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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 through 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 vol.%, 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.

Keywords: Scaffold; Controlled drug delivery; Copolymer; Modelling; Tissue engineering

1. 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 rhBMP2 [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

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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 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 KDa) 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.

2. Materials and methods

2.1. 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 *a*PEG*T*/*b*PBT*c* 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.

2.2. Preparation of protein-loaded polymeric matrices.

2.2.1. 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 and 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 wt.%, with a PEG molecular weight of 300, 1000, 2000 and 4000 g/mol.

2.2.2. 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 h. The resulting films had a thickness ranging from 50 to 110 μm after swelling. A complete description of the swelling determination is presented below.

2.2.3. 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 min, a protein-containing emulsion was cast above this first dense layer. The resulting two-layered films were slowly dried for 10 min. 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 min and freeze-dried for 24 h. 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.

2.2.4. 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 min. 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 h.

2.3. 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 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.

2.4. 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.

2.5. 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:

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

The scaffold pore interconnection before and after coating treatment was quantified using a method that applies Darcy's law, as described elsewhere [16,28–30]. 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.

2.6. 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 wt.% 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 fluorescent microscopy (FITC-Texas red multi-band dual filter, Nikon, Tokyo, Japan). 2 μl of a 1 wt.% 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 fluorescent 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.

2.7. 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 (μBCA). 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 (μBCA) 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 μBCA) could be calculated correctly.

The lysozyme concentration was further confirmed with another detection method based on a *Micrococcus Lysodeikticus* assay [16,27]. 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 min at 15 s 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.

2.8. 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]:

$$\frac{\partial C}{\partial t} = D\left(\frac{\partial^2 C}{\partial x^2}\right). \tag{2}$$

Using the following initial conditions:

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

and boundary conditions:

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

$$C(t, x > l) = 0. \tag{5}$$

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:

$$D(t) = \int_0^t D_{\text{initial}}\left(1 + at + bt^2\right)\mathrm{d}t = D_{\text{initial}}\left(t + \frac{1}{2}at^2 + \frac{1}{3}bt^3\right). \tag{6}$$

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 $a = 1\times10^{-5}\ \mathrm{s}^{-1}$ and $b = 1.3\times10^{-12}\ \mathrm{s}^{-2}$ [27].

For films with a time-dependent diffusion coefficient, Fick's second law can be approximated as:

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

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\exp\left(-\frac{\pi^2 D(t)}{l^2}\right), \quad \text{when} \quad \frac{M_t}{M_\infty} > 0.4, \tag{8}$$

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 Eqs. (9) and (10):

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

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

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 Eq. (11) for films or Eq. (12) for multiple layered constructs:

$$\frac{M_t}{M_\infty} = 4\sqrt{\frac{D_{\text{initial}}t}{\pi l^2}} \quad (11)$$

$$\frac{M_t}{M_\infty} = 2\sqrt{\frac{D_{\text{initial}}t}{\pi l^2}}. \quad (12)$$

3. Results and discussion

3.1. 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 Fig. 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.

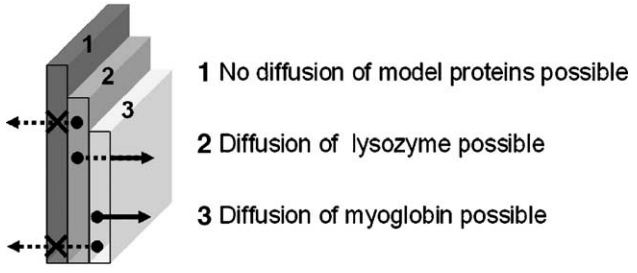

Fig. 1. Schematic representation of the model used to mimic a double coated scaffold. Layer 1 (300PEGT55PBT45) does not contain protein and do not allow diffusion of the model proteins. Layer 2 contains lysozyme while layer 3 contains myoglobin.

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 Fig. 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. 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 KDa, 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.

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

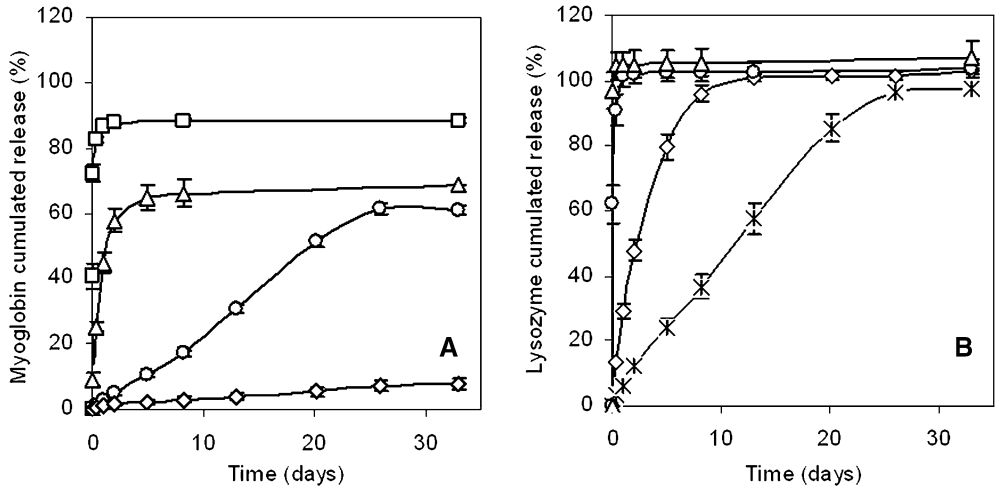

Fig. 2. Cumulated release of Myoglobin (A) and lysozyme (B) from different copolymeric films of different PEGT/PBT compositions: 4000PEGT80PBT20 (□), 2000PEGT80PBT20 (△), 1000PEGT80PBT20 (○), 1000PEGT70PBT30 (◇) and 1000PEGT60PBT40 (✳). ($n=3$; ±s.d.).

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.

3.2. Three-layered constructs and release

The release profiles of myoglobin and lysozyme observed from multiple layered constructs are presented in Fig. 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 on-going 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.

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],

Table 1
Myoglobin and lysozyme initial diffusion coefficients from single PEGT/PBT films ($D_{initial}$, cm^2/s)

	Diffusion coefficient (D, cm^2/s)				
Copolymer composition	1000PEGT60PBT40	1000PEGT70PBT30	1000PEGT80PBT20	2000PEGT80PBT20	4000PEGT80PBT20
Myoglobin	n.a.	$(7\pm1)\times10^{-14}$	$(2.5\pm0.1)\times10^{-12}$	$(1.1\pm0.1)\times10^{-10}$	$(4.6\pm0.1)\times10^{-9}$
Lysozyme	$(1.6\pm0.4)\times10^{-12}$	$(2.4\pm0.3)\times10^{-11}$	$(1.5\pm0.2)\times10^{-9}$	$(4.6\pm0.2)\times10^{-12}$	n.a.

($n=3$ ±s.d.).

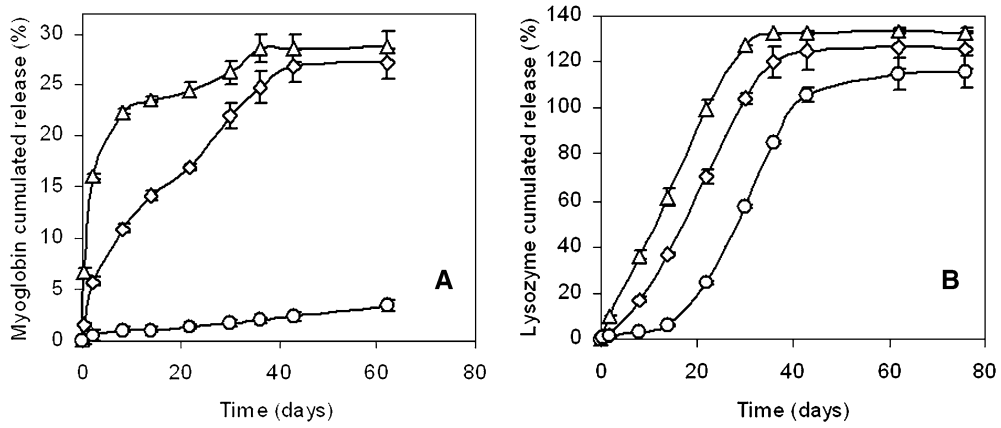

Fig. 3. Cumulated release of Myoglobin (A) and lysozyme (B) from three-layered constructs. The copolymer composition of the second layer (containing lysozyme) was fixed (1000PEGT70PBT30), whereas the third layer (containing myoglobin) was varied: 2000PEGT80PBT20 (△), 1000PEGT80PBT20 (◇) and 1000PEGT70PBT30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

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 (Fig. 4). This clearly indicates that neither layer thickness nor unidirectional release can fully explain the slow release observed.

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

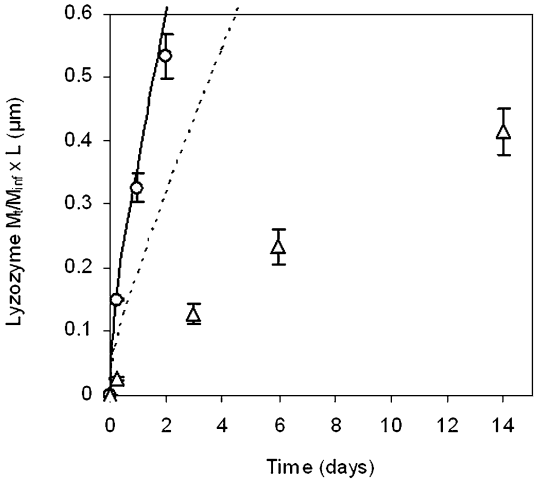

Fig. 4. Modeling of lysozyme release from 1000PEGT70PBT30 single films (○) and second layer (△). The plain line represents a bidirectional release (Eq. (1)), the dashed line a unidirectional release (Eq. (4)) and symbols corresponds to the experimental release. The effect of layer thickness is corrected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

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 ($D_{initial}$) from the second and third layers were quantified using two-layered and three-layered constructs with lysozyme-loaded top layers (Table 2). 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

Table 2
Equilibrium swelling ratios (Q) and lysozyme initial diffusion coefficients ($D_{initial}$, cm^2/s) of single films and superposed layers

	Equilibrium swelling ratio (Q)			
Copolymer composition	300PEGT55PBT45	1000PEGT70PBT30	1000PEGT80PBT20	2000PEGT80PBT20
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		
Third layer		2.14±0.06	2.11±0.03	2.45±0.05
	Diffusion coefficient (D, cm^2/s)			
Copolymer composition	n.a.	1000PEGT70PBT30	1000PEGT80PBT20	2000PEGT80PBT20
Single films		$(2.4\pm0.3)\times10^{-11}$	$(1.5\pm0.2)\times10^{-9}$	$(4.6\pm0.2)\times10^{-11}$
Second layer		$(6.5\pm0.8)\times10^{-12}$	n.a.	
Third layer		$(2.4\pm0.2)\times10^{-11}$	$(1.5\pm0.1)\times10^{-10}$	$(8.6\pm1.1)\times10^{-10}$

($n=3$ ±s.d.).

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.

3.3. 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 fluorescent microscopy, are presented in Fig. 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 fluorescent 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 inhomogeneous as can be seen in Fig. 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.

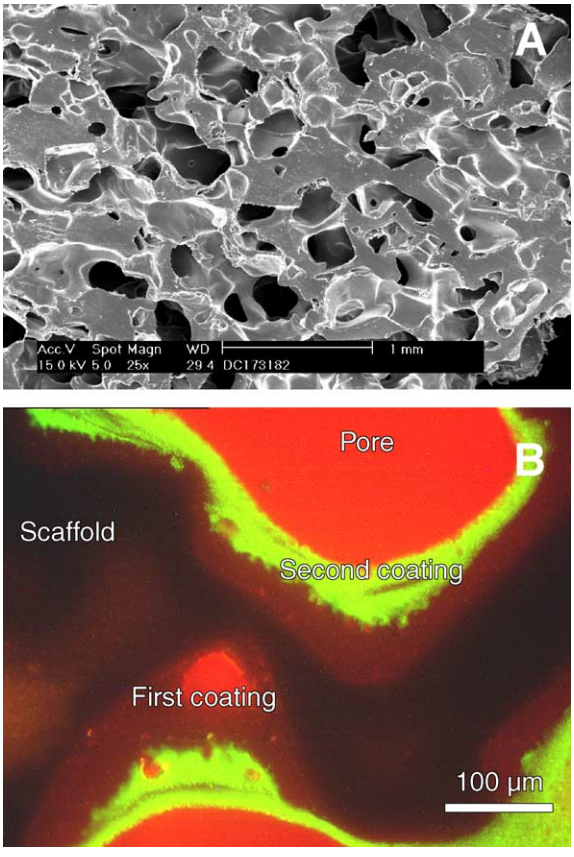

Fig. 5. Cross-sections of porous scaffolds obtained after application of two emulsion coatings, examined by scanning electron microscopy (A) and optical fluorescent microscopy (B). The first emulsion coating applied contained FITC-BSA, the second one and the GMA embedding solution rhodamine B. The scaffold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

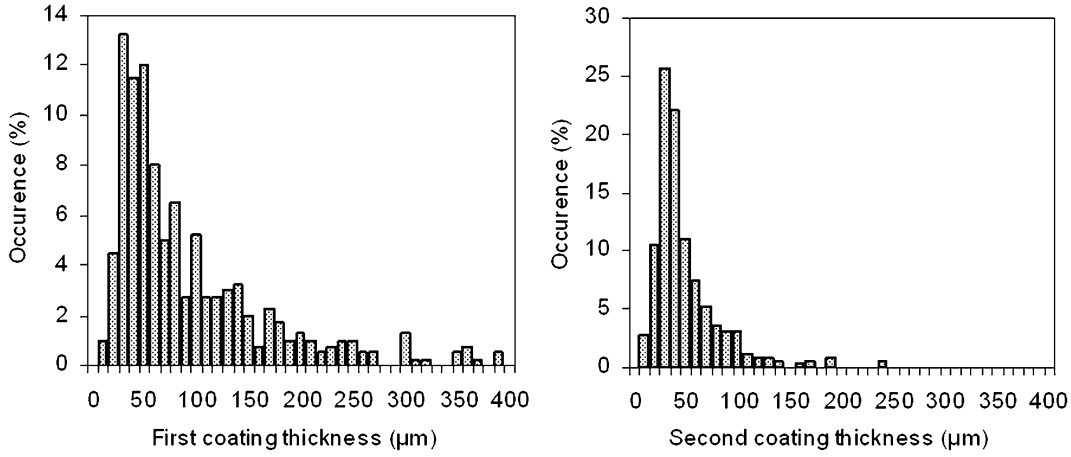

Fig. 6. Thickness distribution of the first coating (A, containing lysozyme) and second coating (B, containing myoglobin) over a porous polymeric scaffold.

The lysozyme and myoglobin release obtained from the double coated scaffolds are presented in the Fig. 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 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:

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

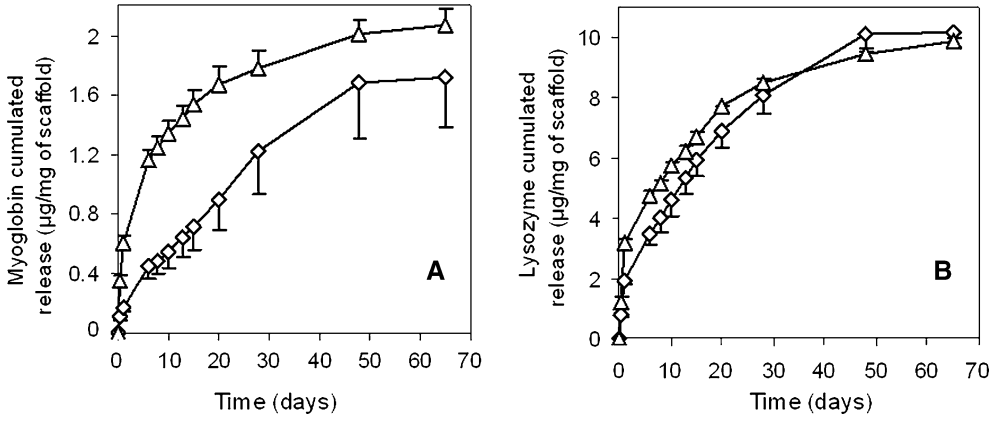

Fig. 7. Cumulated release of Myoglobin (A) and lysozyme (B) from double emulsion-coated scaffolds. The copolymer composition of the first coating (containing lysozyme) was fixed (1000PEGT70PBT30), whereas the second coating (containing myoglobin) was varied: 2000PEGT80PBT20 (△) and 1000PEGT80PBT20 (◇).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

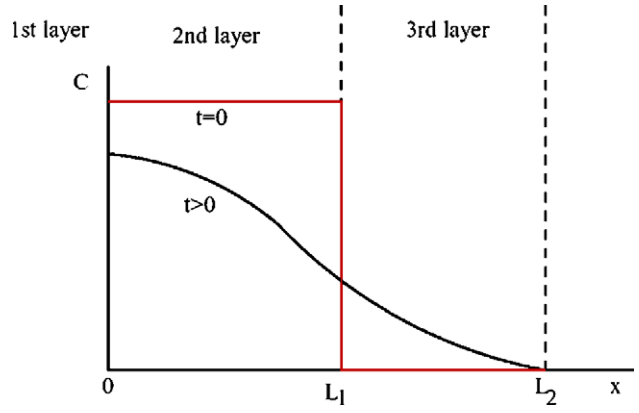

Fig. 8. Schematic representation of the initial and boundary conditions of the three layered construct, for lysozyme. The protein cannot diffuse through the first layer, therefore $\left(\frac{\partial C}{\partial x}\right)_{x=0} = 0$.

using the following initial conditions (see Fig. 8):

$$C(t=0,x) = \begin{cases} 1 & 0 \le x \le L_1 \\ 0 & x > L_1 \end{cases} \quad (14)$$

and boundary conditions:

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

$$C(t, x = L_2) = 0. \quad (16)$$

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:

$$F(t) = 1 - \frac{\int_0^{L_2} C(t,x)dx}{\int_0^{L_2} C(t=0,x)dx}. \quad (17)$$

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 Eqs. (9) and (10), with the averaged measured thicknesses and 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 (Fig. 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).

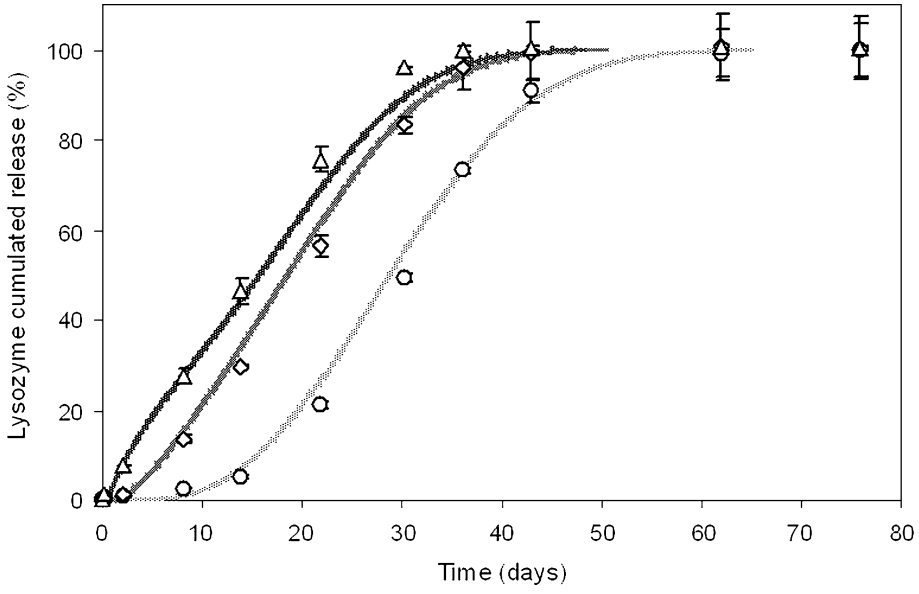

Fig. 9. Modeling of lysozyme release from three-layered constructs. The copolymer composition of the second layer (containing lysozyme) was fixed (1000PEGT70PBT30), whereas the third layer was varied. The symbols correspond to the experimental release curves while the lines represent the simulation results (Eq. (12)) for a third layer composition of 2000PEGT80PBT20 (△, ▬), 1000PEGT80PBT20 (◇, ▒) and 1000PEGT70PBT30 (○, ▒). (*n* = 3; ± s.d.).

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 (39×39), in 10 μm steps. The resulting release profiles obtained for each combination were then averaged using the following formula:

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

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 $(10 \times i)$ and $(10 \times j)$ 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 Fig. 6. As can be seen in Fig. 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.

4. 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

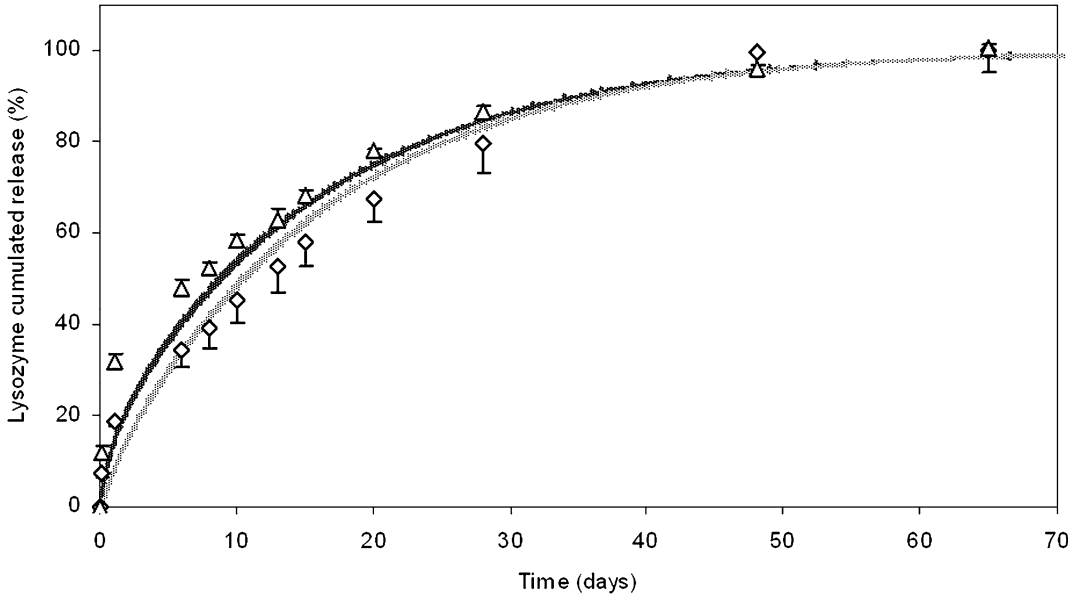

Fig. 10. Modeling of lysozyme release from porous scaffolds. The copolymer composition of the first coating (containing lysozyme) was fixed (1000PEGT70PBT30), whereas the second coating was varied. The symbols correspond to the experimental release curves. The lines represent the simulation results (Eq.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

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.

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