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Tailored release of TGF-β_1 from porous scaffolds 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-β_1 (TGF-β_1) 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-β_1 which were subsequently applied onto prefabricated scaffolds. This approach resulted in defined porous structures (66%) with interconnected porosity, suitable to allow tissue ingrowth. The scaffolds were effectively associated with TGF-β_1 and allowed to tailor precisely the release of the growth factor from 12 days to more than 50 days by varying the copolymer composition of the coating. An incomplete release was measured by ELISA, possibly linked to the rapid concentration decrease of the protein in solution. The released growth factor retained its biological activity as was assessed by a cell proliferation assay and by the ability of the released protein to induce chondrogenic differentiation of bone marrow-derived mesenchymal stem cells. However, exact bioactivity quantification was rendered difficult by the protein concentration decrease during storage. Therefore, this study confirms the interest of poly(ether–ester) multiblock copolymers for controlled release of growth factors, and indicates that emulsion-coated scaffolds are promising candidates for cartilage tissue engineering applications requiring precise TGF-β_1 release rates.

Keywords: Controlled release; Porous scaffolds; Poly(ethylene glycol)-terephthalate/poly(butylene terephthalate); Transforming growth factor beta; Cartilage tissue engineering

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 (Vacanti et al., 1988). In addition to the classical requirement such as high porosity and inter-pore connection, specific mechanical properties and degradation rates (Hutmacher, 2000; Zeltinger et al., 2001), 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 1 and 2, basic fibroblast growth factor, transforming growth factors, and bone morphogenetic proteins) (Mankin et al., 1991; Hiraki et al., 1991).

Promising data were previously reported showing the relevance of local release of various growth factors from scaffolds for bone, cartilage, and angiogenesis (Isobe et al., 1996; Lee et al., 2000; Kim et al., 2003; Murphy et al., 2000). 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 (Uludag et al., 2000; Ikada and Tabata, 1998; Yamamoto

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et al., 2000; Park et al., 1998; Sakano et al., 2002; Aspenberg et al., 1996). 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 (Sherris et al., 1998; Ueda et al., 2003; Steinbrech et al., 2000), angiogenesis (Phillips et al., 1993; Dinbergs et al., 1996), neuroprotection (Blottner et al., 1996) and wound repair (Chin et al., 2004; O'Kane and Ferguson, 1997; Beck et al., 1990). It controls the production of extracellular matrices by stimulating the synthesis of collagens, fibronectin and proteoglycans (Ignotz et al., 1987; Streuli et al., 1993). It also appeared to have positive effects on cartilage differentiation and repair (Hunziker et al., 2001; Miura et al., 2002; Caterson et al., 2001; Mierisch et al., 2002). Nevertheless, this multi-potency 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 (Nimni, 1997), while a too high dosage in cartilaginous sites results in osteophytes formation (Mierisch et al., 2002). 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) (Sohier et al., 2003b). 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 (Bezemer et al., 2000a; van Dijkhuizen-Radersma et al., 2003). It was demonstrated that the protein release was controlled by a combination of diffusion and degradation of the polymeric matrix (Bezemer et al., 2000b; van Dijkhuizen-Radersma et al., 2005).

The resulting scaffolds were evaluated with regard to their structure, TGF-β_1 release ability, stability of the released protein, and their potential interest for cartilage tissue engineering.

2. Materials and methods

2.1. 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 *a*PEGT*b*PBT*c* or *a*PEG(T/S)*b*PB(T/S)*c*(*d*T/*e*S) in which *a* is the PEG molecular weight, *b* the weight percentage (wt%) of PEGT or the combined wt% of PEGT and PEGS, and *c* (=100 − *b*) the wt% of PBT or the combined wt% 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 beta-1 (rhTGF-β_1 later referred as TGF-β_1) 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). Beta-fibroblast growth factor (bFGF) was obtained from VWR international (Roden, The Netherlands). Chloroform, obtained from Fluka chemica (Buchs, Switzerland), was of analytical grade.

2.2. Preparation of TGF-β_1-loaded polymeric scaffolds

2.2.1. 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-β_1 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/g of copolymer (water/polymer ratio = 1 ml/g). The copolymer solution was obtained by dissolving 0.5 g 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 wt%, the PEG MW of 600, 1000 or 2000 g/mol, and the T/S molar ratio varied between 0 and 100%.

2.2.2. Emulsion-coating method

The emulsion-coated scaffolds were obtained as described elsewhere (Sohier et al., 2003b). Briefly, compression molded/salt leached scaffolds were obtained by applying pressure (10,000 PSI during 10 min) 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 h, and then placed in a vacuum

oven (50 °C) for a minimum of 12 h. The PEGT/PBT copolymer used to prepare the scaffold had a PEGT content of 55 wt% 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) (Sohier et al., 2003b). This vacuum was applied for at least 5 min, 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 h.

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.

2.3. 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.

2.4. 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:

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

Three scaffolds pieces were used to determine the porosity of a specific emulsion-coated scaffold.

The scaffold pore interconnection before and after coating treatment was quantified using a method that applies Darcy's law, as described elsewhere (Sohier et al., 2003b; Li et al., 2003; Hui et al., 1996; Grimm and Williams, 1997). 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.

2.5. 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 3 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 (Sohier et al., 2003b). 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.

2.6. 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 min 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.

2.7. TGF-β_1 bioactivity 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) (McKay and Leigh, 1993). CCL64 cells were cultured in DMEM supplemented with 10 vol.% 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 1×10^4 cells/well in 48-well plates and subsequently let to attach for 3 h 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 h, 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 Fig. 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 h and the relative amount of cells was evaluated using an alamar blue assay (200 μl added per well and incubated for 4 h prior reading). 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 8×10^5 cells/well over 40 h. Each assay was performed with

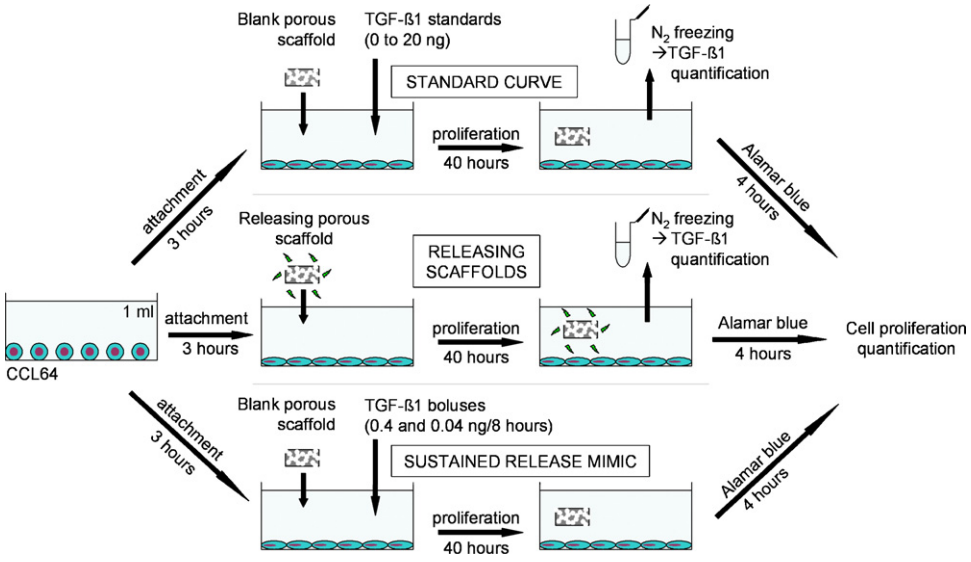

Fig. 1. Schematic representation of the different conditions used to evaluate the bioactivity of the released TGF-β_1 in a CCL64 growth inhibition assay.

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.

2.8. 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 5×10^5 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 2 mM L-glutamine. The medium was refreshed first after 3 days and then twice a week until confluency (8–10 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 confluent in α-MEM supplemented with 15% FBS, 100 UI/ml penicillin, 100 μg/ml streptomycin, 0.1 mM L-ascorbic acid-2-phosphate, and 2 mM L-glutamine (expansion medium).

Passage 3 cells were used to prepare the pellets. After trypsinization, 5×10^5 cells were spun down at 500 g for 2 min 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 h 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. A 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).

3. Results and discussion

3.1. 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 (Sohier et al., 2003b) 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 Fig. 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 (Sohier et al., 2003b), 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 (Li et al., 2003). As can be visually assessed by

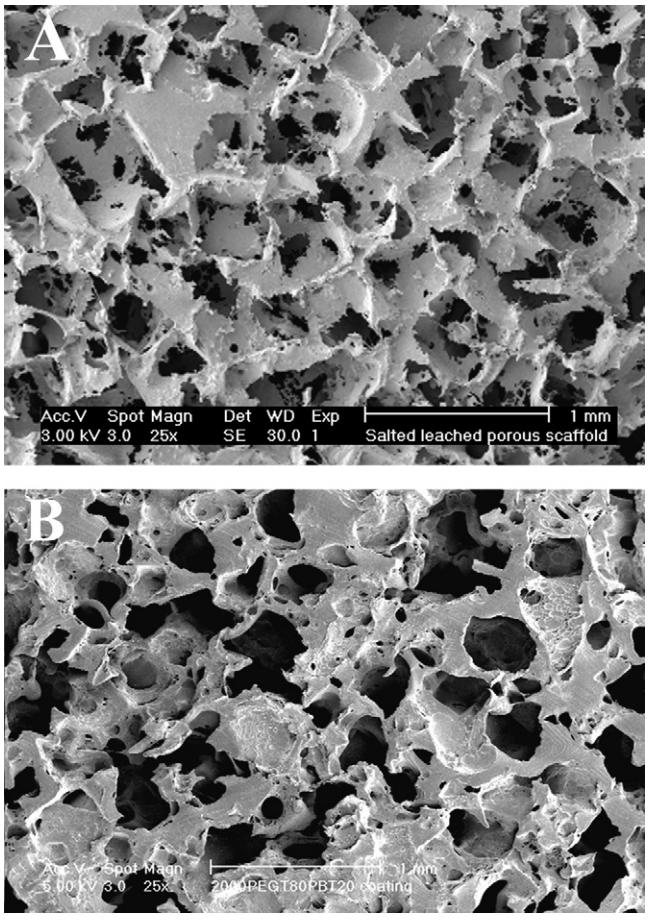

Fig. 2. Cross sections of a porous scaffold obtained before (A) and after application of a TGF-β_1 containing emulsion (B), examined by scanning electron microscopy.

comparing the scaffolds before and after coating (Fig. 2A and B, respectively), 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 (Cucchiarini et al., 2006). After 3 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.

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 ($49 \pm 1\%$).

3.2. 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 Fig. 3,

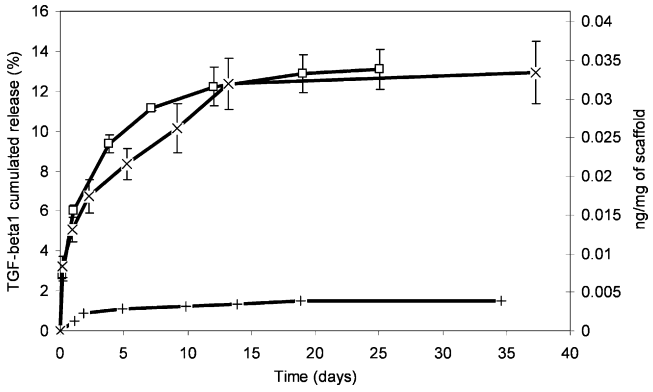

Fig. 3. Cumulated release of TGF-β_1 from porous polymeric scaffolds coated with water-in-oil emulsions of different copolymeric compositions: 2000PEGT80PBT20 (□), 1000PEGT80PBT20 (×) and 600PEGT80PBT20 (+) ($n = 3 \pm$ S.D.).

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 10–20 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 (Bezemer et al., 2000a,b; van Dijkhuizen-Radersma et al., 2002, 2004; Sohier et al., 2003a). 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 (Bezemer et al., 1999). 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.

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 (Bezemer et al., 1999). 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 (van Dijkhuizen-Radersma et al., 2005). 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 (van Dijkhuizen-Radersma et al., 2005). As a consequence, the protein diffusion coefficients are increased by the degree of substitution (van Dijkhuizen-Radersma et al., 2003). As presented in Fig. 4, the use of 1000PEGT70PBT30 or PEG(T/S)PB(T/S) copolymers resulted indeed in intermediate release profiles. A 1000PEGT70PBT30 coated copolymer showed a zero order release still on going

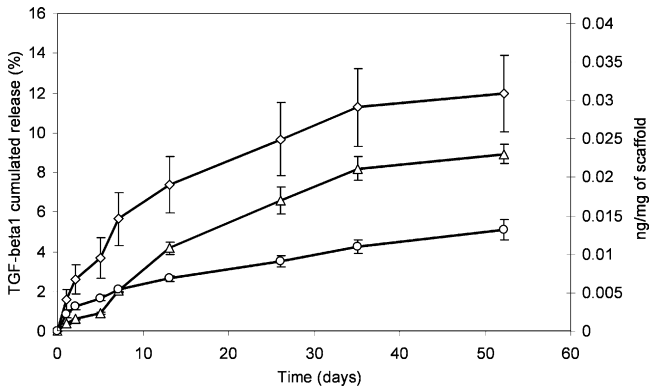

Fig. 4. Cumulated release of TGF-β_1 from porous polymeric scaffolds coated with water-in-oil emulsions of different copolymeric compositions: 1000PEG(0T/100S)70PB(0T/100S) (◊), 1000PEG(50T/50S)70PB(50T/50S) (△) and 1000PEGT70PBT30 (◯) ($n = 3 \pm$ S.D.).

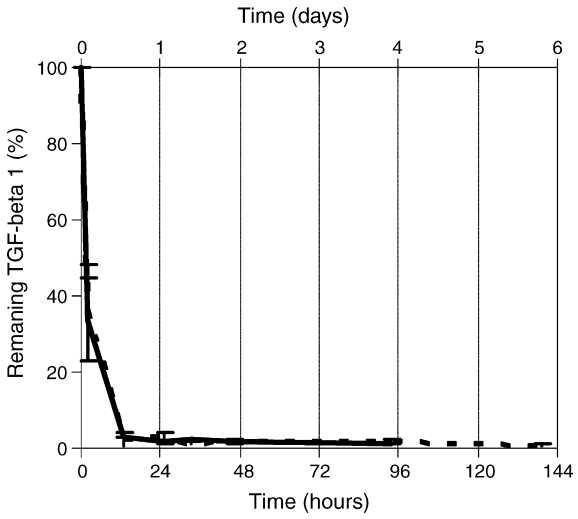

Fig. 5. Concentration decrease of TGF-β_1 in release medium. Two different concentrations were supplemented as a bolus: 5 ng/ml (full line) and 10 ng/ml (dotted line) ($n = 3 \pm$ S.D.).

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-β_1 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 (Sohier et al., 2003b). This discrepancy could be linked to the intrinsic stability of TGF-β_1 in solution. The half-life of TGF-β_1 in vivo is less than 30 min (Dinbergs et al., 1996; Nimni, 1997) when in its active form. In addition, due to its high hydrophobicity, TGF-β_1 tends to adsorb quickly to plastic surface, reducing so the concentration of the protein in solution (McKay and Leigh, 1993). To assess the effective degradation of the protein in our release experiment condition, the concentration decrease of two TGF-β_1 standards was measured over time. As can be seen in Fig. 5, the amount of TGF-β_1 left in the release medium was decreasing rapidly to reach a stable value close to 2.5% after 12 h. Within 20 min, 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 rapid protein depletion renders the release measurement less accurate and hampers the determination of the release completeness. However, the release profile can still be considered to be valid as the protein concentration decrease reached a plateau in 12 h and as each sampling time was separated by more than 24 h.

To determine if the growth factor depletion is linked to adsorption phenomena (as the release medium does not contain proteins), the release of TGF-β_1 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 of BSA (data not shown) suggesting that the protein disappearance 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.

3.3. 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 Fig. 1. The activity of TGF-β_1 directly released from emulsion-coated scaffolds was assessed during the length of the growth inhibition assay (40 h). 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.

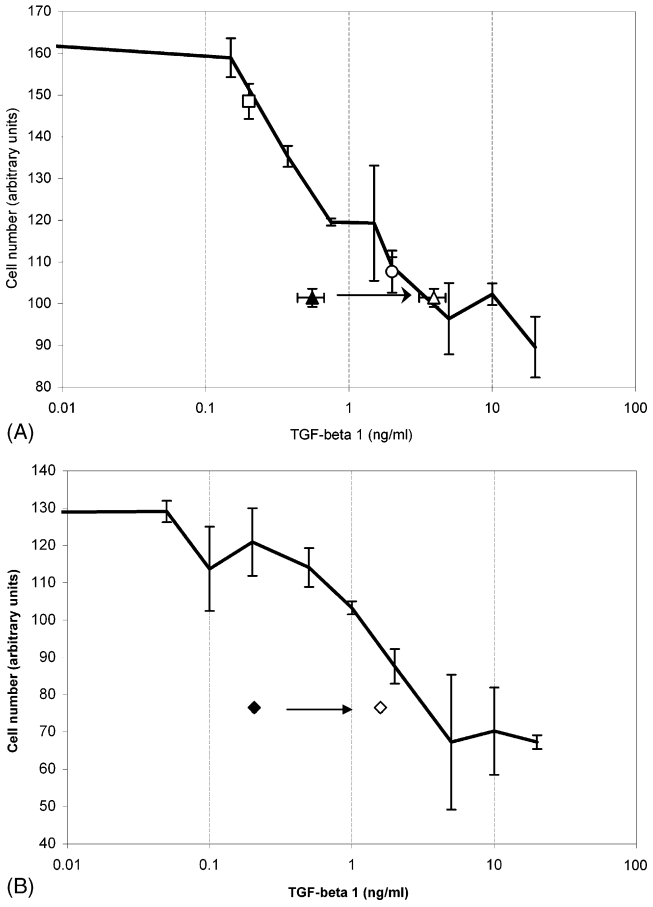

Fig. 6. Activity of TGF-β_1 releasing scaffolds and samples mimicking a sustained release system, measured in a cell growth inhibition assay, based on the growth of CCL64 cells. Increasing values of TGF-β_1 result in a lower number of cells after 40 h of culture (standard curve). The releasing scaffold 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 ng/ml (□) and 2 ng/ml (◯) and were supplemented in the culture medium in five regular time intervals (8 h).

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 (Fig. 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 $1500 \pm 63\%$ 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 CCL4 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 h (five times). As can be seen in Fig. 6A, 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 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 h. 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 $85 \pm 25\%$ for scaffolds coated with a 2000PEGT80PBT20 copolymer and $200 \pm 8\%$ 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 that prohibited accurate concentration measurements and rendered a quantitative interpretation difficult, they tend to indicate that the bioactivity of the TGF-β_1 was at least partly preserved during preparation of the scaffolds.

3.4. 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 (Williams et al., 2003; Johnstone et al., 1998). 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 which showed a slow delivery over 40 days were selected. Considering the fast degradation of TGF-β_1 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-β_1 on cell pellets differentiation after 15 and 21 days was assessed histologically. Fig. 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-β_1, presented no sign of GAG formation, the group containing releasing scaffolds showed an intense safranin O positive stain-

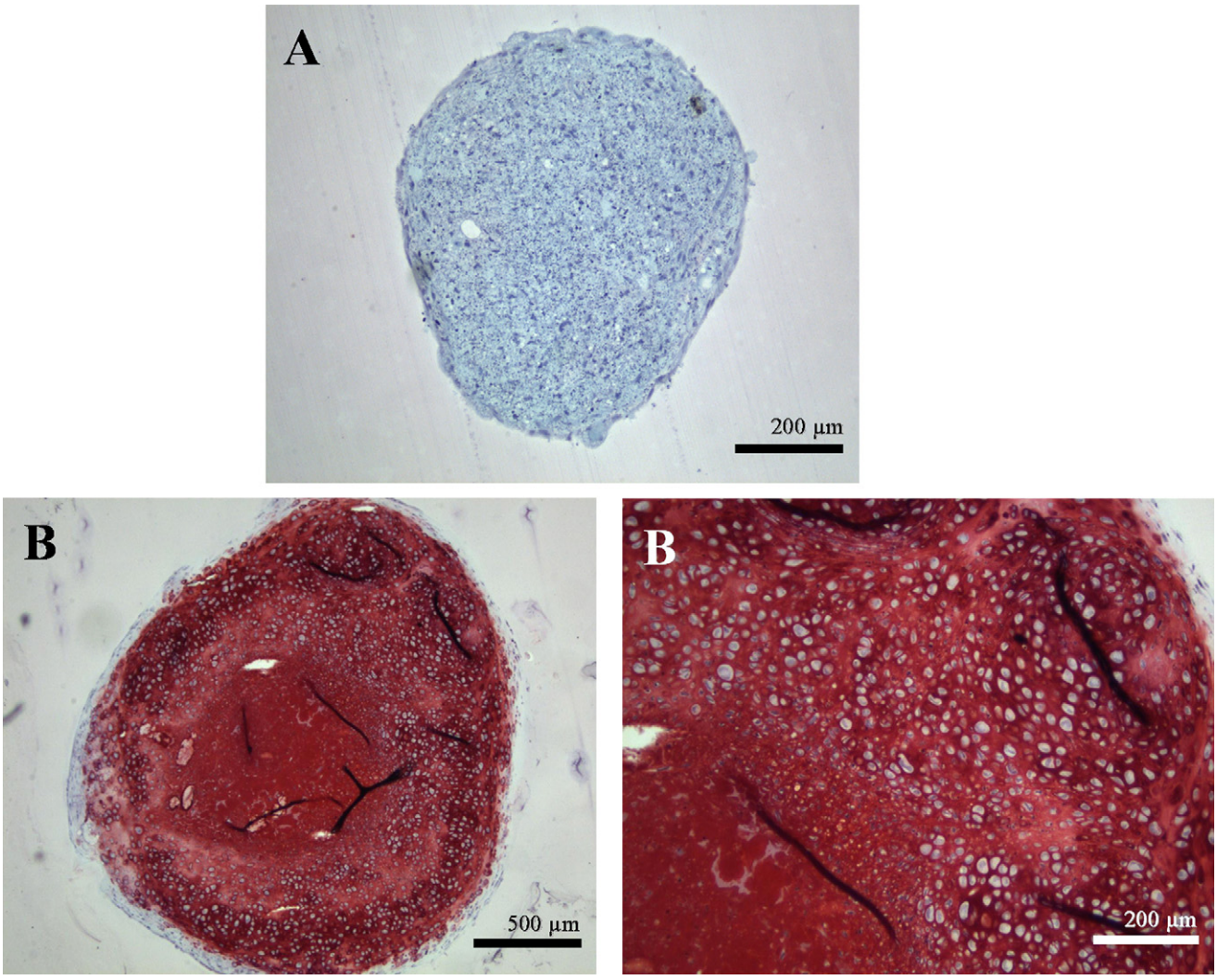

Fig. 7. Histological sections of the bone marrow-derived mesenchymal stem cells pellets cultures during 21 days, in the presence of emulsion-coated porous scaffolds. The cross sections were stained with safranin O/fast green. (A) Pellets cultured in the presence of an unloaded emulsion-coated scaffold,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

ing. 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-β_1 releasing scaffolds to induce cartilage in this cell pellet model confirms qualitatively the activity of the released protein.

4. Conclusions

To associate TGF-β_1 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 and migration of progenitor cells in osteochondral defects. The growth factor was effectively released from the scaffolds. By varying the copolymer composition used as coating, the release rate of TGF-β_1 could be precisely tailored from 12 days to more than 50 days. The apparent incompleteness of the release was possibly linked to the instability of the protein in solution combined with the detection method employed (ELISA) that did not allow a denaturing independent measurement. The released protein appeared not to be fully denaturated by the emulsion-coating process and was shown to retain its bioactivity in a cell inhibition assay, although no exact quantification could be performed due to the inaccurate concentration measurements. This however indicates 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-β_1 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-β_1 release rate on cartilage formation and determine the most optimal release profile in vivo.

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