than 10 ng/ml in the culture medium [34]. Therefore, the superiority of the bolus supplementation could simply be due to the higher amount of growth factor in the medium during the first three days of culture, before the first refreshment. Subsequently, although the amount of protein is important, the time frame where TGF-β_1 is presented to the cells appears important as well.

This can be better understood in the light of the physiological mechanism of action of the growth factor. Studies investigating the signaling pathway of TGF-β_1-mediated chondrogenesis of human MSC pellets reported that its triggering action on the cells was occurring rapidly [52,50]. The TGF-β_1 receptors are saturated within 25 minutes and the expression of N-cadherin (cell adhesion molecule functioning during precartilage mesenchymal condensation leading to chondrogenic differentiation) is up-regulated within one day and down-regulated after 5, while changes in cell morphology and increase of ECM production are concomitant. The same rapid chondrogenic induction of the cells was suggested by the temporal pattern of gene expressions. For all culture conditions the up-regulation of the collagen type 2 and aggrecan genes was seen during the first 12 days of culture. Over the next 9 days (till 21 days of culture), the gene expression decreased or increased not significantly. Similar results were observed when human MSC where seeded on PLA scaffolds and exposed to 50 ng/ml of TGF-β_1 during the first three days of culture [53]. This indicates that the cells are most active in reaching the cartilage phenotype during the early time of culture, after being exposed to TGF-β_1. At later time points, the cartilage phenotype is perhaps partially achieved and the cells are reaching a basal level of gene expression or initiating a de-differentiation towards hypertrophic or fibrous cartilage or a fibrotic tissue as was suggested by the increase of collagen type I expression. Cross sections of pellets from the positive control group after 12 days confirmed that the cartilage phenotype was mostly achieved within this time as they presented staining intensities close to the ones observed after 21 days (data not shown). Additionally, it seems that TGF-β_1 is naturally not presented in a continuous fashion to cells

in the body. Its high hydrophobicity induces a high binding affinity for extracellular matrix component, which is irreversible unless proteolytic cleavage occurs [15]. Rapidly upon secretion from the cell, the major fraction is covalently associated with the extracellular matrix and not available for the cells. A bolus supplementation is therefore closer to the physiological mechanism of action than a sustained delivery.

It is likely that once the cells are induced to the chondrogenic lineage at early culture time, they do not need further differentiation signals. Higher amount of growth factor would trigger more of the undifferentiated cells present towards the cartilage lineage. The triggered cells would then differentiate and proliferate as chondrocytes within the pellet, while those not triggered by TGF-β1 would remain undifferentiated and eventually die (in the pellet core). This hypothesis was further strengthened in a separate experiment evaluating the cartilage formation of GMSC aggregates subjected to different bolus of TGF-β1. A gradual increase in differentiation was seen from single cells up to complete aggregate when exposed from 10 ng/ml to 100 ng/ml for 24 hours (data not shown). Similarly, it was previously reported that the exposure of rabbit periosteal explants (containing undifferentiated mesenchymal stem cells) to TGF-β1 for 30 minutes was sufficient to induce cartilage formation after six weeks in a concentration dependent manner [54].

To conclude, the experiments conducted clearly indicate that a long term delivery of TGF-β1 or its supplementation at regular time interval is not the most optimal way to induce the chondrogenic differentiation of GMSC. TGF-β1 is involved in the cell fate decision, which occurs immediately after exposure, but not in later phases of differentiation. Hence, a single bolus delivery is more effective, granted that all cells are available at that time, which might not be the case when scaffolds are implanted in chondral or osteochondral defects. In addition, this study reveals that the copolymers used as scaffolds have an indirect effect on MSC chondrogenic differentiation via TGF-β1 availability. This is important to consider as the nature of the scaffold can diminish the effect of the growth factor.

The quality of the cell differentiation appears mainly linked to the concentration of the growth factor present at the beginning of the culture. This has important implication for the use of TGF-β1 release systems for cartilage regeneration, as it might not be of interest to develop complex release systems providing a long term delivery. Additionally, it could be helpful to develop tissue engineered systems based on MSC without controlled release. One could think for instance to trigger the cells by a bolus administration and implant them without waiting for their differentiation in vitro. Alternatively, if the totality or part of the cells is to be recruited from the site of implantation in vivo, a bolus or burst delivery might not be the best option as the growth factor will probably be degraded faster than in the confined tube environment and as the number of cells present immediately after implantation might not be sufficient to induce a positive response. It has been reported that osteochondral defects in rabbits could be filled with mesenchymal stem cells about one week after surgery [55]. There, the delayed burst delivery of the growth factor after one week or its sustained release over more than one week days could be of advantage to respectively trigger a sufficient number of cells after one week or to continuously trigger newly recruited cells towards the chondrogenic lineage efficiently.

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Chapter 7

Effect of TGF-β1 released from a scaffold on chondrogenesis in an osteochondral defect model in the rabbit

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Abstract

CHONDROGENESIS in healing articular cartilage defects might be stimulated by the controlled and sustained delivery of growth factors. Transforming growth factor-β1 (TGF-β1) plays an important role in chondrocyte differentiation and proliferation. We tested whether TGF-β1 can be released from a biodegradable polymeric scaffold over a prolonged period of time *in vitro* and whether transplantation of these scaffolds in osteochondral defects modulates articular cartilage repair *in vivo*. TGF-β1 was associated to a poly(ether-ester) copolymeric scaffold by coating the inner pores with a water-in-oil emulsion containing TGF-β1. Unloaded control or TGF-β1 scaffolds were applied to osteochondral defects in the knee joints of rabbits. Cartilage repair was assessed qualitatively and quantitatively at three weeks after implantation. *in vitro*, a cumulative dose of 9 ng TGF-β1 was

released from the scaffold over a period of 4 weeks with an initial fast release over the first week. *in vivo*, there were no adverse pathologic effects on the synovial membrane. Defects treated with TGF-β_1 scaffolds showed no significant difference in individual parameters of chondrogenesis and in the average total score. There was a trend towards a smaller total area of the repair tissue that stained positive for safranin O in defects receiving TGF-β_1 scaffolds, 42.5% lower compared with control defects. The data indicate that TGF-β_1 is released from emulsion-coated scaffolds over a prolonged period of time *in vitro*. The data further suggest that poly(ether-ester) scaffolds releasing a cumulative dose of 9 ng TGF-β_1 applied to osteochondral defects in the knee joints of rabbits do not significantly modulate cartilage repair after three weeks *in vivo*. Future studies need to address the relative importance of TGF-β_1 dose and release rate to modulate chondrogenesis *in vivo*.

Introduction

Traumatic lesions of adult articular cartilage do not regenerate [1,2]. Cartilage defects that penetrate the underlying subchondral bone are repopulated with mesenchymal cells from the bone marrow which differentiate into chondrocytes, deposit a cartilaginous matrix and form a fibrocartilaginous repair tissue [3]. This repair tissue has inferior structural characteristics and degenerates over the course of some months [2]. A variety of approaches is currently used to clinically treat articular cartilage defects, including marrow-stimulating techniques, the transplantation of isolated and expanded autologous chondrocytes in the absence or presence of supportive biodegradable matrices or the transplantation of cylindrical osteochondral autografts to sites of articular cartilage damage. However, even such highly sophisticated procedures do not predictably lead to the formation of articular cartilage that is identical in its structure to the normal cartilage and capable of withstanding mechanical stresses over time [4]. The regeneration of the original hyaline articular cartilage therefore remains a great challenge for orthopaedic researchers and clinicians.

The process of chondrogenesis within a healing articular cartilage lesion can be enhanced by regenerative signals provided to the site of articular cartilage repair [5]. Transforming growth factor-β (TGF-β) plays an important role in the growth and differentiation of articular cartilage and promotes chondrogenic differentiation of mesenchymal cells [6]. However, TGF-β_1, the predominant isoform of TGF-β in articular cartilage, has a very short half-life [7]. Recently, an emulsion-coating method was successfully used to associate TGF-β_1 to porous poly(ether-ester) scaffolds and release the bioactive protein in a controlled fashion [8]. These biodegradable hydrogels are based on poly(ethylene glycol)-terephtalate and poly(butylene terephtalate) (PEGT/PBT), and poly(ethylene glycol)-succinate and poly(butylene succinate) (PEGS/PBS) and have been successfully used to release different proteins [9]. Although PEGT/PBT porous scaffolds have been previously used in tissue engineering applications [10] it remains unknown if transplantation of TGF-β_1 scaffolds to sites of articular cartilage damage would modulate the chondrogenesis and cartilage repair *in vivo*. In the present study, we tested the hypothesis that TGF-β_1 can be released from a biodegradable polymeric scaffold over a prolonged period of time *in vitro*. We further investigated

whether transplantation of these scaffolds in osteochondral defects modulates articular cartilage repair *in vivo*. To determine the effects of TGF-β_1 at the onset of chondrogenesis [3], the time point of three weeks after transplantation was chosen for evaluation.

Materials and methods

Materials

Poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) (PEGT/PBT) and Poly(ethylene glycol)-succinate)/poly(butylene succinate) (PEGS/PBS) multiblock copolymers were obtained from OctoPlus, Leiden, The Netherlands, and were used as received. Vitamin B_{12}, bovine serum albumin (BSA), recombinant human TGF-β_1 were from R&D Systems (Minneapolis, USA). Roswell Park Memorial Institute medium (RPMI 1640) was purchased from Cambrex (East Rutherford, USA). Chloroform, obtained from Fluka (Buchs, Switzerland), was of analytical grade.

Preparation of TGF-β_1-loaded polymeric scaffolds.

The TGF-β_1 protein was associated to the porous scaffolds by means of a water-in-oil emulsion method previously described [11]. TGF-β_1 in a 4 mM HCL aqueous solution containing 1 mg/ml BSA (according to the supplier's protocol) was emulsified with PEGS/PBS copolymer solution in chloroform, using an Ultra-Turrax (T25 Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) for 30 s at 19 krpm. The TGF-β_1 concentration of the aqueous solution was set at 19.8 μg/ml. The volume of the aqueous phase was set to 0.5 ml. The copolymer solution was obtained by dissolving 0.5 gram of copolymer in 3 ml of chloroform. The copolymer compositions used had a PEGS content of 70 weight % and a PEG molecular weight of 1000 g/mol.
Compression molded/salt leached scaffolds were obtained by applying pressure (10000 PSI during 10 minutes) and heat (240 °C) to a homogeneous mix of NaCl salt crystals and copolymer powder in a mold. The volume fraction of salt in the mixture was adjusted to 75 %. After cooling of the resultant dense block, the salt was extracted by successive immersions in RX-water (water conductivity less than 25 μS). Subsequently, the porous blocks were dried in ambient air for at least 24 hours, and then placed in a vacuum oven (50 °C) for a minimum of 12 hours. The PEGT/PBT copolymer used to prepare the scaffold had a PEGT content of 55 weight % and a PEG molecular weight of 300 g/mol. The salt crystals were sieved between 400 and 600 μm. Coated scaffolds were prepared by forcing the TGF-β_1-containing emulsion through a cylindrical prefabricated porous scaffold (1 cm in diameter and 1 cm in length) with the use of vacuum (300 mBars). This vacuum was applied for at least 5 minutes, in order to evaporate as much chloroform as possible from the emulsion, thereby creating a polymeric coating on the scaffold. The resulting TGF-β_1-coated scaffolds

(termed TGF-β_1 scaffolds) were frozen in liquid nitrogen, and freeze-dried at room temperature for 24 hours. Blank scaffolds (termed control scaffolds) were prepared by using a TGF-β_1-free 4 mM HCL solution (with 1 mg/ml BSA) in the same conditions as TGF-b containing scaffolds. For *in vivo* implantation and *in vitro* release determination, 4 mm cylinders were aseptically cored from the coated scaffolds prepared as described above. Each cylinder was subsequently cut to a length of 4 mm.

Scanning electron microscopy

A Philips XL 30 ESEM-FEG was used to evaluate the internal morphology of the scaffolds. The internal porous structure was observed by cutting the scaffolds in the longitudinal axis with a razor blade. All samples were gold sputter-coated using a Cressington 108 auto apparatus before analysis.

Characterization of scaffold porosity and interconnection

The average porosity (%) of the scaffolds was evaluated from their dry weight, dry volume and density of the PEGT/PBT copolymer (density = 1.2 g/ml) according to the following equation:

$$p = 1 - \frac{\text{sample weight}}{\text{sample volume} \times 1.2} \qquad \text{(Equation 1)}$$

Three scaffolds pieces were used to determine the porosity of a specific emulsion-coated scaffold. The scaffold pore interconnection was quantified using a method that applies Darcy's law, as described elsewhere [11]. Briefly, water is forced through the porous samples by applying a constant pressure and the flow rate is measured, from which the sample permeability (κ, 10^{-12} m^2) can be calculated. This parameter reflects the sample porosity and pore interconnection.

in vitro release of TGF-β_1 protein

TGF-β_1 loaded scaffolds (4 x 4 mm cylinders of approximately 19 mg) were incubated in 1 ml RPMI 1640 medium at 37 °C, in polypropylene tubes. All samples were kept under constant agitation (25 rpm). The release medium was entirely refreshed at various time points, immediately frozen in liquid nitrogen and conserved at -20 °C until quantification. TGF-β_1 concentrations were quantified using an ELISA with a detection limit of 76 pg/ml (Quantikine human TGF-β_1 immunoassay, R&D Systems). The TGF-β_1 used for the standards and the preparation of the releasing scaffolds originated from the same batch. Aliquots of different volumes were frozen in liquid nitrogen immediately after reconstituting the protein solution and stored at -20°C. They were thawed immediately prior to use for scaffold

preparation or as standards.
To determine the quantity of emulsion effectively coated on the porous scaffold and establish the amount of protein present, coated scaffolds were prepared in the same conditions using a polymer emulsion containing 10 mg of vitamin B_{12} per gram of polymer. The size of this molecule allows a complete release within three days when entrapped in the copolymers used in this study. The quantity of vitamin released is correlated to the amount of polymer coated onto a given scaffold, as the vitamin is homogeneously distributed through the emulsion. In turn, the amount of polymer coated can be related to the amount of protein associated with the scaffold. The amount of vitamin released was calculated using a standard curve of vitamin B_{12} in phosphate buffered saline and a spectrophotometer (El 312e, BioTek instruments) at 380 nm. This indirect detection method was proven to be accurate for lysozyme [11].
The amount of emulsion effectively coated onto the scaffolds was 47 % of the emulsion applied, while the weight of the coated scaffolds was close to 1 gram. Therefore, the amount of TGF-β_1 incorporated in a scaffold piece of 19 mg was about 85 ng.
To determine the stability of TGF-β_1 in the release medium, the absolute concentration decrease of the growth factor was measured by ELISA. Fresh TGF-b was added to the release medium (1 ml) containing control scaffolds at a concentration of 3 and 7 ng/ml. After one day, the medium was collected in triplicate and assayed for concentration.

Transplantation of PEGT/PBT porous scaffolds to osteochondral cartilage defects in vivo

The transplantation of PEGT/PBT porous scaffolds to articular cartilage defects *in vivo* was performed in a previously described animal model [12-14]. Animal procedures were approved by the Saarland Governmental Animal Care Committee. Chinchilla bastard rabbits (Charles River, Sulzfeld, Germany) were kept in air-conditioned rooms with constant temperatures and a regular light/dark scheme. They were fed a standard diet and received water ad libitum. Six five female rabbits (mean weight: 3.1 $\pm$ 0.2 kg; six animals per group) were anesthetized by intramuscular injection of Rompun (0.2 ml/kg of body weight; Bayer, Leverkusen, Germany) and Ketavet (0.75 mg/kg of body weight; Pharmacia & Upjohn, Erlangen, Germany). The knee joint was entered through a medial parapatellar approach, the patella was dislocated laterally and the knee flexed to 90°. Using a manual cannulated burr (3.5 mm in diameter; Synthes, Umkirch, Germany), a cylindrical osteochondral defect was created in the patellar groove of each knee (n = 12 defects). All defects were washed with PBS to remove debris and blotted dry. PEGT/PBT porous scaffolds were press-fit into the defects. The right and left knees alternately received control or TGF-β_1 scaffolds. After reducing the patella, the knee was put through a range of motion to assure the stability of the scaffolds. Incisions were closed in layers. Immediately postoperatively, animals were allowed full weight bearing without any immobilization.

Histological and immunohistochemical analysis

Three weeks after implantation, rabbits were euthanized with Pentobarbital (150 mg/kg body weight; Merial, Hallbergmoos, Germany) and the knee joints were rinsed with 1 ml PBS, exposed and examined for synovitis, osteophytes, or other reactions. The appearance of the defect (color, integrity, contour) and the articulating surfaces were documented. Distal femurs were retrieved, fixed in 4% phosphate-buffered formalin, trimmed, and decalcified. Paraffin-embedded frontal sections (5 mm) were stained with safranin O – fast green, hematoxylin and eosin according to routine histological protocols [15].
The synovial membrane was evaluated using a previously published scoring system [16,17]. The categories of the score include villus thickening (fibrosis), villus architecture (blunting) and the presence of inflammatory cell infiltrates.
For type-II collagen and type-I collagen immunostaining, deparaffinized sections were submerged for 30 min in 0.3% hydrogen peroxide. After washing with PBS and incubation in 0.1% trypsin for 30 min, sections were washed with PBS and blocked with 3% bovine serum albumin in PBS (blocking buffer) for 30 min. Sections were then incubated with a 1:50 dilution of a monoclonal mouse anti-type-II or type-I collagen IgG (Acris Antibodies, Hiddenhausen, Germany) in blocking buffer for 24 h at 4°C, washed and exposed to a 1:500 dilution of a biotinylated anti-mouse antibody (Vector Laboratories, Grünberg, Germany) for 1 h at room temperature. After washing with PBS, the sections were incubated for 30 min with the avidin-biotin-peroxidase reagent (Vectastain Elite ABC kit; Vector Laboratories), washed, and exposed to diaminobenzidine (Vector Laboratories). To control for secondary immunoglobulins, sections were processed as above, except for the secondary antibody. Immunoreactivity to type-II collagen in the repair tissue was compared to that of the adjacent normal articular cartilage, which served as a positive internal control. Immunoreactivity to type-I collagen in the repair tissue was compared to that of the subchondral bone adjacent to the normal articular cartilage, which served as a positive internal control. A score was given to each knee: –, no immunoreactivity; +, weaker immunoreactivity; ++, similar immunoreactivity; +++, stronger immunoreactivity.
For the quantitative assessment of the repair tissue, serial histological sections of the distal femora were taken at 200 μm intervals. Safranin O-stained sections within approximately 1.0 mm from the center of the defect (n = 9 - 10 per defect) were analyzed using the articular cartilage repair scoring system described by Sellers and co-workers [18,19]. Specific parameters that were evaluated include filling of the defect, integration of the new cartilage, safranin O staining, cell morphology, architecture within the defect and of its surface, restoration of the subchondral bone and tidemark. Scores were combined and resulted in an average total score. Values are ranging from 31 points (empty defect without repair tissue) to 0 points (normal articular cartilage, complete regeneration). A total of 110 sections were independently scored by two individuals without knowledge of the treatment groups. The safranin O-positive area in the new tissue filling the defects was measured on serial histological sections of the distal femora that were taken within approximately 0.6 mm from the center of the defects at 200 μm intervals (n = 3 - 6 per defect). Low-magnification images of the carti-

lage defects were acquired by a solid-state CCD camera mounted on a BX-45 microscope (Olympus, Hamburg, Germany). The image on the monitor was digitalized and the safranin O-positive area was manually outlined by a blinded observer. The safranin O-positive area was calculated using the analySIS program (Soft Imaging System, Münster, Germany). Collagen fibrils were evaluated using polarized light microscopy (Olympus).

Statistical analysis

in vitro data were evaluated using ANOVA and are expressed in terms of the mean ± standard deviation. *in vivo*, points for each category and total score were compared between the two groups using a mixed general linear model with repeated-measures (knees nested within the same animals). All continuous variables were tested for normality using the Kolmogorov-Smirnov goodness-of-fit method and no significant skewness or kurtosis was detected. Therefore, continuous data are expressed in terms of the mean ± 95% confidence interval. A two-tailed $p < 0.05$ was considered statistically significant. Statistical analysis of the data was performed using the SPSS software package (version 12.0, SPSS Inc., Chicago, USA).

Results

Human TGF-β_1 is efficiently released from poly(ether-ester) copolymeric scaffolds over at least 4 weeks in vitro

The ability of the scaffold to release TGF-β_1 over a prolonged period of time is a prerequisite for the localized delivery of the protein to cartilage defects *in vivo*. Accordingly, we prepared scaffolds coated with TGF-β_1 using an emulsion-coating method. The morphology of these scaffolds prior to implantation, evaluated by Scanning Electron Microscopy (SEM), is presented in Figure 1.

The porosity of the control and TGF-β_1 scaffolds was in average 65 ± 4 %. Pores were of various sizes and shapes, ranging from 80 to 650 μm, and were visually interconnected. The inter-pore connection of the scaffolds was reflected by their high permeability towards water (κ, [20]) which was in average 78 ± 14 x 10^{-12} m^2.

The *in vitro* release of TGF-β_1 from the porous scaffolds is depicted in Figure 2. A fast release was observed during the first 8 days, followed by a slow and linear release over the following three weeks. The total cumulative amount of growth factor released, as measured by ELISA, was in average 7.5 ng after 8 days and 9 ng after 28 days. Noticeably, the amount of TGF-β_1 released over 28 days only reached 9% of the amount effectively entrapped in the scaffold (85 ng).

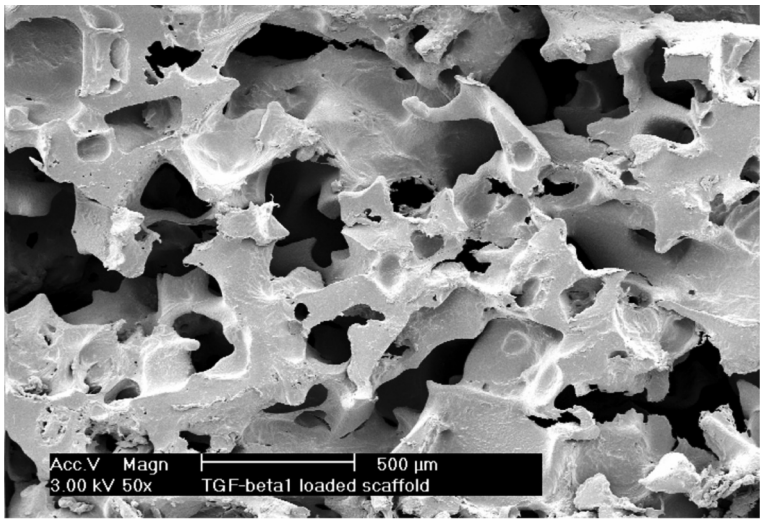

Figure 1: Cross sections of a TGF-β_1-loaded porous scaffold examined by scanning electron microscopy.

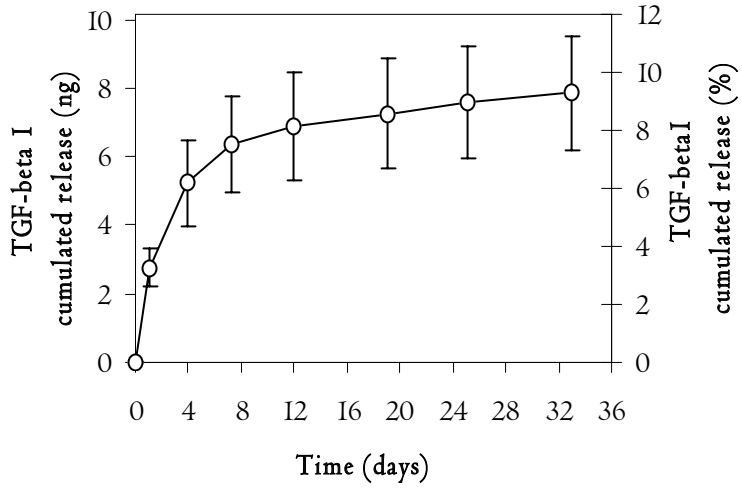

Figure 2: Cumulated release of TGF-β_1 from 4 x 4 mm cylindrical porous polymeric scaffolds. At each time point, the release medium was collected and the amount of growth factor released quantified by ELISA. (n = 3 ± s.d.).

Spatial delivery of human TGF-β_1 does not induce a synovial inflammatory response in vivo

Three weeks following implantation in osteochondral defects in each patellar groove of rabbits, there were no macroscopic signs of inflammation or hematoma. The new tissue in these

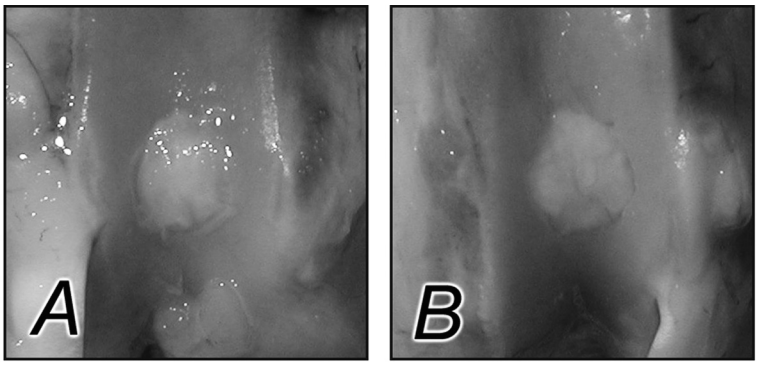

Figure 3: Macroscopic view of femoral condyles receiving a control scaffold (left; A) or a TGF-β_1 scaffold (right; B). Defects in both groups are filled with a new white tissue that is distinguishable from the neighboring normal articular cartilage.

defects had a white color and was still distinguishable from the neighboring normal articular cartilage (Figure 3).

TGF-β_1 concentrations were monitored in the lavage fluid of knees. After 3 weeks, TGF-β_1 concentrations were below the detection limit of the assay in knees receiving control or TGF-β_1 scaffolds ($n = 6$). We next investigated whether the released TGF-β_1 has an effect on the synovial membrane. Analysis of the thickness, architecture of synovial villi, and presence of inflammatory cell infiltrates was performed using a previously published score [17]. The data revealed no significant differences between knees receiving control or TGF-β_1 scaffolds at 3 weeks ($p > 0.05$, $n = 6$) (Table I).

Table I. Histological grading of the synovium at 3 weeks in vivo.

Category	Control Scaffolds	TGF-β_1 Scaffolds	*p* value
Villus thickening	0.45 ± 0.42	0.44 ± 0.54	> 0.05
Villus architecture	0.29 ± 0.43	0.27 ± 0.42	> 0.05
Inflammatory cell infiltrate	0.12 ± 0.37	0.13 ± 0.41	> 0.05
Average total score	**0.86 ± 0.17**	**0.84 ± 0.18**	**> 0.05**

Poly(ether-ester) copolymeric scaffolds remain present in the osteochondral defect for at least three weeks in vivo and allow cartilage formation

After three weeks *in vivo*, the scaffolds remained in a subchondral location (Figure 4; A, B). Both control and TGF-β_1 scaffolds were filled with a tissue consisting of undifferentiated mesenchymal cells, histiocytotic cells and new bone, its trabeculae surrounding the biomaterial (Figure 5; A - D). Polarized microscopy confirmed the presence of trabecular bone (Data not shown). There were few signs of scaffold degradation.

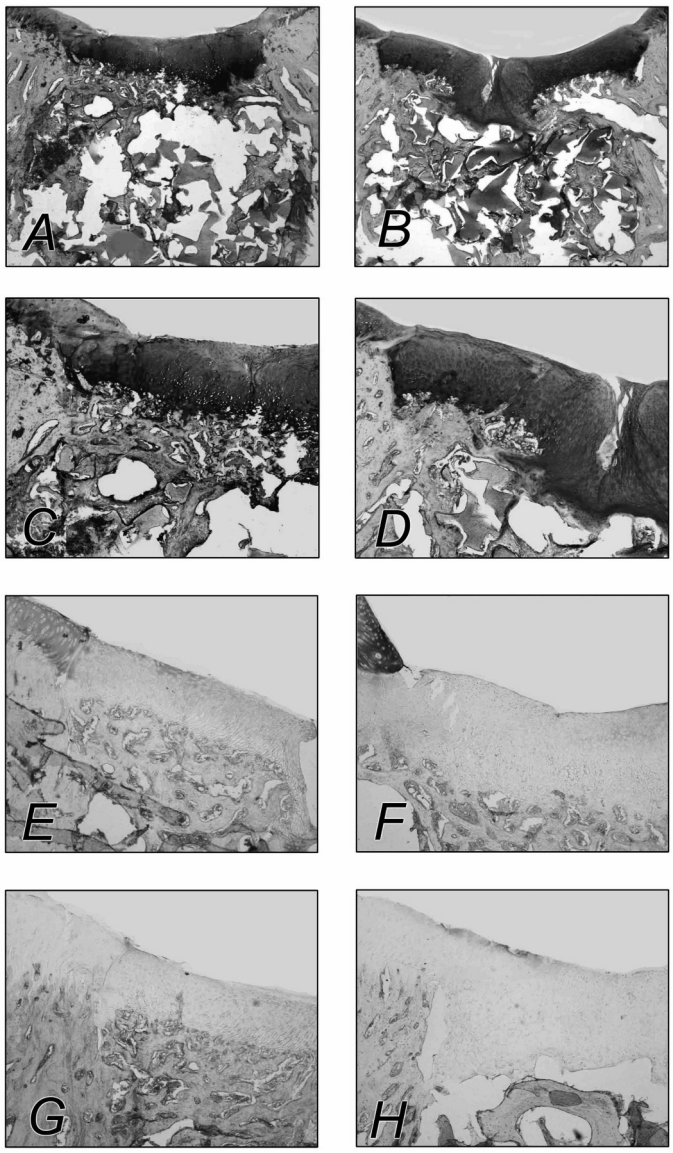

Figure 4. Effect of TGF-β_1 scaffolds on chondrogenesis in articular cartilage defects 3 weeks after transplantation in vivo. Histological appearance of osteochondral defects following implantation of a single control scaffold (left; A, C, E, G) or a TGF-β_1 scaffold (right; B, D, F, H) stained with safranin O – fast green (A - D), a monoclonal mouse anti-human type-I collagen IgG (E, F) or a monoclonal mouse anti-human type-II collagen IgG (G, H). Images C and D are magnified views of the left side of images A and B. Normal articular cartilage can be identified on the far left side of Images (A – H) including the area of integration between the repair tissue (right side of each picture) with the adjacent normal articular cartilage (left side of each picture). The scaffolds remain in a subchondral location and can be identified by their brown color (A, B). Photomicrographs were obtained using standardized photographic parameters, including light intensity. Original magnifications ×10 (A, B), x 40 (C - H).

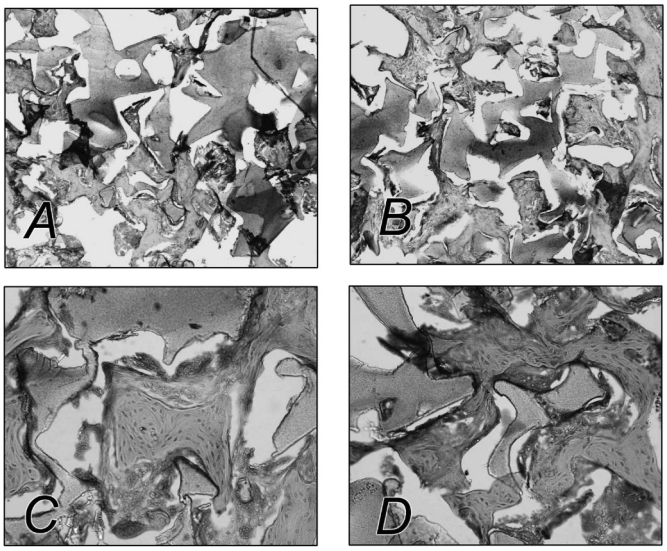

Figure 5: Effect of TGF-β_1 scaffolds on new bone formation in the subchondral space 3 weeks after transplantation in vivo. Histological appearance of the subchondral bone below the osteochondral defects that has been filled with a single control scaffold (left; A, C) or a TGF-β_1 scaffold (right; B, D) stained with safranin O – fast green (A - D). New subchondral bone has formed within the pores of the scaffold, its trabeculae surrounding the biomaterial (C, D). Photomicrographs were obtained using standardized photographic parameters, including light intensity. Original magnifications ×40 (A, B), x 100 (C, D).

Effect of TGF-β_1 released from copolymeric scaffolds on chondrogenesis in vivo

The new tissue in the cartilage defects was analyzed by immunohistochemistry for the presence of type-II collagen, a major component of the extracellular matrix of hyaline articular cartilage (Figure 4; E, F) (Table 2). Three weeks after transplantation, immunoreactivity to type-II collagen was more pronounced in defects receiving TGF-β_1 scaffolds than in defects receiving control scaffolds.

Table 2: Semiquantitative analysis of type-II collagen immunoreactivity in the repair tissue after 3 weeks in vivo.

Animal number	Control scaffolds	TGF-β_1 scaffolds
1	+	+
2	++	+++
3	+++	++
4	+	++
5	+	++
6	++	++
Range	**+ to +++**	**+ to +++**

Type-II collagen immunoreactivity in the repair tissue of the defect was compared to that of the normal articular cartilage adjacent to the defect, used as a positive internal control. Type -II collagen immunoreactivity was scored as follows: –, no immunoreactivity; +, weaker immunoreactivity; ++, similar immunoreactivity; +++, stronger immunoreactivity compared to the normal artic ular cartilage.

Type-I collagen is mainly expressed in fibrocartilage and in the subchondral bone. Immunohistochemical analysis of the repair tissue revealed more type-I collagen in the repair tissue of defects receiving TGF-β_1 scaffolds than in control defects (Figure 4; G, H) (Table 3).

To study the effects of TGF-β_1 on articular cartilage repair *in vivo*, the newly formed repair tissue within the defect was evaluated using a previously published grading system [18] that consists of eight individual parameters. When combined, values are ranging from 31 points (indicating an empty defect without repair tissue) to 0 points (indicating the complete regeneration of normal articular cartilage). No complete articular cartilage regeneration (0 points) was achieved at three weeks for defects treated with control or TGF-β_1 scaffolds. Interestingly, nearly all individual categories of defects treated with TGF-β_1 scaffolds received higher score values than defects treated with control scaffolds, indicative of a lesser grade of cartilage repair than the control group. For example, the scores for the filling of the defect and for the staining of the new tissue with safranin O, an indicator of proteoglycans,

Table 3. Semiquantitative analysis of type-I collagen immunoreactivity in the repair tissue after 3 weeks in vivo.

Animal number	Control scaffolds	TGF-β_1 scaffolds
1	+	+
2	+	++
3	+	+
4	++	+++
5	-	+
6	++	++
Range	**- to ++**	**+ to +++**

Type-I collagen immunoreactivity in the repair tissue of the defect was compared to that of the subchondral bone adjacent to the normal articular cartilage, used as a positive internal control. Type-I collagen immunoreactivity was scored as follows: –, no immunoreactivity; +, weaker immunoreactivity; ++, similar immunoreactivity; +++, stronger immunoreactivity compared to the normal articular cartilage.

Table 4: Effects of TGF-β_1 on the histological grading of the repair tissue after three weeks.

Category	Control scaffolds mean (95% CI)	TGF-β_1 scaffolds mean (95% CI)	*p* value
Filling of the defect	0.55 (0.08 – 1.02)	1.41 (0.01 – 3.33)	0.34
Integration	1.56 (1.07 – 2.05)	1.87 (1.24 – 2.50)	0.34
Matrix staining	0.36 (0.11 – 0.61)	0.94 (0.00 – 2.28)	0.33
Cell morphology	2.83 (2.14 – 3.51)	2.91 (1.55 – 4.26)	0.85
Architecture of defect	3.09 (1.93 – 4.23)	3.08 (2.14 – 4.03)	0.99
Architecture of surface	2.05 (1.76 – 2.33)	2.21 (1.85 – 2.57)	0.47
New subchondral bone	2.86 (1.91 – 3.81)	3.18 (2.83 – 3.52)	0.34
Tidemark	3.98 (3.90 – 4.04)	3.79 (3.37 – 4.20)	0.31
Average total score	**17.3 (14.6 – 19.8)**	**19.4 (13.5 – 25.3)**	**0.42**

Each category and total score is based on the average of 2 independent evaluators. Means indicate estimated scores in points for each category (lower scores indicate better healing). Comparisons were made by repeated-measures ANOVA (knees within the same animal). CI = confidence interval. No statistically significant differences were observed for any category (all $p > 0.30$).

were 2.6-fold worse in defects treated with TGF-β_1 scaffolds than in the control group (Table 4). Although the average total score of TGF-β_1 treated defects was 12% higher (worse) than the average total score of control defects, statistical significance was not reached (Table 4).

The area that was safranin O-positive was 1.137.686 ± 140.494 μm^2 in defects receiving control scaffolds and 798.229 ± 687.286 μm^2, 42.5% more than in defects receiving TGF-β_1 scaffolds ($p > 0.005$).

Each category and total score is based on the average of 2 independent evaluators. Means indicate estimated scores in points for each category (lower scores indicate better healing). Comparisons were made by repeated-measures ANOVA (knees within the same animal). CI = confidence interval. No statistically significant differences were observed for any category (all $p > 0.30$).

Discussion

The difficult accessibility of the cartilage defect to agents that modulate chondrogenesis is a major obstacle in the development of strategies to regenerate articular cartilage lesions. In the perspective of delivering a chondrogenic growth factor to an articular cartilage defect, we developed a system that allowed for a controlled release of TGF-β_1 from porous supportive structures. We tested the hypothesis that TGF-β_1 can be released from these polymeric scaffolds over a prolonged period of time *in vitro*. We further evaluated whether transplantation of such TGF-β_1 scaffolds into osteochondral defects in the patellar groove of rabbits modulates articular cartilage repair *in vivo*. The data indicate that human TGF-β_1 is efficiently released from poly(ether-ester) copolymeric scaffolds over at least 4 weeks *in vitro*. The data further suggest that delivery a cumulative dose of about 9 ng TGF-β_1 in four weeks via these scaffolds is not sufficient to modulate articular cartilage repair *in vivo*.

The release of TGF-β_1 over a prolonged period of time is a prerequisite for the safe and localized delivery of the protein to cartilage defects *in vivo*. The scaffolds employed in the present study released a total cumulative amount of 7.5 ng TGF-β_1 after eight days and 9 ng TGF-β_1 after twenty-eight days, confirming the suitability of poly(ether-ester) copolymer to release proteins [21,22]. The mechanism of release from these hydrogel copolymers is a combination of diffusion and matrix degradation. Over the first eight days of release, the release is likely to be mainly driven by diffusion mechanisms as the degradation of the coated copolymer is minimal after one week [9]. The cumulated amount of TGF-β_1 released over this period of time was relatively low when compared to the total amount entrapped in the scaffolds. This reduced recovery may be linked to the denaturation of the protein during the scaffold preparation or during release. It was previously demonstrated that TGF-β_1 and lysozyme are not denaturated during the emulsion-coating process and that different release rates can be obtained with the same scaffold [11,8]. Therefore, the released amount of TGF-β_1 is possibly linked to the release profile of the scaffold and the intrinsic stability of the protein in the release medium between sampling. TGF-β_1 is not a stable protein and its half life time *in vivo* is inferior to 30 minutes when in its active form [7]. In our experiment, the

absolute depletion over one day of TGF-β_1 in the release medium (RPMI 1640) was 93 ± 2%. Consequently, the cumulative amount of TGF-β_1 measured by ELISA may reflect only a part of the TGF-β_1 that was effectively released. Nevertheless, the amounts of TGF-β_1 released in the present study are about 50-fold lower than applied by Abe and colleagues in liposomes via intraarticular injection [23] and about 5 – 50-fold lower than applied by Mierisch encapsulated in alginate spheres into articular cartilage defects [24]. Additional studies need to test higher doses of TGF-β_1 using these scaffolds. We designed the slow release system employed in the present study in order to maximize the duration of exposure to the growth factor of the defect while avoiding undesired intraarticular side-effects. For example, when 500 ng TGF-β_1 encapsulated in liposomes were applied by intraarticular injection, extensive fibroblastic hyperplasia was seen [23]. Injection of high doses of TGF-b2 led to synovial hyperplasia and cartilage loss [25]. When an adenoviral vector carrying a TGF-β_1 cDNA was injected intraarticularly to naive and arthritic rabbit knee joints, a dose-dependent stimulation of glycosaminoglycan release and nitric oxide production, and induction of fibrogenesis and muscle edema were observed [26]. In addition, chondrogenesis within the synovial lining was induced [26]. These results suggest that TGF-β_1 may stimulate cartilage degradation and may serve to caution the intraarticular application of high doses. The absence of elevated TGF-β_1 levels in the synovial fluid in the present study is probably due to the containment of the protein within the new tissue. Such a lack of elevated intraarticular TGF-β_1 and of inflammatory changes of the synovial membrane may be advantageous in a clinical setting, in order to avoid undesired effects of the therapeutic factor.

After three weeks *in vivo*, the scaffolds remained underneath the original articular cartilage without any signs of adverse reactions. They allowed cartilage formation and the formation of new subchondral trabecular bone. These features are important as the scaffold integrated with the bony compartment. As the implanted scaffolds were devoid of cells, the permeability towards fluids is important to allow the progenitor cells present in the bone marrow to reach the cartilage zone and to allow tissue ingrowth after implantation in osteochondral defects. This was effectively seen *in vivo*, as the scaffolds (either control or TGF-β_1-scaffolds) showed tissue growth in the pores present in the bone area, while cartilage was formed on top of the scaffolds. Scaffold fragments could be seen in all animals, which indicates that the scaffolds dissolves very slowly and thereby have value to act as a support for the new bone and articular cartilage. These features of poly(ether-ester) copolymeric scaffolds may be desirable in tissue engineering and in a clinical application, as some biomaterials have been shown to induce undesired effects when applied intraarticularly [27].

No difference in articular cartilage repair was seen in the present study following transplantation of TGF-β_1 scaffolds compared to control scaffolds. This was somewhat surprising, as previous studies reported improved cartilage repair following application of TGF-β_1 [24,23]. In defects receiving TGF-β_1 scaffolds, trends towards an inhibition of chondrogenesis were seen without reaching significance. In particular, categories indicative of proteoglycan synthesis such as matrix staining and the area that stained positive for safranin O were inferior to defects treated with control scaffolds. When TGF-β_1 encapsulated in liposomes was intraarticularly injected into the knee joints of rabbits at one week after creation of

osteochondral cartilage defects, the early repair of these defects was accelerated [23]. Similarly, when alginate spheres containing 50 or 500 ng TGF-β_1 (per sphere) were applied to osteochondral defects in the trochlear grooves of rabbits, better repair compared with controls was seen [24]. Others reported a trend towards improvement of articular cartilage repair without statistical significance when polymer oligo(poly(ethylene glycol) fumarate scaffolds containing TGF-β_1 were applied to osteochondral defects in rabbits [28]. A separate study perfomed prior to these experiments demonstrated that the TGF-b released from the scaffold is bioactive and is capable to modulate chondrogenesis *in vitro* [8]. One of the reasons for the lack of a significant effect may be that the concentration of the TGF-β_1 was 5 – 50-fold lower than in other reports [24,23]. It is also possible but unlikely that the effect of TGF-β_1 treatment would have become more evident at later time points. Using gene-based treatments, significant improvements in articular cartilage repair were already present after 3 weeks *in vivo* [12-14] in an identical animal model.

Although mainly considered as a stimulator of articular cartilage repair, TGF-β_1 has also been implicated in inhibiting chondrocyte maturation *in vitro* and in inducing alterations of skeletal morphogenesis *in vivo* [29]. Galera and colleagues described differential effects of TGF-β_1 on major components of extracellular matrix, collagen, and proteoglycans, depending on the differentiation state of the cells [30]. Recently, it was reported that TGF-β_1 can inhibit type II collagen biosynthesis in primary articular chondrocytes at transcriptional levels [31]. In a study by Hunziker and Rosenberg, application of 6 ng/ml TGF-β_1 in a fibrin matrix to partial-thickness (chondral) defects in Yucatan minipigs did not lead to cartilage formation [32].

In summary, the results of the present study demonstrate that TGF-β_1 is released from emulsion-coated scaffolds over a prolonged period of time *in vitro*. Scaffolds releasing a cumulative dose of 9 ng TGF-β_1 that are applied to osteochondral defects in the knee joints of rabbits do not significantly modulate articular cartilage repair at 3 weeks *in vivo*. In the future, it will be important to address the relative importance of TGF-β_1 dose and release kinetics of the scaffold employed. It will be further essential to better characterize the effect of TGF-β_1 on chondrogenesis, perhaps in cartilage defects in a larger, clinically more relevant animal model over an extended period of time. We are currently investigating these possibilities. In addition, it will be critical to test other therapeutic factors to enhance articular cartilage repair. A better understanding of the role of TGF-β_1 within the context of a cartilage lesion will lead to improved strategies for articular cartilage defects.

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Can two different proteins be released from a scaffold in a controlled fashion ?

An essential aspect of creativity is not being afraid to fail

Isaac Newton (1643 - 1727)

Chapter 8

Dual release of proteins from porous polymeric scaffolds

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Abstract

To create porous scaffolds releasing in a controlled and independent fashion two different proteins, a novel approach based on protein-loaded polymeric coatings was evaluated. In this process, two water-in-oil emulsions are forced successively through a prefabricated scaffold to create coatings, containing each a different protein and having different release characteristics. In a first step, a simplified three-layered system was designed with model proteins (myoglobin and lysozyme). Poly(ether-ester) multiblock copolymers were chosen as polymer matrix, to allow the diffusion of proteins though the coatings. The model system showed the independent release of the two proteins. The myoglobin release was tailored from a burst to a linear release still on-going after 60 days, while the lysozyme release rate was kept constant. Macro-porous scaffolds, with a porosity of 59 volume %, showed the same ability to control the release rate of the model proteins independently. The relation between the coatings properties and their release characteristics were investigated with the use of a mathematical diffusion model based on Fick's second law. It confirmed that the multiple coated scaffolds are biphasic system, where each coating controls the release of the protein that it contains. This approach could be of value for tissue engineering applications.

Introduction

The repair of damaged or worn out tissues is an increasing concern in western societies where life span is constantly expanding. By combining different approaches taken from biotechnology, biology and material science, tissue engineering aims to provide efficient tools to reach this goal [1]. Although extensive research is currently on going, many difficulties remain to achieve successful and complete tissue regeneration. A novel approach in this field consists of combining porous supportive structures with bioactive molecules such as growth or differentiation factors to guide the tissue regeneration more efficiently. Promising data were reported for bone [2-4], cartilage [5], and angiogenesis [6-8], where single growth factors were used. Nevertheless, a well-timed delivery of the bioactive compounds from the scaffold is necessary to reach the desired effect, as was shown for rhBMP-2 [9,10], bFGF [11], TGFβ$_1$ [12], and platelet-derived growth factor (PDGF) [13]. Growth factors concentration is also of high importance, as wrong dosages can lead to inhibitory effects [14,15]. It is therefore important to be able to modulate precisely the amount and release rate of bioactive compound released from porous structures. A suitable method for this purpose, based on the coating of a protein-containing polymeric emulsion on top of a prefabricated scaffold, has been reported [16].

However, the natural tissue repair process involves multiple growth factors and signaling molecules, in a time and concentration-dependent fashion, as it is clearly established for bone repair [17-19]. Accordingly, the porous supporting structure should optimally allow the release of multiple growth factors in a controlled and orchestrated fashion. Different attempts have been already made to release different proteins from a single release system, in the shape of rods [20], hydrogels [21], or gelatin layers [22]. Porous scaffolds as reservoir for multiple proteins were so far obtained only by assembly and fusion of microspheres [23,24] or by associating them with pre-existing porous structures [25]. It is therefore of interest to investigate new approaches to deliver multiple proteins.

The aim of this study is therefore to develop a method allowing to control the release rate of two different model proteins from defined porous scaffolds in an independent fashion, the proteins being intrinsic part of the scaffolds. To achieve this, a novel approach consisting of applying successive protein-loaded polymeric coatings on top of a prefabricated scaffold was evaluated. In this approach, it is crucial that the top coated layer does not hinder the release of proteins present in underlying coatings. In other words, the polymeric system chosen for the coatings should allow the diffusion of proteins situated in coated layers beneath. This consideration implies that the selected polymers have a release mechanism based on diffusion rather than degradation. This excludes the use of Poly (lactic acid) polymers and copolymers. Instead, a poly(ether-ester) multiblock hydrogel copolymer was used to prepare emulsions and prefabricated scaffolds. This biodegradable hydrogel, based on poly(butylene terephtalate) and poly(ethylene glycol) (PEGT/PBT), is successfully used as protein release system [26] as it allows to tailor release rates easily by varying the copolymer composition. It was demonstrated that the protein release was controlled by a combination of mainly diffusion and degradation of the polymeric matrix [27]. As first approximation, it is expected that that

copolymers containing a higher hydrophilic content (PEG) will result in a faster protein release.
In a first step, a simplified three-layered model system was designed to study the potential independent release of two model proteins: lysozyme and myoglobin. These proteins were selected for their approximately similar molecular weight (respectively 14 and 17 kD) to evaluate the ability of the system to modulate independently the release of proteins of comparable sizes. In addition, myoglobin in solution can be measured by direct absorbance, which facilitates its detection in a mixture of lysozyme and myoglobin solution. The relations between the observed release and three layered-construct properties were then investigated using a mathematical diffusion model based on Fick's second law. Finally, scaffolds were prepared with the same model proteins to validate the concept of multiple coatings. The resulting porous structures were studied with regards to structure and release properties and compared to the model mentioned above.

Materials and methods

Materials

Poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) (PEGT/PBT) multiblock copolymers were obtained from Octoplus, Leiden, The Netherlands, and were used as received. Polymers are indicated as aPEGTbPBTc in which a is the PEG molecular weight, b the weight percentage (weight %) of Poly(ethylene glycol)-terephthalate, and c (=100-b) the weight % of PBT. Lysozyme from chicken egg white (3x crystallized, dialyzed and lyophilised), Myoglobin from horse heart, fluoroisothiocyanate labelled bovine serum albumin (FITC-BSA), Rhodamine B, vitamin B_{12} were purchased from Sigma Chem. corp. (St. Louis, USA). Phosphate buffered saline (PBS), pH 7.4 was obtained from Life Technologies Ltd (Paisley, Scotland). Glycol methacrylate (GMA) and Cryomatrix embedding solutions were respectively purchased from Technovit (Heraeus Kulzer, Germany) and Thermo Shandon (Pittsburgh, USA). Chloroform, obtained from Fluka chemica (Buchs, Switzerland), was of analytical grade.

Preparation of protein-loaded polymeric matrices.

Emulsion preparation

The protein-loaded films and scaffolds were prepared using a water-in-oil (w/o) emulsion method. An aqueous solution of lysozyme or myoglobin in PBS was emulsified with a PEGT/PBT copolymer solution in chloroform, using an Ultra-Turrax (T25 Janke & Kunkel, IKA-Labortechnik) for 30 s at 19 krpm. The protein concentration of the aqueous solution

was fixed at 50 mg/ml for lysozyme and 40 mg/ml for myoglobin. The volume of the aqueous phase was set to 1 ml per gram of copolymer used (water/polymer ratio = 1 ml/g). The copolymer solution was obtained by dissolving one gram of copolymer in 6 ml of chloroform. Six different PEGT/PBT copolymer compositions were used in which the PEGT content was varied from 55 to 80 weight %, with a PEG molecular weight of 300, 1000, 2000 and 4000 g/mol.

Preparation of protein-loaded films

Emulsions of protein and copolymer solutions, prepared as described above, were cast on a glass plate using a casting knife (set at 700 μm). The solvent was slowly evaporated at room temperature and, subsequently, the films were removed from the glass plate and freeze-dried for 24 hours. The resulting films had a thickness ranging from 50 to 110 μm after swelling. A complete description of the swelling determination is presented below.

Preparation of three-layered protein-loaded films

As basis for the multiple-layered films, a 300PEGT55PBT45 solution in chloroform (1 g/6 ml), exempt from protein, was cast on a glass plate with a film applicator (set at 700 μm). After slow evaporation of the solvent for 10 minutes, a protein-containing emulsion was cast above this first dense layer. The resulting two-layered films were slowly dried for 10 minutes. Subsequently, a second emulsion was cast on top of the previous one. The final three-layered films were removed from the glass plate after 10 minutes and freeze-dried for 24 hours. The emulsion preparation and casting of the different layers were done as described above. The second layer contained lysozyme while the third one contained myoglobin. At each emulsion-casting step, a part of the previous film(s) was left untreated for thickness and equilibrium swelling ratio determination of each layer after swelling for at least three days. The swollen three-layered films obtained had a total thickness close to 300 μm. After swelling, the first layer was on average 58 μm thick, the second one 95 μm. The thickness of the third layer was 137 μm for the 1000PEGT70PBT30 and 1000PEGT80PBT20 copolymers and 159 μm for the 2000PEGT80PBT20 one.

Preparation of double emulsion-coated scaffolds

The prefabricated scaffolds were obtained by compression molding-salt leaching method, using salt crystals sieved between 400 and 600 μm and 300PEGT55PBT45 copolymer granules, as reported in detail elsewhere [16].
Coated scaffolds were prepared by forcing successively two protein containing emulsions (prepared as mentioned above) through a prefabricated porous scaffold with the use of vacuum (300 mBars). The vacuum and resulting air flow through the scaffold was applied for at

least 5 minutes. This resulted in a rapid evaporation of chloroform from the emulsion, thereby creating a polymeric coating. The first coating contained lysozyme while the second one contained myoglobin. The resulting coated scaffolds were frozen in liquid nitrogen, and freeze-dried at room temperature for 24 hours.

Swelling of protein-loaded films

The swelling behavior of the different protein-loaded films was determined by immersing dry film pieces (1.77 cm^2 discs) of known weight in PBS at 37°C in a shaking bath. After 3 days, the weight of the swollen films was determined after residual surface water was removed by blotting the surface on a tissue. A time period of three days was previously shown to be sufficient to reach a swelling equilibrium of PEGT/PBT films of comparable thickness [27]. The water uptake (in ml per gram of polymer) was calculated from the weight increase. The equilibrium swelling ratio Q was determined from the weight of the swollen scaffolds using a density of 1.2 g/ml for all PEGT/PBT copolymers.
The swelling of each layer comprised in each three-layered film was estimated by measuring the individual water uptake of single, double and triple-layered films (1.77 cm^2 discs). These films were obtained during preparation of each triple-layered film, by leaving part of the successive layers uncovered by the cast emulsion. As the single, double or triple-layered films used for swelling determination were of similar size, it was possible to deduce the water uptake of each construct top layer. For instance, the water uptake of the second layer of a double-layered film was obtained by subtracting the water uptake of the bottom layer (measured using a single-layered film obtained from an uncovered part of the double-layered film) from the one of the double-layered construct. It was assumed that more hydrophilic copolymer compositions did not influence the swelling of less hydrophilic ones. Each swelling determination was done in triplicate. The homogeneity of each single layer or multiple construct was assessed from the variations seen in the dry and swollen weight of the samples used for swelling determination (measured in triplicates). The minimal and maximal variations observed were respectively of 0.39 and 7.05 %, indicating a homogeneous weight of the layers and therefore homogeneous thickness.

Microscopic evaluation of multiple layered constructs

Cross sections (300 μm) of multiple layered films embedded in PMMA were made using a Leyca saw microtome (sp 1600). Subsequently, the cross sections were observed by polarized light microscopy.

Characterization of scaffold porosity

The average porosity (p, %) of the scaffolds was evaluated from their dry weight, dry volume and density of the PEGT/PBT copolymer (density = 1.2 g/ml) according to:

$$p = 1 - \frac{\text{sample weight}}{\text{sample volume} \times 1.2} \qquad \text{(Equation 1)}$$

The scaffold pore interconnection before and after coating treatment was quantified using a method that applies Darcy's law, as described elsewhere [28-30,16]. In brief, water is forced through the porous samples by applying a constant pressure and the flow rate is measured, from which the sample permeability (κ, m^2) can be calculated. This parameter reflects the sample porosity and pore interconnection; therefore, it can be used to compare different scaffolds.

Microscopic evaluation of coated scaffolds

The internal morphology of the scaffolds was observed by scanning electron microscopy (Philips XL 30 ESEM-FEG). The internal porous structure was observed by cutting the scaffolds in the longitudinal axis with a razor blade. All samples were gold sputter-coated using a Cressington 108 auto apparatus before analysis.

The coatings were evaluated using Fluoroisothiocyanate labelled bovine serum albumin (FITC-BSA) and rhodamine as incorporated proteins in the first and second coating (respectively 12.5 mg/ml of PBS and 23.5 µl of 1 weight % rhodamine B alcoholic solution). Samples were embedded in GMA and 10 µm cross-sections made by using a Microm microtome (HM 355 S). Subsequently, the cross-sections were observed by fluorescence microscopy (FITC-Texas red multi-band dual filter, Nikon, Tokyo, Japan). 2 µl of a 1 weight % rhodamine B solution in water was added to 5 ml of GMA-A solution (prior polymerization) to distinguish the embedding matrix from the prefabricated scaffold under fluorescent light.

To evaluate the distribution of the coatings after swelling in PBS, the scaffolds were embedded in Cryomatrix and 10 µm cross sections made by using a cryotome (Cryostat, Shandon, Pittsburgh, USA). The cross sections were subsequently immersed in PBS for three days to leach out the embedding material and allow the swelling of the scaffolds sections. Two cross sections were then observed under fluorescence microscope as described above and the thicknesses distribution of each layer was measured using image analysis software (Bioquant nova prime, Nashville, USA). For each layer, approximately 400 measurements were used to obtain the thickness distribution.

In vitro protein release

Protein loaded films or scaffolds (10 mg of films, 30 mg of three-layered films, and 50 mg of coated scaffolds) were incubated in 1 ml PBS (pH 7.4) at 37 °C. All samples were kept under constant agitation (25 rpm). Samples of the release medium were taken at various time points and the medium was refreshed after sampling. When the release medium was containing one protein, the myoglobin and lysozyme concentrations were respectively quantified by direct absorbance using a spectrophotometer (405 nm) or using a standard protein assay (μ BCA). When the release medium was containing both proteins, the myoglobin concentration in the refreshed medium was quantified by direct absorbance and a standard curve of mixed myoglobin and lysozyme solutions in PBS at a 50/50 weight ratio. The total protein concentration was quantified using a standard protein assay (μ BCA) and a standard curve of mixed myoglobin and lysozyme solutions at a 50/50 weight ratio. The amount of lysozyme released was deduced from the two previous values. It was noticed that the two proteins have different reactivity towards the μBCA assay. Lysozyme causes a higher signal as compared to myoglobin at the same concentration. Therefore, solutions of different lysozyme and myoglobin ratios would result in an incorrect total protein amount when measured with a standard curve of mixed myoglobin and lysozyme at a fixed 50/50 weight ratio. To correct for the different reactivity of each protein towards the μ-BCA assay, different solutions were prepared with the same total protein content (100, 50 and 25 μg/ml) but of different lysozyme/myoglobin weight ratios (from 100 to 0 %). These solutions were measured with a standard curve of mixed myoglobin and lysozyme at a 50/50 weight ratio. The linear decrease of the total protein content measured when increasing the weight ratio of myoglobin was characterized (slope and intercept). Therefore, the amount of lysozyme deduced from the amount of myoglobin and the total amount of protein (respectively measured by direct absorbance and μ BCA) could be calculated correctly.
The lysozyme concentration was further confirmed with another detection method based on a *Micrococcus Lysodeikticus assay* [27,16]. To 150 μl of the lysozyme release medium, a suspension of *M. Lysodeikticus* (100 μl, 2.3 mg/ml), was added in a 96-wells microplate. The decrease in turbidity at 37 °C was measured at 450 nm, during 4 minutes at 15 seconds intervals. The initial kinetic rate (OD slope at t=0) was measured for each samples and the protein effective concentration deducted from a fresh standard curve. The lysozyme concentrations obtained for release time points up to 12 days were similar to the ones obtained with the previous method, confirming its validity. The use of this enzymatic assay allowed to confirm as well the bioactivity of the protein.

Modeling of lysozyme release from films and determination of lysozyme diffusion coefficient

To investigate the lysozyme release from films and multiple-layered films, mathematical models for the diffusion of drugs from polymeric films were used, that successfully described the

release of lysozyme from PEGT/PBT films and microspheres [27]. These models are based on Fick's second law [31]:

$$\frac{\partial C}{\partial t} = D\left(\frac{\partial^2 C}{\partial x^2}\right) \qquad \text{(Equation 2)}$$

Using the following initial conditions:

$$C(t=0,x) = \begin{cases} C_0 & 0 \le x \le l \\ 0 & x > l \end{cases} \qquad \text{(Equation 3)}$$

and boundary conditions:

$$\left(\frac{\partial C}{\partial x}\right)_{x=0} = 0 \qquad \text{(Equation 4)}$$

$$C(t, x > l) = 0 \qquad \text{(Equation 5)}$$

In these equations C(t,x), is the concentration at time t and at position x. D is the protein diffusion coefficient from the polymeric matrix. As was previously reported [27], the diffusion coefficient of lysozyme through PEGT/PBT matrices is a function of time due to polymer degradation. To account for the increase of diffusion over time, the following empirical relation was used:

$$D(t) = \int_0^t D_{initial}(I + at + bt^2)dt = D_{initial}\left(t + \frac{I}{2}at^2 + \frac{I}{3}bt^3\right) \qquad \text{(Equation 6)}$$

In which a and b are constants determined by the empirical relation drawn between polymer degradation (molecular weight, Mn) as a function of time and effect of polymer molecular weight on lysozyme diffusion coefficients. For lysozyme releasing from 1000PEGT70PBT30 films, these constants were $a = 1 \times 10^{-5}\ s^{-1}$ and $b = 1.3 \times 10^{-12}\ s^{-2}$ [27].
For films with a time-dependent diffusion coefficient, Fick's second law can be approximated as:

$$\frac{M_t}{M_\infty} = 4\sqrt{\frac{D(t)}{\pi l^2}} \text{ when } \frac{M_t}{M_\infty} < 0.6, \text{ and} \qquad \text{(Equation 7)}$$

$$\frac{M_t}{M_\infty} = I - \frac{8}{\pi^2}\exp\left(-\frac{\pi^2 D(t)}{l^2}\right) \text{when } \frac{M_t}{M_\infty} > 0.4, \qquad \text{(Equation 8)}$$

where l is the film thickness.
In the current study, copolymer compositions and geometries (films) were similar to the ones used previously (see ref [27]). Therefore the mathematical model summarized above is relevant to describe the release from multiple layered films. However, in case of double, triple-layered films and coated scaffolds, the release is unidirectional. A way to incorporate a unidirectional release to these models consists of considering the thickness of the film as doubled

(l becoming 2l), resulting in equations 9 and 10:

$$\frac{M_t}{M_\infty} = 2\sqrt{\frac{D(t)}{\pi l^2}} \text{ when } \frac{M_t}{M_\infty} < 0.6, \text{ and} \qquad \text{(Equation 9)}$$

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\exp\left(-\frac{\pi^2 D(t)}{4l^2}\right) \text{when } \frac{M_t}{M_\infty} > 0.4. \qquad \text{(Equation 10)}$$

The permeability of PEGT/PBT copolymers used in this study towards lysozyme was estimated by plotting the cumulative release versus the square root of time. The initial diffusion coefficient can be calculated from the linear first part of the curve using equation 11 for films or equation 12 for multiple layered constructs:

$$\frac{M_t}{M_\infty} = 4\sqrt{\frac{D(t)}{\pi l^2}} \qquad \text{(Equation 11)}$$

$$\frac{M_t}{M_\infty} = 2\sqrt{\frac{D(t)}{\pi l^2}} \qquad \text{(Equation 12)}$$

Results and discussion

Characterization of single films

To prepare porous polymeric scaffolds containing and releasing two different growth factors in a controlled and orchestrated manner, a novel approach was evaluated. By applying successive coatings of protein-containing emulsions on top of a prefabricated scaffold, the release of the different proteins could be controlled independently, based on the release properties of each coating. This concept was first assessed using a simplified three-layered model system. The use of a model allows to study the system parameters in a more simple and defined way as compared to porous scaffolds. A schematic representation of this model is presented in Figure 1. The first layer corresponds to the prefabricated scaffold on which the coatings are successively applied and is protein-free. The copolymer composition used (300PEGT55PBT45) prevents any significant diffusion of lysozyme and myoglobin, due to its low degree of swelling. Indeed, previous studies demonstrated that the release of lysozyme from PEGT/PBT films was linked to their swelling. It has been shown that a material with limited degree of swelling, like 600PEGT55PBT45, did not allow any release of lysozyme [32]. As the swelling of 300PEGT55PBT45 films is lower than that of 600PEGT55PBT45 films (respectively 1.1 and 1.4 [33]), it can be assumed that no lysozyme diffusion through a 300PEGT55PBT45 film is possible. The second and third layers contain respectively two different model proteins (lysozyme and myoglobin) and are obtained by casting successively two water-in-oil (w/o) emulsions on top of the first layer. These two layers mimic the successive coatings applied on the prefabricated scaffold.

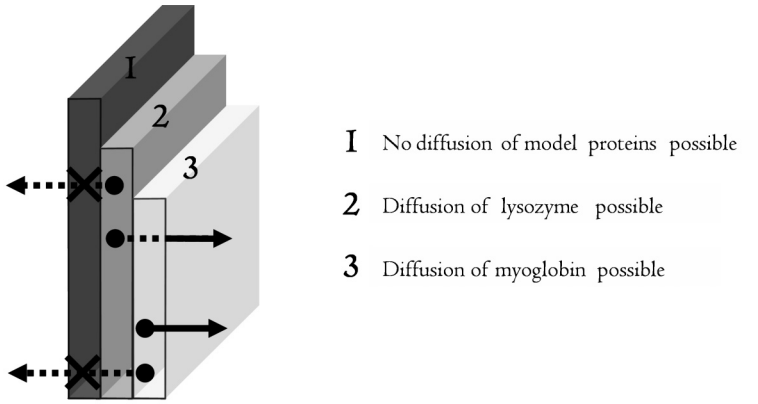

Figure 1: Schematic representation of the model used to mimic a double coated scaffold. Layer 1 (300PEGT55PBT45) does not contain protein and do not allow diffusion of the model proteins. Layer 2 con-

To release lysozyme (present in the second layer) from the construct, the third layer should be permeable for lysozyme. As the release of protein from PEGT/PBT copolymers is mainly diffusion driven and controlled by the copolymer composition [27], a careful selection of the third layer composition is necessary. To select suitable copolymers, single films loaded with lysozyme and myoglobin were prepared from different PEGT/PBT compositions, and characterized by the diffusivity of the proteins.

The release profiles measured for the two proteins are presented in Figure 2. By varying the PEG molecular weight (MW) and PEGT/PBT weight ratio (wt-%), the release rates of both proteins were varied from a burst-like fashion (completed in less than one day) to a linear release over 26 days. The influence of the PEG MW was clearly seen for myoglobin, as an increase in MW from 1000 to 4000 g/mol resulted in increasing release rates of the protein (from a release over more than 30 days to a completion in one day). The PEGT/PBT ratio of the copolymer had a similar effect on lysozyme release, where higher amount of PEGT resulted in faster release.

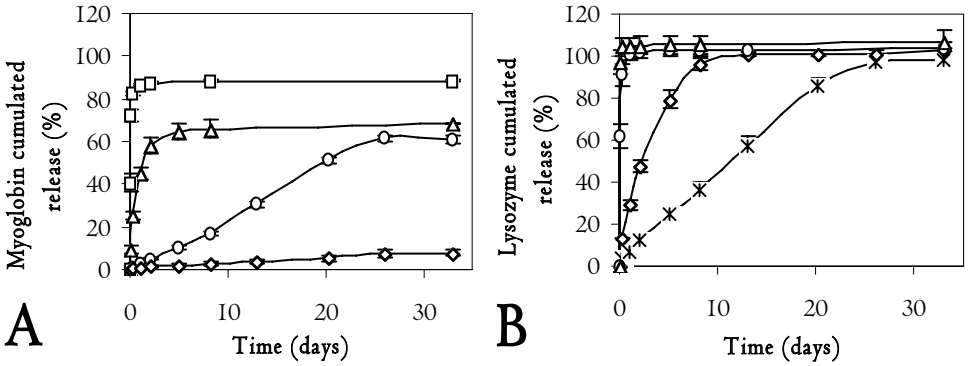

Figure 2: Cumulated release of Myoglobin (A) and lysozyme (B) from different copolymeric films of different PEGT/PBT compositions: 4000PEGT80PBT20 (□), 2000PEGT80PBT20 (△), 1000PEGT80PBT20 (○), 1000PEGT70PBT30 (◇) and 1000PEGT60PBT40 (✳). (n=3; ± s.d.)

Interestingly, for similar copolymer compositions, the release of myoglobin was slower than that of lysozyme. The initial diffusion coefficients of both proteins from the different copolymeric films confirmed this trend, as can be seen in Table 1.
The diffusion coefficient of myoglobin and lysozyme respectively varied from 7.10^{-14} to 5.10^{-9} and from 2.10^{-12} to 5.10^{-9} cm^2/s by increasing the copolymer PEG MW or PEGT content. These results are in agreement with previous work which showed that increasing values of PEG MW and PEGT wt-% were related to an increase of swelling and hydrogel mesh size, resulting in a faster diffusion of the incorporated protein [32]. However, the discrepancy seen between lysozyme and myoglobin diffusion coefficients within the same copolymer composition is surprising, considering that the two proteins have an almost similar molecular weight and hydrodynamic radius (respectively 14 and 17 kD, and 41 and 42.4 Å for lysozyme and myoglobin [33]). This suggests that an extra mechanism plays a role in the release of the myoglobin.

Table 1: Myoglobin and lysozyme initial diffusion coefficients from single PEGT/PBT films ($D_{initial}$, cm^2/s). (n=3 ± s.d.).

Copolymer composition	Initial diffusion coefficient (D, cm ²/s)				
	1000PEGT60PBT40	1000PEGT70PBT30	1000PEGT80PBT20	2000PEGT80PBT20	4000PEGT80PBT20
Myoglobin	n.a.	(7 ± 1) x10^{-14}	(2.5 ± 0.1) x10^{-12}	(1.1 ± 0. 1) x10^{-10}	(4.6 ± 0.4) x10^{-9}
Lysozyme	(1.6 ± 0.4) x10^{-12}	(2.4 ± 0.3) x10^{-11}	(1.5 ± 0.2) x10^{-9}	(4.6 ± 0.2) x10^{-9}	n.a.

In contrast to lysozyme, the release of myoglobin was incomplete, suggesting protein instability. The copolymer composition appeared to have an influence on the total amount released, as faster release rates showed higher protein recovery. This could be due to the intrinsic stability of the protein in the release buffer (phosphate buffered saline (PBS)). It was noticed that precipitates were formed during the storage of myoglobin solutions. It is therefore possible that the protein forms also aggregates in the polymeric matrix over time. Accordingly, faster release would allow a higher protein recovery. The instability of myoglobin hampers the interpretation of the myoglobin release. Although the total amount released is not equal to the total amount incorporated, the release kinetics were based on the amount of soluble protein in solution (not irreversibly aggregated within the matrix).
Based on the release profiles and diffusion coefficients obtained from single films, copolymer compositions were selected for the preparation of multiple layered constructs. With the aim to release the two proteins independently, the copolymer composition of the second layer was fixed, while the one of the third layer was varied. A 1000PEGT70PBT30 copolymer was selected as second layer, which provided a release over 10 days from films. The compositions selected for the third layer (1000PEGT70PBT30, 1000PEGT80PBT20 and 2000PEGT80PBT20) should allow the diffusion of lysozyme, as single films showed a complete release within hours up to 10 days. In addition, the release of myoglobin should be tailored from 5 to more than 25 days, as was seen from films. The diffusion of myoglobin towards the second layer should be prevented by the low diffusion coefficient of this protein in 1000PEGT70PBT30 copolymer.

Three-layered constructs and release

The release profiles of myoglobin and lysozyme observed from multiple layered constructs are presented in Figure 3.

As was expected, the release rate of myoglobin (third layer) was tailored by varying the PEG molecular weight and PEGT/PBT ratio. A burst followed by a slow release over 30 days was obtained for the composition of highest PEG content (2000PEGT80PBT20). The composition of lowest PEG content (1000PEGT70PBT30) showed a very slow release still ongoing after 60 days. The release appeared slower than what was previously seen from single films of the same composition. In addition, myoglobin release was incomplete. In comparison to single films, the recovery was lower, which confirms the aggregation of myoglobin as a function of time, as was suggested in the previous section. The release of lysozyme from the construct second layer was characterized by the appearance of a lag-time, which increased from 0 to 15 days by lowering the PEG content of the third layer from 2000PEGT80PBT20 to 1000PEGT70PBT30. After the lag-time, the release rate of lysozyme was similar for the different third layers, indicating that the release of protein present in the second layer is not restricted by the third one. The lag time can be attributed to the time necessary for lysozyme to cross the third layer. Accordingly, it depends on the protein diffusion coefficient in the third layer. As a result, the lag time can be controlled by varying the third layer copolymer composition or thickness.

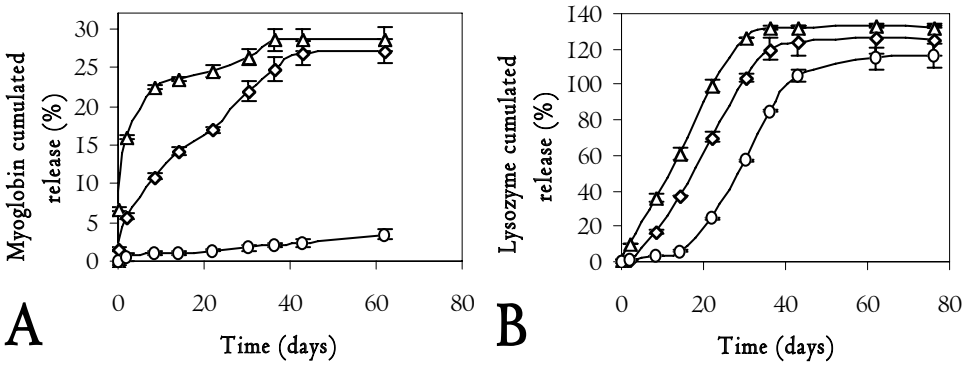

Figure 3: Cumulated release of Myoglobin (A) and lysozyme (B) from three-layered constructs. The copolymer composition of the second layer (containing lysozyme) was fixed (1000PEGT70PBT30), whereas the third layer (containing myoglobin) was varied: 2000PEGT80PBT20 (△), 1000PEGT80PBT20 (◇) and 1000PEGT70PBT30 (○). (n=3; ± s.d.)

However, a question remains regarding the release mechanism taking place in these systems, which leads to a slower release of myoglobin and lysozyme than from single films of similar copolymer compositions. In contrast to single films, for which proteins can diffuse in both directions, the second and third layers only allow a unidirectional diffusion, which could explain the longer release observed. To assess this and to exclude the effect of the third layer on lysozyme release, two-layered constructs were prepared, exempt from the third layer. The

release of lysozyme was measured and compared to a mathematical diffusion model of drug release from PEGT/PBT films, based on Fick's second law [31,34]. The validity of this model for PEGT/PBT films has been discussed elsewhere [27], and was successfully applied to describe the release of lysozyme from 1000PEGT70PBT30 films. The model is based on a time dependent diffusion coefficient to account for the effect of polymer degradation on the protein release rate. Taken into account the thickness of the films, the release of lysozyme from 1000PEGT70PBT30 single films was adequately described by the model. Nevertheless, the unidirectional model predicted a faster release than the one seen for stacked layers (Figure 4). This clearly indicates that neither layer thickness nor unidirectional release can fully explain the slow release observed.

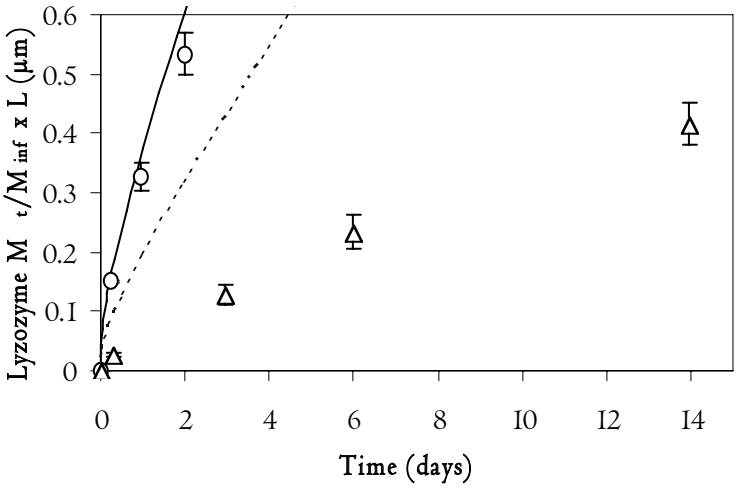

Figure 4: Modeling of lysozyme release from 1000PEGT70PBT30 single films (○) and second layer (□). The plain line represents a bidirectional release (equation 1), the dashed line a unidirectional release (equation 4) and symbols corresponds to the experimental release. The effect of layer thickness is corrected by multiplying the cumulated releases by respective thicknesses. (n=3; ± s.d.)

A mixing phenomenon of the different layers could occur upon evaporation of the solvent, causing change in the polymeric matrix. However, the examination of the interfaces between layers upon polarized light (which reveals the intrinsic crystallinity of the copolymers) showed that no mixing was taking place (data not shown). Another factor that might contribute to the slow release resides in potential interactions of the multiple layers with each other. As stated above, the release rate of proteins from PEGT/PBT copolymers can be related to their swelling. In general, increasing swelling values result in higher protein diffusion coefficient. Therefore, the equilibrium swelling ratio (Q) of each superposed layer was determined and compared to single films. In addition, the initial diffusion coefficients of lysozyme (Dinitial) from the second and third layers were quantified using two-layered and three-layered constructs with lysozyme-loaded top layers (Table 2).

Table 2: Equilibrium swelling ratios (Q) and lysozyme initial diffusion coefficients ($D_{initial}$, cm^2/s) of single films and superposed layers. (n=3 ± s.d.).

Copolymer composition	300PEGT55PBT45	1000PEGT70PBT30	1000PEGT80PBT20	2000PEGT80PBT20
	Equilibrium swelling ratio (Q)			
Single films	1.06 ± 0.02	2.19 ± 0.05	2.23 ± 0.01	3.02 ± 0.04
First layer	1.06 ± 0.02	n.a.		
Second layer	n.a.	2.01 ± 0.03	n.a.	
Third layer		2.14 ± 0.06	2.11 ± 0.03	2.45 ± 0.05
	Initial diffusion coefficient (D, cm^2/s)			
Single films	n.a.	$(2.4 \pm 0.3) \times 10^{-11}$	$(1.5 \pm 0.2) \times 10^{-9}$	$(4.6 \pm 0.2) \times 10^{-9}$
Second layer		$(6.5 \pm 0.8) \times 10^{-12}$	n.a.	
Third layer		$(2.4 \pm 0.2) \times 10^{-11}$	$(1.5 \pm 0.1) \times 10^{-10}$	$(8.6 \pm 1.1) \times 10^{-10}$

n.a.: not applicable

As expected, an increasing content of PEG resulted in increasing value of the equilibrium swelling ratio of single films, from 1 (300PEGT55PBT45) to 3 (2000PEGT80PBT20). The second and third layers showed lower equilibrium swelling ratios in comparison to single films of the same composition. The relative difference varied with the copolymer composition. Interestingly, when second and third layers were of the same composition (1000PEGT70PBT30), the swelling of the second layer was reduced, but the third layer swelled similarly to single films. The initial diffusion coefficients of lysozyme measured for the different layers reflected the equilibrium swelling ratio variations. The effect of the underlying layer, either 300PEGT55PBT45 or 1000PEGT70PBT30 was visible. This caused a decrease in lysozyme initial diffusion coefficient up to 4-fold in comparison to single films. Although the swelling differences observed between single films and layers appear small, the swelling is known to have a large influence on lysozyme initial diffusion coefficient [26]. This hypothesis was further underlined as the protein diffusion coefficient reached a value close to the one of single films when the equilibrium swelling ratio of the third layer was similar to the one of single films.

The swelling variations can be ascribed to the relative effect of the lower layers on the top ones. For instance, the low swelling of the first layer (300PEGT55PBT45) will hamper the swelling of the second one, simply by retaining its physical expansion. The same phenomenon occurs in the third layer influenced by the second one that swells less. The lower swelling of the layers results in lower lysozyme diffusion coefficients as compared to the ones of single films.

These results confirm that a multiple layered system containing different proteins enables an independent controlled release rate of two proteins by careful selection of PEGT/PBT copolymer compositions. As a final evaluation of the concept of multiple polymeric releasing layers with more complex structures, porous scaffolds were prepared.

Porous scaffolds releasing two proteins

The scaffolds were obtained by coating prefabricated porous scaffolds with successively two different w/o emulsions. Copolymer compositions similar to the ones used in the three-layered constructs were selected for the different coatings, with the aim to obtain a fixed release profile of lyzozyme while varying the one of myoglobin. Therefore, two different scaffolds were prepared in which the copolymer composition of the first coating (containing lysozyme) was fixed (1000PEGT70PBT30) while the second one (containing myoglobin) was varied (1000PEGT80PBT20 and 2000PEGT80PBT20).

An overview of the resulting scaffolds and emulsion coated layers morphology, as evaluated by scanning electron microscopy (SEM) and fluorescence microscopy, are presented in Figure 5. The porosity of the scaffolds was decreased by the successive coatings application from 77 % to 66 % in average after the first coating, and to 59 % after the second one. In parallel, the permeability of the scaffolds toward water was modified by the coatings. κ increased from 60 to 140 μm^2 in average after the first coating application and decreased to 110 μm^2 after the second one. High κ values indicate a high inter-pore connection. As was previously reported for single emulsion coated scaffolds [16], the coated layers partly filled the pores and consequently decreased porosity. The increase of scaffold permeability after the first coating is due to the opening of (partly) closed pores which were present in the prefabricated compression molded-salt leached scaffolds [16]. When the emulsion flows through the scaffold during the coating process, the solvent present in the emulsion dissolves the thin polymeric,membranes between the pores. The small decrease in permeability observed after the second coating application suggests that the second coating blocks some pores.

The coatings reflected the order of their application on the scaffolds. They did not molecularly mix and were clearly identified as two separate polymer layers, without mixing of the proteins, as was assessed by fluorescence microscopy. Although the majority of the scaffold surface was covered by the two successive coatings, some areas presented only the first one (containing lysozyme). The distribution of the coatings regarding thickness was inhomoge-

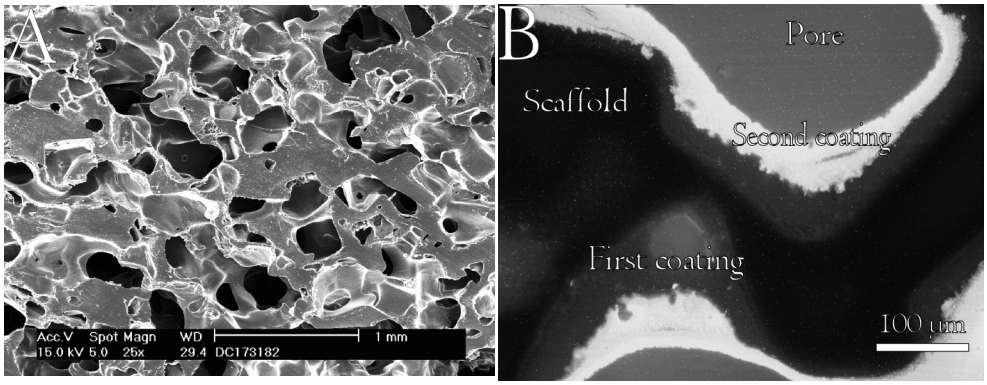

Figure 5: Cross sections of porous scaffolds obtained after application of two emulsion coatings, examined by scanning electron microscopy (A) and optical fluorescent microscopy (B). The first emulsion coating applied contained FITC-BSA, the second one and the GMA embedding solution rhodamine B. The scaffold appears as black.

neous as can be seen in Figure 6. The first coating applied (containing lysozyme) broadly ranged from 3 to 400 μm while the one (containing myoglobin) ranged from and 3 to 240 μm.

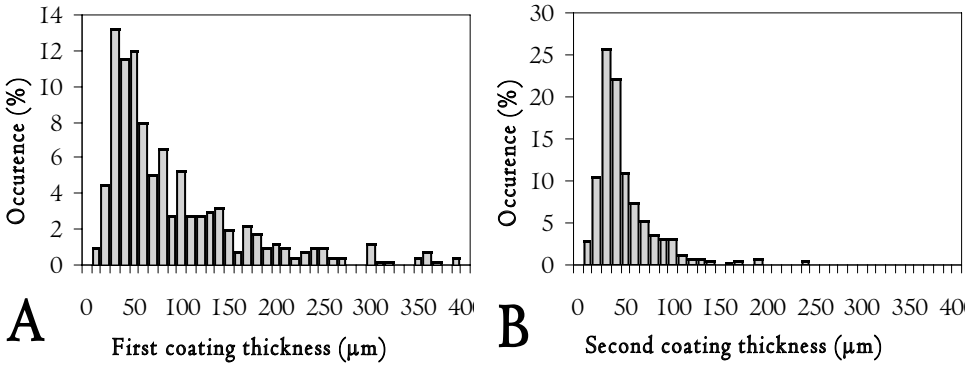

Figure 6: Thickness distribution of the first coating (A, containing lysozyme) and second coating (B, containing myoglobin) over a porous polymeric scaffold.

The lysozyme and myoglobin release obtained from the double coated scaffolds are presented in the Figure 7. The release of myoglobin was tailored from a close to zero order release to a first order release by varying the middle coating copolymer composition from 1000PEGT80PBT20 to 2000PEGT80PBT20. The release obtained from both compositions was completed within 50 days, which is slightly slower than for three-layered constructs of same compositions. The lysozyme release was characterized by a burst during the first hours of release, which contrasts with the release obtained from three-layered constructs. In addition, the release was completed within 50 to 65 days, which is slower than expected. The release profile seemed slightly influenced by the second coating composition, as a more

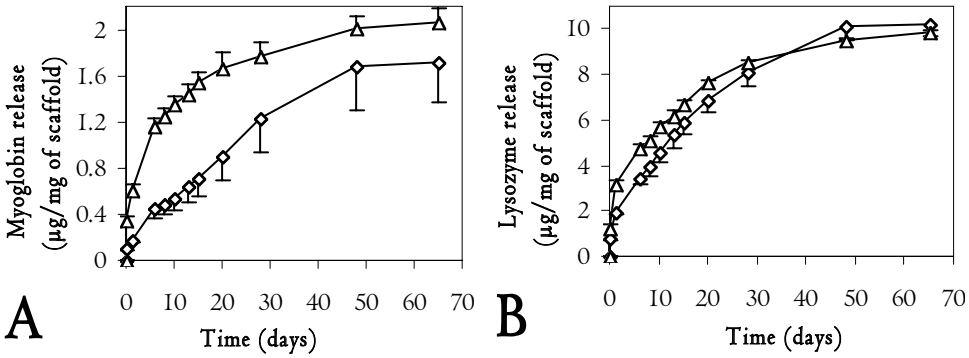

Figure 7: Cumulated release of Myoglobin (A) and lysozyme (B) from double emulsion-coated scaffolds. The copolymer composition of the first coating (containing lysozyme) was fixed (1000PEGT70PBT30), whereas the second coating (containing myoglobin) was varied: 2000PEGT80PBT20 (△) and 1000PEGT80PBT20 (◇). (n=3; ± s.d.)

hydrophilic second coating (2000PEGT80PBT20) showed a higher burst. Beside the burst, the two lysozyme release profiles were similar.
The released lysozyme was not significantly denaturated during the coating process or release, as it presented an activity close to 100 % during the first 12 days of release (data not shown). Due to the evident instability of myoglobin in PBS, a potential effect of the coating process could not be evaluated.
These results confirm the suitability of multiple coatings to create scaffolds of defined properties, containing and releasing two different proteins in an independent way. As was expected from simple three-layered constructs models, the first coating (which was similar for both scaffolds) determined the release of lysozyme while the second emulsion (which was varied) controlled the one of myoglobin, without altering the lysozyme release profile significantly.
Nevertheless, differences appeared regarding the shape and velocity of lysozyme release, in comparison to the three-layered model. To elucidate the reasons for these differences, a model was designed using the data obtained from the three-layered constructs (release profiles, layers thicknesses and diffusion coefficients measured previously for each layer). As previously stated, the release of protein from PEGT/PBT copolymers is due to a combination of diffusion and matrix degradation, and can be modelled using Fick's second law of diffusion. To account for the situation of lysozyme, Fick's second law was numerically solved:

$$\frac{\partial C}{\partial t} = \frac{\partial}{\partial x}\left(D(t,x)\frac{\partial C}{\partial x} \right) \qquad \text{(Equation 13)}$$

using the following initial conditions (see Figure 8):

$$C(t=0,x) = \begin{cases} I & 0 \le x \le L_I \\ 0 & x > L_I \end{cases} \qquad \text{(Equation 14)}$$

and boundary conditions:

$$\left(\frac{\partial C}{\partial x}\right)_{x=0} = 0 \qquad \text{(Equation 15)}$$

$$C(t, x = L_2) = 0 \qquad \text{(Equation 16)}$$

In these equations C(t,x), is the concentration at time t and at position x. When the lysozyme release from the constructs stopped, the cumulated release value at that point was considered as 100 %. This correction could be applied as single films clearly indicated that lysozyme was fully released from PEGT/PBT matrices. The diffusion coefficient D(t,x) is also a function of time and position, as the diffusivity of each layer is different.
During the simulation, the fraction of material released from the system (F) is calculated as a function of time using:

$$F(t) = I - \frac{\int_0^{L_2} C(t,x)dx}{\int_0^{L_2} C(t=0,x)dx} \qquad \text{(Equation 17)}$$

The numerical integration was carried out using the Crank-Nicholson scheme [35]. To obtain the a and b empiric constants necessary to determine the time dependent diffusion coefficient, the lysozyme release measured from the second and third layers was fitted with the model obtained from equations 9 and 10, with the averaged measured thicknesses and

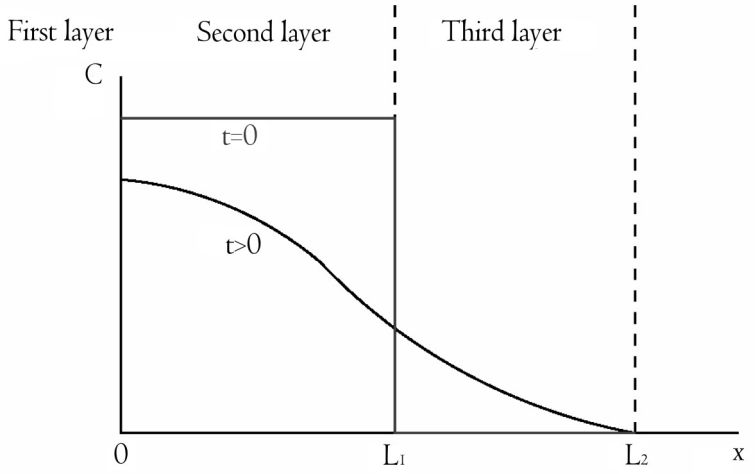

Figure 8: Schematic representation of the initial and boundary conditions of the three layered construct, for lysozyme. The protein cannot diffuse through the first layer, therefore.

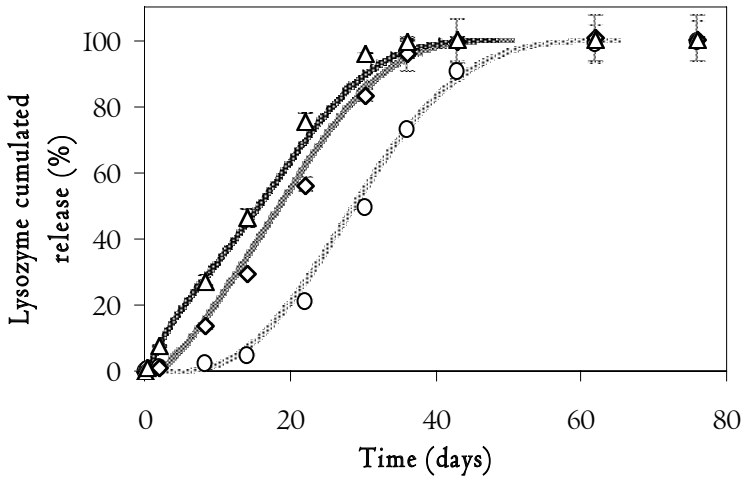

Figure 9: Modeling of lysozyme release from three-layered constructs. The copolymer composition of the second layer (containing lysozyme) was fixed (1000PEGT70PBT30), whereas the third layer was varied. The symbols correspond to the experimental release curves while the lines represent the simulation results (equation 13) for a third layer composition of 2000PEGT80PBT20 (△), 1000PEGT80PBT20 (◇) and 1000PEGT70PBT30 (○). (n=3; ± s.d.)

lysozyme diffusion coefficients for each layer. The best fit was obtained when $a=6 \times 10^{-7}$ and $b=1.2 \times 10^{-12}$ for both second and third layer.

The resulting model showed a god fit with the experimental release (Figure 9). The model mathematically corroborates that, within the conditions of the three-layered constructs, the lysozyme release rate is tailored by the second layer properties (diffusion coefficient and layer thickness) while the lag time is controlled by the third layer. This could explain the lack of lag time and slow release profile obtained from the coated scaffolds. Indeed, the model predicts that decreasing the third layer thickness or lysozyme diffusion coefficient will result in a decrease of the lag time, while the release rate of lysozyme is kept constant (data not shown). Using the copolymer compositions of the coated scaffolds, the lag time would fully disappear for thicknesses lower than 30 μm. Similarly, variations of the middle layer thickness or diffusion coefficient will result in different lysozyme release rates while the lag time is constant (data not shown).

Although the successive emulsion coatings can be considered as films distributed on the scaffold pores, they are not of homogenous thicknesses, which is likely to induce a different release profile. To confirm this, and further validate the model with more complex structures, it was applied to the conditions of the porous scaffolds and its prediction compared with the experimental release. The variable thicknesses of each coating were taken into account by solving the partial differential equations corresponding to each combination of first and second coating thicknesses (39x39), in 10 μm steps. The resulting release profiles obtained for each combination were then averaged using the following formula:

$$F(t) = \frac{\sum_{i=1}^{39}\sum_{j=1}^{39} W_{ij}\, f(t,ij)}{\sum_{i=1}^{39}\sum_{j=1}^{39} W_{ij}} \qquad \text{(Equation 18)}$$

In which W_{ij} is the weight of each coating thickness combination relatively to the complete release profile. The thickness of the first and second coatings are respectively (10 x i) and (10 x j) μm. The independence of each layer thickness was assumed ($W_{ij}=W_i \times W_j$) and W_i and W_j are taken from Figure 6. As can be seen in Figure 10, the forecasted release profiles obtained from the model were close to the experimental release seen from the porous scaffolds. This further underlines the model suitability to describe the release of lysozyme from complex multiple layered polymeric release systems, and mathematically confirms that the longer release profile of lysozyme observed from the porous scaffolds is mainly due to the thickness distribution of the first coating. The little influence of the second coating on the lysozyme release is due to its narrower and smaller thickness distribution. Similarly to the three-layered constructs, by increasing the second coating thickness distribution or decreasing its diffusion coefficient towards lysozyme, the model predicts the apparition of a lag time in the lysosyme release (data not shown). The ability to control this lag time could be of interest for tissue engineering applications which require a sequential release of growth factors at defined rate.

Considering the results obtained from the model, the multiple coated scaffolds can be seen as a biphasic system in which each coating controls the release of the protein that it contains. In addition, the second coating tailors the lag time of the protein present in the first one. By careful selection of copolymer composition and thickness for the first and second coatings, the release profile of the proteins can be separately and independently adjusted regarding release rate and apparition of a lag time. Further experiments will focus on the application of the model to design scaffolds of complex release profiles, with proteins relevant for tissue engineering applications.

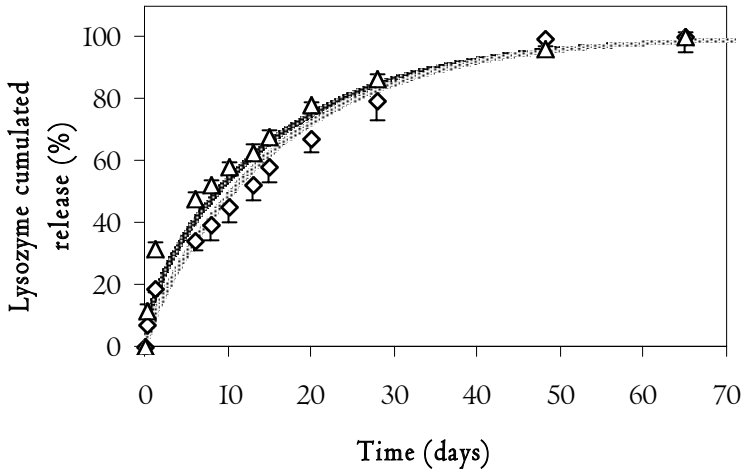

Figure 10: Modeling of lysozyme release from porous scaffolds. The copolymer composition of the first coating (containing lysozyme) was fixed (1000PEGT70PBT30), whereas the second coating was varied. The symbols correspond to the experimental release curves. The lines represent the simulation results (equation 13) for a second coating composition of 2000PEGT80PBT20 (△) and 1000PEGT80PBT20 (◇). The cumulated release was corrected for 100 %. (n=3; ± s.d.)

Conclusions

To create porous scaffolds releasing in a controlled and independent fashion two different proteins, a novel approach based on successive protein-loaded polymeric coatings was evaluated. Each coating containing a different protein and having different release characteristics, it was hypothesized that the release rate of each protein would be tailored separately. To evaluate the effectiveness of this concept, a simplified three-layered model system was designed with model proteins (myoglobin and lysozyme). It showed the suitability of the method, as the release of myoglobin was varied while the one of lysozyme could be kept constant. A lag time in the lysozyme release was present due to the time necessary for the protein to diffuse through the overlaying coating. Scaffolds with a porosity of 59 volume % were prepared accordingly and showed the same ability to control independently the proteins release.

However, no lag time was noticed. A mathematical diffusion model based on Fick's second law was developed to better understand the relations between coatings properties and release profiles. It indicated that the multiple coated scaffolds can be considered as a biphasic system, where each coating controls the release of the protein that it contains. Therefore, a careful selection of coatings copolymer composition and thicknesses virtually allows to obtain a wide range of independent release profiles and lag times. Future experiment will focus on the application of this novel method to relevant growth factors for tissue engineering.

Acknowledgement

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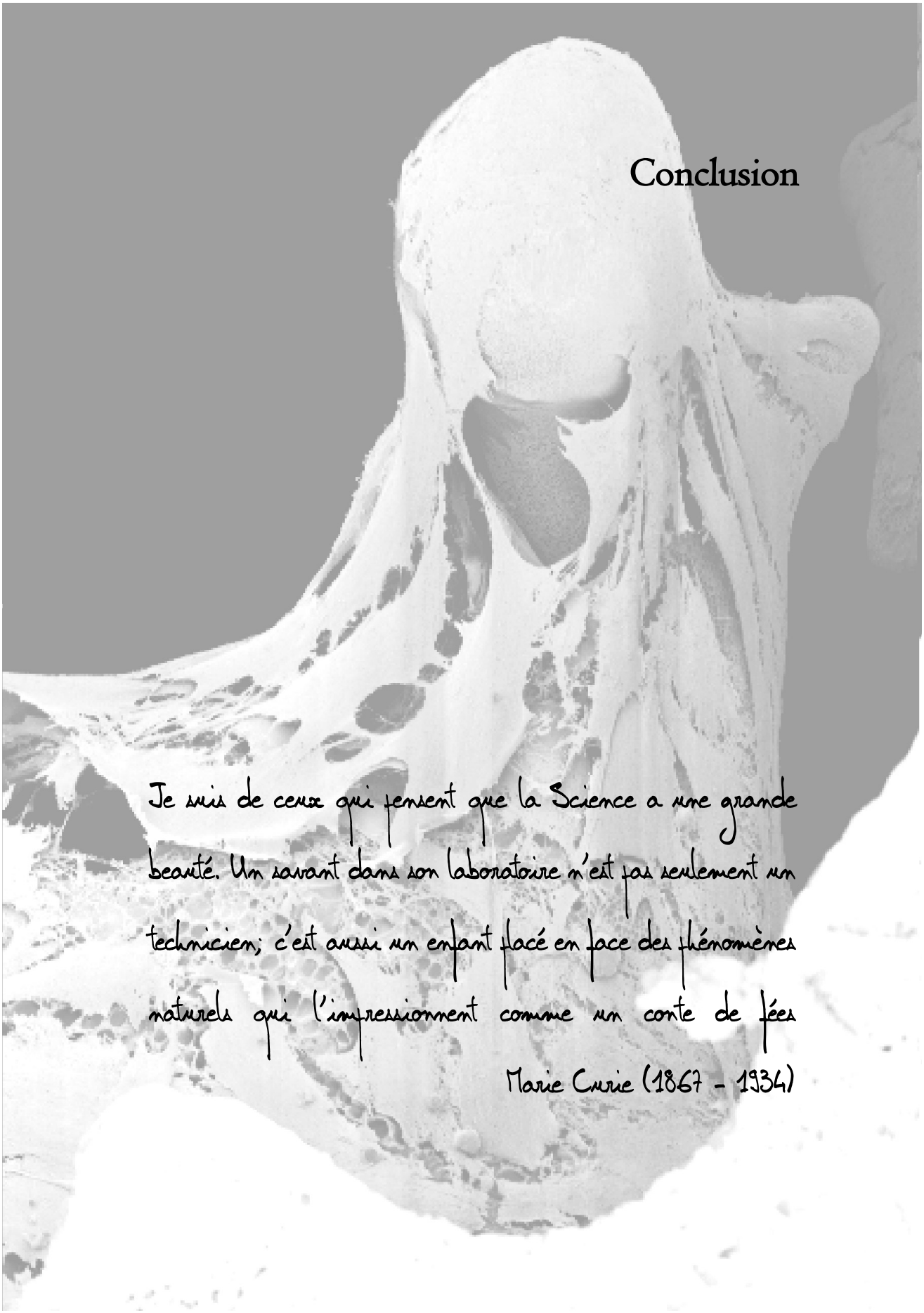

Conclusion

Je suis de ceux qui pensent que la Science a une grande beauté. Un savant dans son laboratoire n'est pas seulement un technicien; c'est aussi un enfant placé en face des phénomènes naturels qui l'impressionnent comme un conte de fées

Marie Curie (1867 - 1934)

Chapter 9

Conclusions

SINCE 100 years, the life span of western society has been continuously and rapidly extended. If this increase of life expectancy is a sign of medical and social progress, it induces as well negative consequences with regard to public health. Due to the finite regenerative capacities of our body, the prevalence of various organ diseases and disorders has increased as a result of the ageing of the population. Articular cartilage is among the organs particularly touched by this phenomenon, as it has low self-repair capabilities. This complex avascular and non-innerved tissue assures the freedom of movement of the joints, which make any disorder incapacitating and painful for the affected persons. There is therefore a need for effective methods to repair and regenerate worn-out or damaged cartilage. Tissue engineering aims to reconstruct tissues both structurally and functionally by combining cells, biomaterials mimicking extracellular matrix (scaffolds) and regulatory signals such as growth factors. Although extensive research has been conducted to determine the most suitable cell source and scaffold architecture to use, complete and stable cartilage regeneration has not yet been achieved. The opportunity to associate growth factors to scaffolds might allow to further enhance cartilage tissue engineering. The controlled release of signaling proteins from the scaffolds is an appealing way to orientate and maintain in a more effective way tissue formation. However, from the literature reviewed in Chapter 2, it can be concluded that many hurdles still have to be overcome to successfully use the combination of growth factors and scaffolds *in vitro*. Most of the processing methods developed to create scaffolds of defined and appropriate properties for cartilage tissue engineering do not allow the incorporation of labile proteins such as growth factors, due to the use of excessive temperature, pressure or organic solvents. As a result, the attempts to evaluate the beneficial effect of growth factor release from scaffolds are often restricted to surface adsorption, which allows a limited control on the release rates of the proteins. Concomitantly, although various growth factors have been characterized and identified to be effective to promote cartilage formation and maintenance, important knowledge is still lacking regarding the most effective rate at which they should be delivered from the scaffolds. Therefore, the possibility to create scaffolds associated with growth factors in a harmless way and allowing to control their release precisely requires further investigation. Once such scaffolds are available, the relations between growth factor release rate and cartilage formation can be further studied and unveiled.

In this context, the objective of this thesis was to answer the following predefined questions:

> A - Can proteins be combined with porous scaffolds of defined properties and released in a well controlled way without loosing their biological activity ?

In drug delivery applications, water-in-oil emulsion (w/o) is the most common method to associate proteins with polymers matrices (in the shape of films or microspheres) and release them in a controlled fashion. However, w/o emulsions have rarely been used to produce porous scaffolds. This might be due to the fact that w/o emulsions were so far mainly used with poly lactic acids polymers (PLA) and poly(L-lactic-co-glycolic acid) copolymers (PLGA), which are know to cause protein aggregation and activity decrease during emulsification. Over the past decade, another copolymer has been investigated as matrix for protein delivery applications, using w/o emulsions. Poly(ether-ester) biodegradable copolymer based on repeating blocks of poly(butylene terephtalate) and poly(ethylene glycol)-terephtalate (PEGT/PBT), were successfully used to release in a controlled fashion various proteins from films or microspheres prepared by w/o emulsions. The advantage of PEGT/PBT copolymers over polyesters such as PLA and PLGA is that they are known to prevent formation of aggregates when used in w/o emulsions. Therefore, the opportunity to use w/o emulsions based on these copolymers to prepare scaffolds were investigated in Chapter 3 and 4. In Chapter 3, a potential approach to circumvent the problematic incorporation of proteins in a scaffold matrix (due to potential denaturation during the preparation process) was evaluated. An attempt was made to dissociate scaffold preparation from protein incorporation by means of w/o emulsions coated on the pore surface of pre-existing scaffolds. Compression molded-salt leached scaffolds were used as prefabricated scaffolds, through which w/o emulsions containing a model protein (lysozyme) were forced by applying a vacuum. After solvent evaporation, a polymer film containing the protein was created on the porous scaffold surface. This approach resulted in an effective, homogeneous and adjustable coating, while the structure of the porous scaffold was modified by the process. The scaffold porosity was decreased due to the coating application whereas the pore interconnection was increased due to the dissolution of membranes present between the prefabricated scaffolds pores. Microporosity appeared on the pores surface, possibly due to the vacuum applied. The release of lysozyme from the coated scaffolds could effectively be tailored from 3 days to more than 2 months by varying the emulsion copolymer composition or water to polymer ratio (w/p). An increase of PEGT weight percentage and w/p ratio resulted in increasing release rates of the protein. Upon increase of PEGT content, the volume swelling and matrix network mesh size of the coated copolymer increases and allows a faster diffusion of the protein. However the lysozyme release from scaffolds was slower as compared to films of similar PEGT/PBT copolymer composition. The effect of emulsion w/p ratio can be related to the formation of interconnected aqueous domains within the emulsion that allow the protein to diffuse more rapidly within the polymer matrix. This was further confirmed in a separate experiment (Appendix to chapter 3) that investigated the release of a different model protein (bovine

serum albumin, BSA) from coated scaffolds. The protein could be released from PEGT/PBT coatings only in an incomplete and burst-like fashion. Conversely to lysozyme, the variation of the PEGT/PBT ratio did not allow to tailor release rates while the increase of w/p ratio only resulted in an increase of the amount of protein released in a burst fashion. This lack of control was linked to the protein size, which is larger than the polymer network mesh sizes. Increase of w/p ratio only allowed a higher amount of protein released through the increasing interconnected aqueous domains. Large proteins such as BSA could be released in a sustained fashion from emulsion-coated scaffolds when using succinate-substituted copolymers (PEG(T/S)/PB(T/S)). The substitution of aromatic groups by aliphatics results in higher swelling properties and faster degradation rates, which allowed the release of BSA over 40 days in a close to zero order release. The applied proteins (lysozyme and BSA) were not denaturated by the emulsion and coating process, as lysozyme activity remained close to 100 % over the release periods and BSA was not aggregated. This indicates the safety of PEGT/PBT w/o emulsions with regard to protein stability. Overall, the emulsion-coating method was found attractive to create scaffolds releasing native proteins in a controlled fashion. The release of different model protein could be obtained and different copolymers could be used. However, a considerable amount of w/o emulsion, and consequently of protein, was lost during the coating process, which hampers the use of expensive growth factors.

To overcome this problem while using PEGT/PBT w/o emulsions, which proved to be effective to entrap proteins, two other scaffold preparation methods were evaluated and compared to emulsion-coating in Chapter 4. These methods were based on the combination of w/o emulsions (containing lysozyme) and of paraffin spheres as porogen, in an organized or unorganized template. Upon exposition to hexane (a non-solvent for the copolymer but solvent for paraffin), porous scaffolds containing lysozyme were obtained by simultaneous precipitation of the copolymer and leaching of the paraffin. As hexane and water are not miscible, the protein was prevented from contact with hexane in the emulsion and no w/o emulsion (and therefore no protein) was lost during the process. When an organized paraffin template was used, porous scaffolds of high porosity and pore interconnection were obtained while unorganized templates resulted in scaffolds of lower porosity and low interconnection. Possibly due to the fast precipitation of the copolymer in hexane, the pores surfaces were dense while the internal structure of the scaffolds was micro-porous. This contrasts with emulsion coated scaffolds which showed the opposite structure. The mechanical properties of the scaffolds indicated that the dynamic stiffness of the scaffolds was related by a power law to the porosity. As a result, scaffolds obtained from organized paraffin templates were highly flexible, which might hamper their use for cartilage applications. The release of lysozyme from the scaffolds could not be tailored by varying the copolymer composition. All scaffolds prepared with paraffin template showed a burst release of the protein within three days, while emulsion-coated scaffolds of similar composition allowed a sustained release up to one month. This discrepancy was related to the internal micro-porous structure of the paraffin templated scaffolds, which shortens the diffusion length within the polymeric matrix and prevent an effective control of the protein release rate. The intact activity of the released protein indicated that lysozyme was not irreversibly aggregated or denaturated during the scaffold

preparation or the release period. This confirms the suitability of PEGT/PBT emulsions to entrap protein in a harmless way. However, the lack of control of lysozyme release from paraffin templated scaffolds hampers their use to obtain a long term release of growth factors. Therefore, emulsion-coated scaffolds based on poly(ether-ester) copolymers were selected as tool to further study the influence of relevant growth factor delivery on cartilage tissue formation.

B - Are PEGT/PBT and PEG(T/S)/PB(T/S) copolymers suitable for the incorporation and controlled release of growth factors ?

Previous investigations on PEGT/PBT and PEG(T/S)/PB(T/S) copolymers as matrix for protein delivery mainly focused on the release of relatively stable model or therapeutic proteins. As growth factors are highly unstable and labile, the suitability of PEGT/PBT-coated scaffolds to release active TGF-β_1 in a controlled way was investigated in Chapter 5. TGF-β_1 was selected for its reported positive effects on cartilage differentiation and repair. It is as well known to induce undesired side effects such as osteophytes formation if present at too high dosage in cartilaginous site and its half-life is short. These properties make it an appealing candidate for sustained release in view of cartilage tissue engineering applications. Emulsion-coated scaffolds containing TGF-β_1 were prepared using PEGT/PBT and PEG(T/S)/PB(T/S) copolymers. The resulting scaffolds were similar to lysozyme and BSA-coated scaffolds with regard to their architecture. The porosity of the scaffolds was decreased while the pore interconnection was increased. By slightly modifying preparation parameters such as vacuum and w/o emulsion volume, the loss of emulsion could be reduced to 50 %. Similarly to lysozyme or BSA-loaded scaffolds, the scaffolds allowed to tailor the release rate of TGF-β_1 *in vitro* from 10 to 40 days by varying the PEGT/PBT ratio or by increasing the degree of succinate substitution. However, only 14 % of the incorporated protein was appeared effectively released from the scaffolds. This low amount of protein recovery was linked to the extremely fast disappearance of the growth factor in the release medium, partly due to adsorption phenomena. The amount of growth factor measured by ELISA at each medium refreshment therefore corresponded to a small fraction of the amount effectively released. The activity of the growth factor released from the scaffolds, measured in a cell growth inhibition assay, indicated that the protein was still active. The bioactivity of the released TGF-β_1 and potential interest for cartilage applications was further investigated by evaluating the capacity of the releasing scaffolds to induce cartilage formation in bone marrow-derived mesenchymal stem cell pellets. After 15 days of culture in the presence of TGF-β_1-releasing scaffolds, histological evaluation of the pellets showed an intense GAG formation which was further enhanced after 21 days. Conversely, the absence of released growth factor showed no GAG formation. It was concluded that PEGT/PBT and PEG(T/S)/PB(T/S) copolymers were suitable for the incorporation and controlled release of active TGF-β_1 and that they allowed the preparation of releasing porous scaffolds by emulsion-coating. In addition, the wide range of release profiles obtained from the scaffolds allowed to investigate the relations between cartilage formation and release rates.

C – What is the impact of growth factor controlled release on cartilage formation and is a sustained delivery a real benefit ?

Considering the short half life and high potency of growth factors, one often believes that a sustained delivery of the protein is beneficial for tissue formation. However, such relation is not necessarily true as growth factors regulate cells proliferation and differentiation by complexes mechanisms. In addition, commercially available growth factors are expensive; therefore, the most optimal way to employ them should be better defined. In Chapter 6, the influence of different TGF-β_1 release profiles from porous scaffolds and different supplementation rates on the cartilage formation of pellets was investigated *in vitro*. Two TGF-β_1 release profiles completed within 12 and 40 days were compared to the instant (bolus) and repeated supplementation every three days of the same total amount of growth factor (positive control). The amount of growth factor released or supplemented was based on commonly used protocol to induce the chondrogenic differentiation of bone marrow-derived mesenchymal stem cells. It clearly appeared that the sustained delivery of TGF-β_1, either released or supplemented, was not the most effective approach to induce the cells towards the chondrogenic phenotype. As was assessed by histology and GAG/DNA quantification, the amount and quality of the cartilage formed was increased when fast delivery rate of the growth factor were used and the best condition was found in a bolus supplementation of the growth factor. The gene expression of cartilage markers (collagen type 2 and aggrecan) indicated that their upregulation occurred mainly over the first 12 days of culture. This suggests that supplementation or release after this period was not useful. As a matter of fact, the differentiation of the cells was triggered during the first 3 days of culture, as the bolus supplementation resulted in the strongest chondrogenic differentiation. The different amount of TGF-β_1 present during the first days of culture and linked to the supplementation and release rates therefore explains the differences seen in differentiation. Faster deliveries result in higher amounts of growth factor during the first three days of culture and better chondrogenic differentiations. Unexpectedly, the different copolymers used as coatings had an effect on differentiation. This was linked to the effect of the copolymers on the growth factor depletion rate in the culture medium. The copolymer that retained a higher growth factor concentration resulted in a more important and homogeneous chondrogenic differentiation. The benefit of a fast delivery of TGF-β_1 could be linked to its physiologic mode of action. Once secreted in the body, the growth factor is rapidly bound to the extracellular matrix and not available for the cells. A fast supplementation is therefore closer to the physiological mechanism of action than a sustained delivery. This fact has important implications concerning the use of TGF-β_1 release systems for cartilage tissue engineering applications. *in vitro*, a fast release covering the recruitment period of bone marrow-derived mesenchymal stem cells (1 week) might be the optimal approach.

This hypothesis was investigated in Chapter 7, which evaluated the effect of TGF-β_1 releasing scaffolds implanted in rabbit osteochondral defects. The scaffolds prepared as previously presented a biphasic release in which the majority of the protein was released over 8 days *in*

vitro. After 21 days of implantation, the scaffolds did not induce signs of inflammation or hematoma, confirming their biocompatibility. In addition, the scaffolds architecture was suitable to allow tissue ingrowth and migration of pluripotent cells as they were filled with a tissue consisting of undifferentiated mesenchymal cells, histiocytotic cells and new bone. However, the release of TGF-β_1 did not enhance articular cartilage formation and showed a lower grade of cartilage repair as compared to implanted unloaded scaffolds (negative control), although not significantly. This lack of effect was surprising and might be attributed to different factors. The amount of release growth factor might not have been sufficient to induce a visible response. This is likely considering the amount measured as released by ELISA *in vitro*. However, as the depletion of the growth factor is fast, the quantity incorporated in the scaffolds should be considered instead. In this case, the role of growth factor dose is unlikely as it is similar to previously reported studies. The fact that the negative control induced a relatively good cartilage restoration could indicate that the animal model selected (rabbit) was not sensitive enough to evidence an effect of the releasing scaffolds. However the benefit of injected TGF-β_1 has been shown with this species. Finally, the release rate obtained from the scaffolds could explain the lack of effect. Although stem cells are know to be present after one week in osteochondral defects, the release might have been too rapid to trigger efficiently a sufficient number of recruited bone marrow-derived mesenchymal stem cells. This chapter further underlines the difficulty to apply growth factor release to clinical applications as optimal release conditions are still unknown.

D – Can two different proteins be released from a single scaffold in an independent and controlled fashion ?

Although the transposition of growth factor controlled release to *in vitro* applications is still not successful, enhancements of releasing scaffolds should be investigated for future applications. The natural tissue repair process involves multiple growth factors and signaling molecules, in a time and concentration-dependent fashion. Accordingly, the porous supporting structure should optimally allow the release of multiple growth factors in a controlled and orchestrated fashion. Accordingly, the aim of Chapter 8 was to develop a method allowing to control the release rate of two different model proteins from defined porous scaffolds in an independent fashion. This could be achieved by applying successive protein-loaded polymeric coatings on top of a prefabricated scaffold. Each coating having different release characteristics, controlled by the copolymer composition, it was hypothesized that the release rate of each protein would be tailored separately. To evaluate the effectiveness of this concept, a simplified three-layered model system was designed with model proteins (myoglobin and lysozyme). It showed the suitability of the method, as the release of myoglobin was varied while the one of lysozyme could be kept constant. A lag time in the lysozyme release was present due to the necessary time for the protein to diffuse through the overlaying coating. The unidirectional release and lower swelling of the coated layers was responsible for the slower protein release rates observed. Scaffolds with a porosity of 59 volume % were prepared accordingly and showed the same ability to control independently the proteins release.

However, no lag time was noticed. A mathematical diffusion model based on Fick's second law was developed to better understand the relations between coatings properties and release profiles. It indicated that the multiple coated scaffolds can be considered as a biphasic system, where each coating controls the release of the protein that it contains. As a result, a careful selection of coatings copolymer composition and thicknesses would virtually allow a wide range of independent release profiles and lag times.

To conclude, this thesis introduces an novel way to combine porous supportive structures and controlled growth factor delivery. The use of PEGT/PBT or PEG(T/S)/PB(T/S) copolymers in w/o emulsions and the dissociation of scaffold preparation and protein incorporation steps allowed an effective entrapment and release of single or multiple proteins. Using the ability of the emulsion-coated scaffolds to release TGF-β_1 in a broad way, the complex relations between release profiles and cartilage formation were investigated. Interestingly, it clearly appeared that a long-term sustained delivery of TGF-β_1 is not the most effective approach. Instead, a relatively fast (12 days) or instant exposure induce a better chondrogenic differentiation of progenitor cells. This knowledge is important to design effective porous scaffolds that will recruit and induce progenitor cells after implantation to form new cartilage. However, upon *in vitro* implantation, the advantage of a TGF-β_1 release over 7 days could not be evidenced. This suggests that more insight is necessary regarding optimal dosage and release rate of growth factors to benefit from their high potency.
Overall, although significant progresses are constantly achieved in cartilage tissue engineering, the path is still long to fully regenerate a functional articulation. May the work presented in this thesis bring sufferers one step closer to relief.

Summary

OVER the last century, life expectancy has increased at a rapid pace resulting in an increase of articular cartilage disorders. To solve this problem, extensive research is currently performed using tissue engineering approaches. Cartilage tissue engineering aims to reconstruct this tissue both structurally and functionally by combining cells and biomaterials mimicking extracellular matrix (scaffolds). Although significant progress has been achieved over the last decade, the complete regeneration of cartilage is not yet at hand. The opportunity to release growth factors from porous scaffolds in a controlled way might allow to further enhance cartilage tissue engineering. However, from the literature reviewed in Chapter 2, it can be concluded that many hurdles still have to be overcome to allow the safe incorporation of labile proteins such as growth factors to scaffolds. As a result, the attempts to release growth factors from scaffolds are often restricted to surface adsorption, which only allows a limited control on the release rates. In addition, important knowledge is still lacking regarding the most effective rate at which relevant growth factors should be delivered.

Therefore, the aim of this thesis was to design polymeric scaffolds containing and releasing growth factors in a safe and controlled way to further study the relations between release rate and cartilage formation.

To prepare porous scaffolds containing and releasing proteins, water-in-oil emulsion methods (w/o) was evaluated in chapters 3 and 4. In combination with poly(ether-ester) biodegradable copolymers, based on repeating blocks of poly(butylene terephtalate) and poly(ethylene glycol)-terephtalate, or poly(butylene succinate) and poly(ethylene glycol)-succinate), a novel method was developed. This method consist of coating prefabricated scaffolds with protein-containing emulsions to obtain scaffolds of defined porosity and pore interconnection. They allowed the release of non-denaturated model proteins (lysozyme and bovine serum albumin) from 3 days to more than 2 months, in a close to zero order release, by varying the emulsion copolymer composition or water to polymer ratio (w/p). The release mechanisms, based on diffusion and degradation, were characteristic of these copolymers.

The emulsion coating method was then evaluated with a relevant growth factor for cartilage tissue engineering, TGF-β_1, in Chapter 5. The resulting scaffolds were similar to the one obtained with model proteins and allowed to tailor the release rate of TGF-β_1 *in vitro* from 10 to 40 days by varying the PEGT/PBT ratio or by increasing the degree of succinate substitution. Although a low protein recovery was noticed, possibly linked to rapid denaturation of the protein in the release medium, the released growth factor was bioactive. The suitability and potential interest of the TGf-β_1 releasing scaffolds for cartilage applications was assessed using bone marrow-derived mesenchymal stem cell (BMSC) pellets. The presence of the releasing scaffold in the vicinity of the cells induced their differentiation towards the cartilage lineage.

In chapter 6, the relations between TGF-β_1 release profiles or supplementation rates and cartilage formation was further investigated *in vitro*. Two TGF-β_1 release profiles were compared to the instant and the repeated supplementation of the same total amount of growth factor. It was apparent by histology, GAG/DNA quantification and gene expression of cartilage markers that the sustained delivery of TGF-β_1, either released or supplemented, was not the most effective approach to induce the cells towards the chondrogenic phenotype. The best conditions were found in the fast release and instant delivery of the growth factor, possibly because of their similarity to TFG-β_1 physiologic mode of action. This fact has important implications concerning the use of TGF-β_1 release systems for cartilage tissue engineering applications. *in vivo*, a fast release covering the recruitment period of bone marrow-derived mesenchymal stem cells (1 week) might be the optimal approach.

This hypothesis was investigated in Chapter 7, which evaluated the effect of TGF-β_1 releasing scaffolds implanted in rabbit osteochondral defects. The scaffolds showed a biphasic release in which the majority of the protein was released over 8 days *in vitro*. After 21 days of implantation, the release of TGF-β_1 did not enhance articular cartilage formation as compared to implanted unloaded scaffolds. This lack of effect could be attributed to an insufficient amount of growth factor released to induce a visible response. Additionally, the animal model selected (rabbit) might not have been sensitive enough to evidence an effect of the releasing scaffolds. Finally, the release rate obtained from the scaffolds could have not been suitable. Although stem cells are know to be present after one week in osteochondral defects, the release might have been too rapid to trigger efficiently a sufficient number of bone marrow-derived mesenchymal stem cells to the cartilage phenotype.

Although the transposition of growth factor controlled release to *in vivo* applications was not successful, enhancements of releasing scaffolds were investigated for future applications in Chapter 8. A novel method allowing to release two model proteins from scaffolds was developed to mimic the natural tissue repair process, which involves multiple growth factors in a time and concentration-dependent fashion. Protein-loaded coatings of different release characteristics were successively applied on top of scaffolds. This approach allowed a controlled and independent release of 2 model proteins.

Overall, this thesis presents an effective new way to combine growth factor release and porous scaffolds. Using the ability of the emulsion-coated scaffolds to release TGF-β_1 in a wide range, the complex relations present between release profiles and cartilage formation were underlined. However, the advantage of sustained TGF-β_1 release could not be evidenced *in vivo*. This suggests that more knowledge is necessary regarding optimal dosage and release rate of growth factors for clinically relevant applications.

Samenvatting

De verhoogde levensverwachting in de laatste eeuw heeft geresulteerd in een groter aantal articulaire (=gewrichts) kraakbeen defecten. Voor een mogelijk alternatieve behandelings wordt momenteel veel onderzoek gedaan naar kraakbeenherstel met behulp van 'tissue engineering'. Kraakbeen 'tissue engineering' richt zich op het structureel en functioneel reconstrueren van weefsel door cellen te combineren met biomaterialen die de extracellulaire matrix nabootsen (scaffolds). Hoewel en aanzienlijke voouitgang is geboekt in de laatste 10 jaar is volledige kraakbeen regeneratie nog steed niet mogelijk. Het op een gecontroleerde manier vrij laten komen ('releasen') van groeifactoren uit poreuze scaffolds kan kraakbeen tissue engineering mogelijk verder verbeteren. Uit het literatuur overzicht van hoofdstuk 2 kan echter worden geconcludeerd dat er nog vele hindernissen genomen moeten worden om een veilige incorporatie van onstabiele eiwitten, zoals groeifactoren, in scaffolds mogelijk te maken. In het algemeen wordt de afgifte van groeifactoren gelimiteerd door oppervlakte-adsorptie wat slechts beperkte controle van de afgifte sneheid toelaat. Bovendien ontbreekt kennis over de meest effectieve snelheid waarmee relevante groeifactoren zouden kunnen worden afgegeven.

Het doel van dit proefschrift was daarom onderzoek te doen aan polymere scaffolds die groeifactoren bevatten en deze afgeven op een veilige en gecontroleerde manier om de relatie tussen de afgifteheid en kraakbeen vorming te bestuderen.

Om poreuze scaffolds te maken die eiwitten bevatten en afgeven, zijn water-in-olie emulsie methoden (w/o) onderzocht in de hoofdstukken 3 en 4. In combinatie met biodegradeerbare poly(ether-ester) copolymeren, gebaseerd op repetrende blokken van poly(butyleen teraphtalate) (PBT) en poly(ethyleen glycol)-teraphtalaat (PEGT) of poly(butyleen succinaat) (PBS) en poly(ethyleen glycol)-succinaat) (PEGS), is een nieuwe methode ontwikkeld. Deze methode is gebaseerd op het coaten van geprefabriceerde scaffolds met een eiwit-bevattende emulsie waardoor scaffolds met een gedefinieerde porositeit en porie-interconnectie werden verkregen. Door het variëren van de copolymeer compositie van de emulsie of de water/polymeer ratio (w/p), werd een afgifte verkregen van niet gedenatureerde model eiwitten (lysozyme en bovine serum albumine) van 3 dagen tot meer dan 2 maanden volgens een nulde-orde afgifte. Het afgiftemechanisme is gebaseerd op diffusie en degradatie hetgeen een kenmerk is van deze copolymeren.

In hoofdstuk 5 is de emulsie coating methode geëvalueerd met een relevante groeifactor voor kraakbeen tissue engineering, namelijk TGF-β_1. De resulterende scaffolds waren vergelijkbaar met de scaffolds die verkregen zijn met de model eiwitten. De afgiftesnelheid van TGF-β_1 *in vitro* kon aangepast worden van 10 tot 40 dagen door het variëren van de PEGT/PBT ratio of door het verhogen van de succinaat substitutie. Hoewel een lage eiwitconcentratie werd gemeten, waarschijnlijk als gevolg van aan een snelle denaturatie van het eiwit in het afgifte medium, was de afgegeven groeifactor bioactief. De geschiktheid en het potentiële belang van

de TGF-β_1 'releasing' scaffolds voor kraakbeentoepassingen is bepaald met behulp van uit beenmerg verkregen mesenchymale stamcel (BMSC) pellets. De aanwezigheid van de releasing scaffold in de nabijheid van deze cellen induceerde differentiatie tot kraakbeen.
In hoofdstuk 6 is de relatie tussen TGF-β_1 afgifteprofielen of supplementatie snelheid en kraakbeenvorming verder *in vitro* bestudeerd. Twee TGF-β_1 afgifteprofielen zijn vergeleken met een eenmalige toediening en herhaalde toediening van een vergelijkbare totale hoeveelheid groeifactor. Met behulp van histologie, GAG/DNA kwantificatie en gen expressie met kraakbeen markers werd duidelijk dat langdurige toediening van TGF-β_1, niet de meest effectieve benadering is om cellen te induceren tot een chondrogeen (=kraakbeen) fenotype. De beste condities bleken de snelle afgifte en eenmalige toediening van de groeifactor, waarschijnlijk doordat ze dichter bij de fysiologische werkwijze van TGF-β_1 liggen. Dit feit heeft belangrijke implicaties betreffende het gebruik van TGF-β_1 vrijlatingssystemen voor kraakbeen tissue engineering toepassingen. *in vivo* kan een snelle afgifte gedurende 1 week (overeenkomstig de recruiteringsperiode van BMSCs) de optimale benadering zijn.
Deze hypothese is verder onderzocht in hoofdstuk 7, waar het effect van de TGF-β_1 releasing scaffolds, geïmplanteerd in osteochondrale defecten in konijnen, is geëvalueerd. De scaffold liet een tweefasige afgifte zien waarin de meerderheid van het eiwit *in vitro* was afgegeven in 8 dagen. Na 21 dagen implantatie, gaf de afgifte van TGF-β_1 geen verhoogde articulaire kraakbeen vorming vergeleken met geïmplanteerde onbeladen scaffolds. Dit gebrek aan effect kan mogelijk worden toegeschreven aan een te lage hoeveelheid afgegeven groeifactor om een zichtbare respons te induceren. Bovendien is het mogelijk dat het gekozen diermodel (konijn) niet gevoelig genoeg is om een effect te vinden van de releasing scaffolds. Tot slot kan het zijn dat de afgiftesnelheid uit de scaffolds niet geschikt is. Hoewel bekend is dat stamcellen al na 1 week aanwezig zijn in osteochondrale defecten, kan de afgiftesnelheid toch te snel zijn geweest om een efficiënt aantal BMSC aan te zetten tot chondrogeen fenotype differentiatie.
Hoewel de gecontroleerde vrijlating van groeifactoren *in vivo* niet succesvol was, zijn de verbeteringen van releasing scaffolds voor toekomstige toepassingen onderzocht in hoofdstuk 8. Een nieuwe methode die de afgifte van twee model eiwitten uit één scaffold toestaat, werdt ontwikkeld om een natuurlijk weefselherstelproces na te bootsen, welke meerdere groeifactoren betreft in een tijd- en concentratie-afhankelijke manier. Scaffolds worden bedekt met twee eiwit-beladen coatingen met verschillende afgiftekarakteristieken. Met deze benadering kon de afgiftesnelheid van twee eiwitten afzonderlijk van elkaar worden gecontroleerd.
Dit proefschrift laat een nieuwe effectieve manier zien om het afgifte van een groeifactor en poreuze scaffolds te combineren. De complexe relatie tussen afgifte profielen en kraakbeen vorming word benadrukt, gebruik makend van de TGF-β_1 afgifte capaciteiten van de emulsie gecoated scaffolds. Hoewel met *in vivo* implantatie het voordeel van aanhoudende TGF-β_1 afgifte niet kon worden aangetoond, zijn er suggestie dat meer informatie noodzakelijk is. Dit in betrekking tot de optimale dosis en afgiftesnelheid van groeifactoren voor klinisch relevante toepassingen.

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Jérôme

Curriculum Vitae

JÉRÔME Sohier was born on May 8th, 1976 in Dakar, Senegal. His childhood was spent between various parts of France and Niger where he received his primary and secondary school education. After his graduation from the Gérard-Philipe high school (Bagnols sur cèze, France) in 1996, he applied for two years to the competitive entry examination of the Medicine University of Montpellier. There, he realized that his interest was in engineering and joined the Health Engineering Institute of Montpellier (I.U.P. Santé), whereby he was awarded a Licence (Bachelor equivalent) and Maîtrise (Honor's bachelor's degree equivalent) on Biomedical and Biodegradable Polymers Engineering, with upper second class honors and first in one's year, in 1999 and 2000. To complete his studies, he performed a 6 month traineeship in IsoTis N.V., Bilthoven, The Netherlands, where he studied and developed the formulation of polymeric microspheres for the controlled delivery of therapeutic peptides. In October 2000, He was hired as a junior research scientist by IsoTis N.V. where he worked for two years on various controlled release systems for proteins. In September 2002, he started as a PhD student in Chienna B.V., Bilthoven, The Netherlands, a drug delivery company which was rapidly acquired by OctoPlus, Leiden, The Netherlands. The research concerning the elaboration and study of growth factor releasing scaffolds for tissue engineering applications was performed under the supervision of Prof. K. de Groot, Prof. C.A. van Blitterswijk and Dr. J.M. Bezemer, the results of which are described in this thesis.

Selected colour figures

Chapter 3

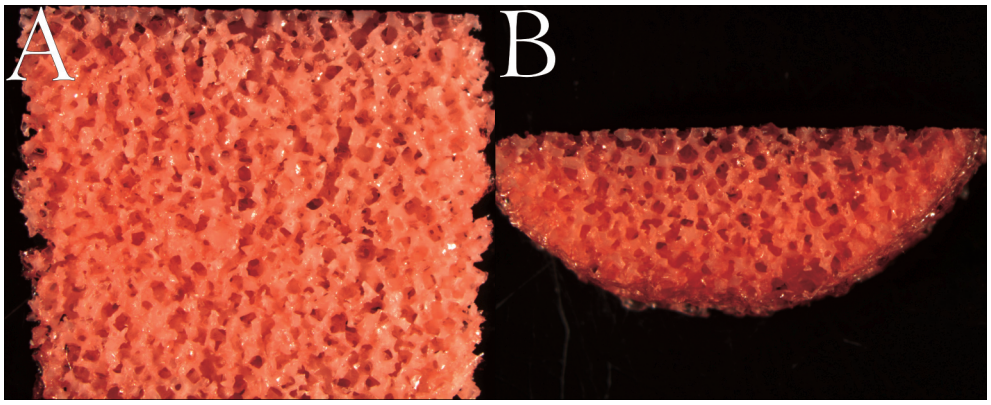

Figure 1: Coating homogeneity as assessed by using a vitamin B12-containing coating. The emulsion was made of 1 ml vitamin B12 solution (10 mg/ml PBS) and 1 gram of 1000PEGT80PBT20 in 6 ml of chloroform; the vacuum applied was 300 mBar. The cross sections were made longitudinally (A) and laterally (B).

Chapter 4

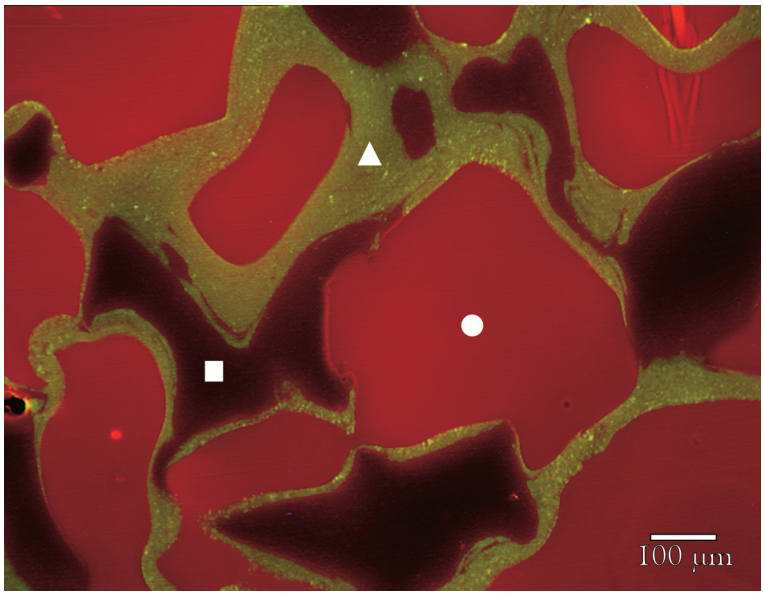

Figure 4: Optical fluorescent micrograph of cross sections of emulsion-coated scaffolds. The emulsion coating contained FITC-BSA (▲) and the GMA embedding rhodamine (●). The polymer appears as black (■).

Chapter 5

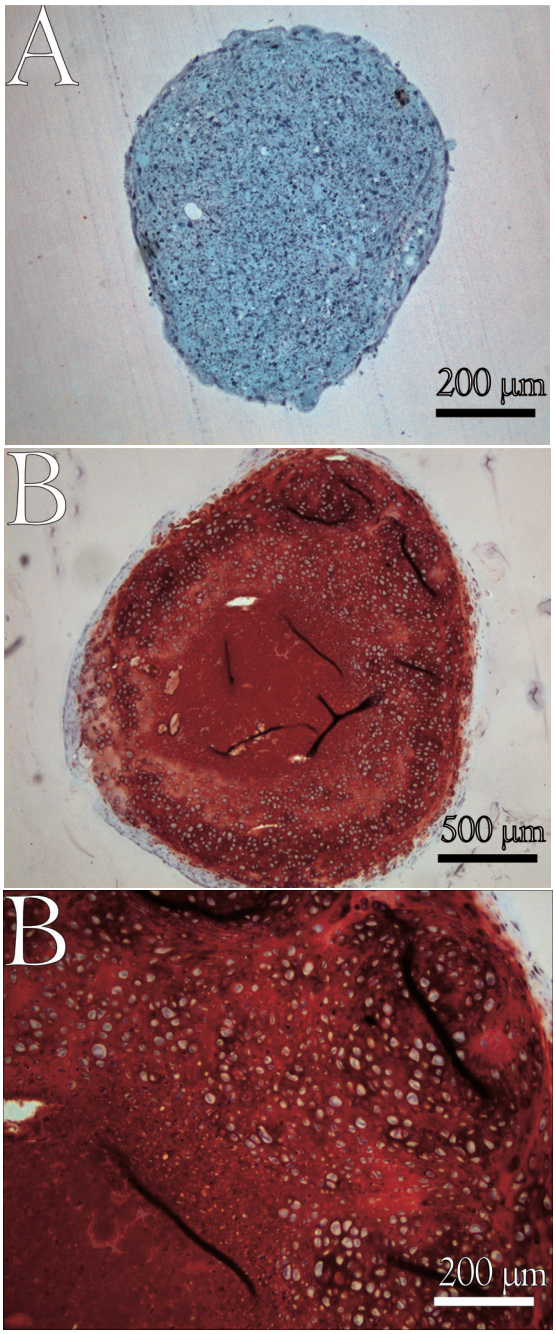

Figure 7: Histological sections of the bone marrow-derived mesenchymal stem cells pellets cultures during 21 days, in the presence of emulsion-coated porous scaffolds. The cross sections were stained with safranin O/fast green.A: pellets cultured in the presence of an unloaded emulsion-coated scaffold, B: pellets cultured in the presence of a releasing TGF-β_1 loaded scaffold.

Figure 2. Gross evaluation and histological cross sections of GMSC pellets cultured in the presence of porous scaffolds of different TGF-β_1 release profiles and different coating copolymers: A: 1000PEGS70PBS30 coating, B: 2000PEGS80PBS30 coating. The cross sections were stained with safranin O/fast green. The negative control pellets were not exposed to TGF-β_1. The positive control consists of unloaded scaffold where constant amounts of TGF-β_1 are added to the culture medium regularly (10 ng/3 days) while in the bolus groups 70 ng of growth factor was added at the beginning of the culture. (n=2)

Chapter 7

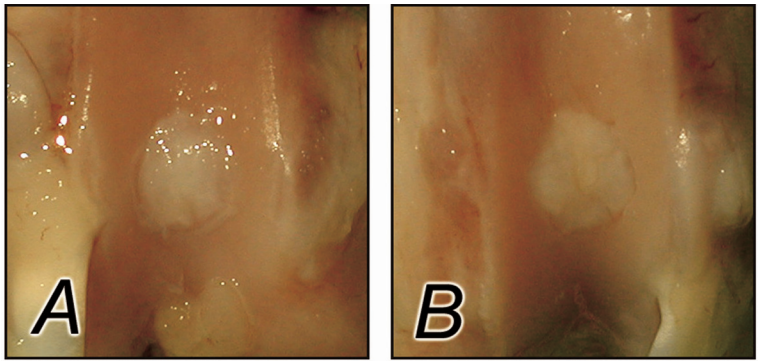

Figure 3: Macroscopic view of femoral condyles receiving a control scaffold (left; A) or a TGF-β_1 scaffold (right; B). Defects in both groups are filled with a new white tissue that is distinguishable from the neighboring normal articular cartilage.

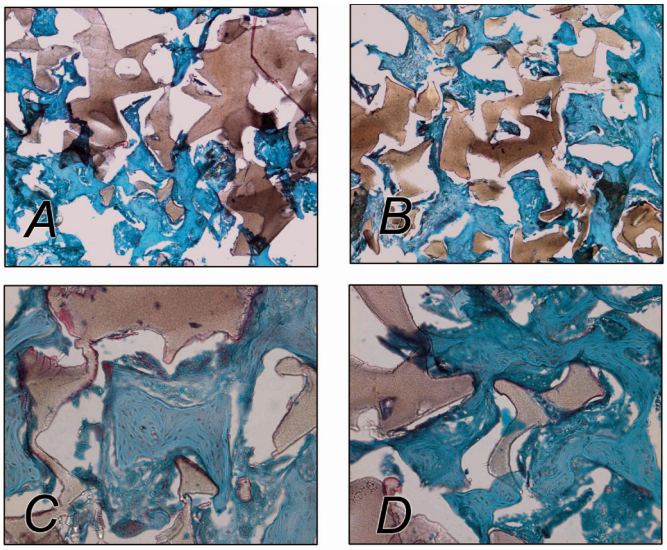

Figure 5. Effect of TGF-b1 scaffolds on new bone formation in the subchondral space 3 weeks after transplantation in vivo. Histological appearance of the subchondral bone below the osteochondral defects that has been filled with a single control scaffold (left; A, C) or a TGF-b1 scaffold (right; B, D) stained with safranin O – fast green (A - D). New subchondral bone has formed within the pores of the scaffold, its trabeculae surrounding the biomaterial (C, D). Photomicrographs were obtained using standardized photographic parameters, including light intensity. Original magnifications ×40 (A, B), x 100 (C, D).

Chapter 7

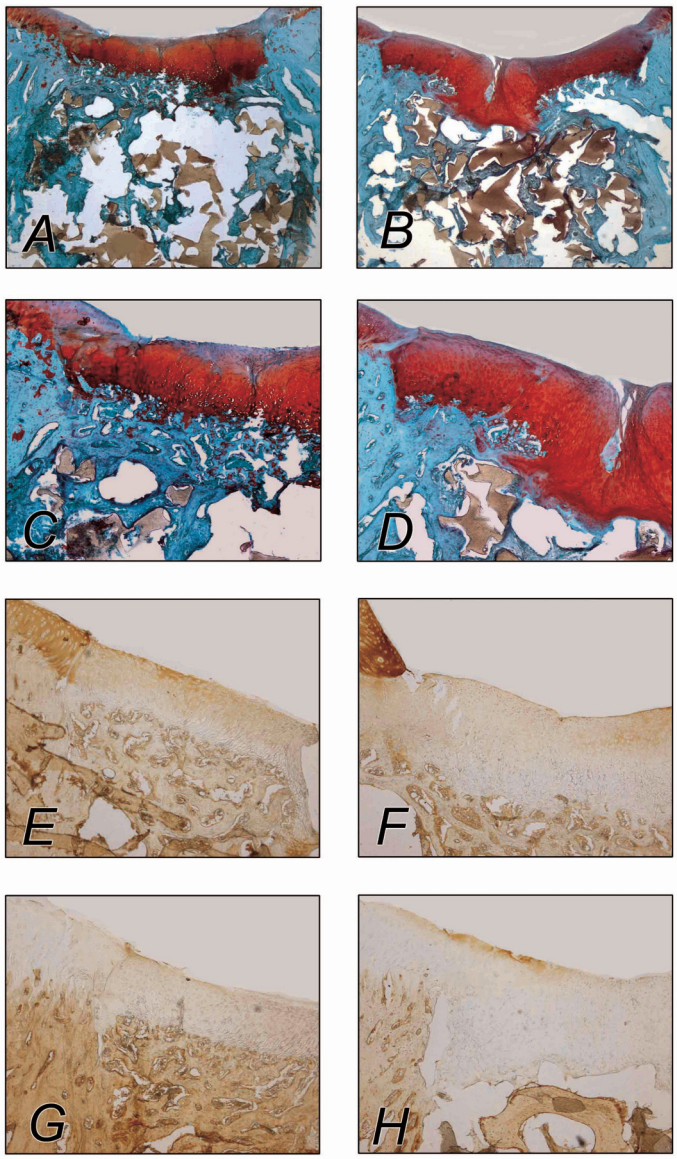

Figure 4. Effect of TGF-b1 scaffolds on chondrogenesis in articular cartilage defects 3 weeks after transplantation in vivo. Histological appearance of osteochondral defects following implantation of a single control scaffold (left; A, C, E, G) or a TGF-b1 scaffold (right; B, D, F, H) stained with safranin O – fast green (A - D), a monoclonal mouse anti-human type-I collagen IgG (E, F) or a monoclonal mouse anti-human type-II collagen IgG (G, H). Images C and D are magnified views of the left side of images A and B. Normal articular cartilage can be identified on the far left side of Images (A – H) including the area of integration between the repair tissue (right side of each picture) with the adjacent normal articular cartilage (left side of each picture). The scaffolds remain in a subchondral location and can be identified by their brown color (A, B). Photomicrographs were obtained using standardized photographic parameters, including light intensity. Original magnifications ×10 (A, B), x 40 (C - H).

Chapter 8

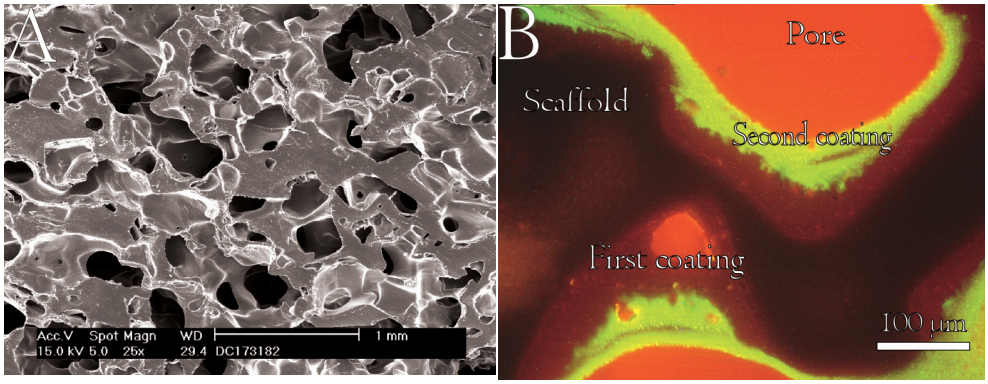

Figure 5: Cross sections of porous scaffolds obtained after application of two emulsion coatings, examined by scanning electron microscopy (A) and optical fluorescent microscopy (B). The first emulsion coating applied contained FITC-BSA, the second one and the GMA embedding solution rhodamine B. The scaffold appears as black.