In Vitro/in Vivo Correlation for ^{14}C-Methylated Lysozyme Release from Poly(Ether-Ester) Microspheres

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Purpose. The purpose of this study was to obtain an *in vitro/in vivo* correlation for the sustained release of a protein from poly(ethylene glycol) terephthalate (PEGT) / poly(butylene terephthalate) (PBT) microspheres.

Methods. Radiolabeled lysozyme was encapsulated in PEGT/PBT microspheres via a water-in-oil-in-water emulsion. Three microsphere formulations varying in copolymer composition were administered subcutaneously to rats. The blood plasma was analyzed for radioactivity content representing released lysozyme at various time points post-dose. The *in vitro* release was studied in phosphate-buffered saline.

Results. The encapsulation efficiency, calculated from the radioactivity in the outer water phase of the emulsion, varied from 60–87%. Depending on the PEG segment length and wt% PEGT, the lysozyme was released completely *in vitro* within 14 to 28 days without initial burst. ^{14}C-methylated lysozyme could be detected in the plasma over the same time courses. The *in vitro/in vivo* correlation coefficients obtained from point-to-point analysis were greater than 0.96 for all microsphere formulations. In addition, less then 10% of administered radioactivity remained at dose site at 28 days for the microsphere formulations, indicating no notable retention of the protein at the injection site.

Conclusion. The *in vitro* release in phosphate-buffered saline and the *in vivo* release in rats showed an excellent congruence independent of the release rate of ^{14}C-methylated lysozyme from PEGT/PBT microspheres.

KEY WORDS: controlled release; *in vitro/in vivo* correlation; poly(ether ester); protein; radiolabeled.

INTRODUCTION

With the increasing number of therapeutic proteins on the pharmaceutical market, extensive investigations have been conducted on biodegradable polymers for controlled release systems for these proteins (1). Most of this work has focused on matrices composed of poly(lactide-co-glycolide) (PLG) because of their good biocompatibility, low toxicity, and biodegradation characteristics (2). However, at present there is considerable concern about the effects of PLG on protein stability (3–6). With respect to the release kinetics, frequently an initial burst has been reported for protein release from PLG matrices followed by a plateau and subsequently result in incomplete release (6–8). Recently, a series of biodegradable poly(ether ester) multiblock copolymers composed of repeating blocks of hydrophilic poly(ethylene glycol) terephthalate (PEGT) and hydrophobic poly(butylene terephthalate) (PBT) was introduced as a matrix for controlled release systems (9,10). Many *in vivo* and *in vitro* studies have proven the biocompatibility of these PEGT/PBT multiblock copolymers (11,12) and in 2000, a degradable cement restrictor composed of PEGT/PBT obtained market clearance from the Food and Drug Administration.

In previous publications, PEGT/PBT copolymers were shown to be a successful matrix for the controlled release of proteins (9,10). An important advantage of this system is that through modulation of the copolymer composition, the required release profile can be obtained. *In vitro* experiments showed a complete release of lysozyme varying from minutes to months depending on the amount and length of the PEG segments in the matrix (9,10). Through the release mechanism, which is based on diffusion of the protein caused by swelling and degradation of the matrix, zero-order release profiles could be obtained without an initial burst. In addition, the *in vitro*-released protein retained its integrity (13) and was fully active (9).

However, for many release systems, the *in vivo* release behavior is very often not predicted by the *in vitro* release (7,14–16). In case of subcutaneous injection of protein-loaded polymer microspheres, the protein input into the systemic circulation depends not only on the release rate, but also on the diffusion rate of the protein through the subcutaneous tissue (6,17) or the lymphatic transport system (18,19). A free protein formulation should always be included as a reference in the *in vivo* evaluations of sustained-release formulations so that one is able to obtain the elimination rate of the protein in the body. However, it cannot be excluded that the rate of clearance for the slow release system differs from the bolus injection (6,20). After taking into account the reference, in most cases the *in vivo* release still differs from the *in vitro* release concerning profile shape and/or time-course (7,14–16). This can be caused by instability of the protein in the *in vitro* release medium (4,16), difference in degradation of the polymer matrix *in vitro* and *in vivo* (21,22), and/or the *in vitro* release test set-up (e.g., sink conditions, centrifugation, type of tubes; Refs. 8,23). Various release media without resemblance to the *in vivo* situation have been used to overcome the discrepancy which resulted in a proper *in vitro/in vivo* correlation (4,7).

In this *in vitro/in vivo* correlation study, radiolabeled lysozyme was used as a model protein as our polymer system has been evaluated extensively for lysozyme (9,10). We selected ^{14}C-methylated lysozyme as the relatively long half-life of the radiolabel ($T_{1/2}$ is 5730 years) means that the activity is essentially constant over the test period. We did not consider the commonly used ^{125}I label ($T_{1/2}$: 60 days; Refs. 4,24) suitable for sustained release experiments, as after 4 weeks only 72% of the initial radioactivity remains and more importantly the radiolabel readily dissociates from the protein *in vivo*, making data interpretation difficult. ^{14}C-methylated lysozyme-loaded PEGT/PBT microspheres were prepared by a water-in-oil-in-water emulsion. Loaded microspheres of various copolymer compositions were administered subcutane-

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ously to rats. The blood plasma was analyzed for radioactivity concentrations at several time points. The *in vitro* release was studied in phosphate-buffered saline (PBS). Lysozyme release in PBS was in good agreement with the *in vivo* release from crosslinked gelatin hydrogels (25), although an incomplete lysozyme release in PBS was observed from PLG microspheres (4).

MATERIALS AND METHODS

Materials

A series of poly(ethylene glycol) terephthalate/poly(butylene terephthalate) (PEGT/PBT) copolymers were obtained from IsoTis NV (Bilthoven, The Netherlands). The poly(ether-ester) copolymers varied in PEGT/PBT weight ratio (60/40–77/23) and in PEG segment length (600–1000 g/mole) and are indicated as **a**PEGT**b**PBT**c**, in which **a** is the PEG molecular weight, **b** the wt% PEG-terephthalate and **c** (=100 – b) the wt% PBT (composition determined by nuclear magnetic resonance). The average weight molecular weight (M_w) determined by GPC (relative to PMMA standards) was approximately 85 kg/mole. PBS (pH 7.4) was purchased from Life Technologies Ltd (Paisley, Scotland). Fluka Chemie GmbH (Buchs, Switzerland) and Rathburn Chemicals Ltd (Walkerburn, Scotland) supplied the chloroform (analytical grade). Hen egg white lysozyme (3× crystallized, dialyzed, and lyophilized) was obtained from Sigma (St. Louis, MO, USA). ^{14}C-methylated lysozyme in 0.01 M sodium phosphate solution (41 μCi/mg, 5 μCi/mL) was supplied by Nycomed Amersham plc (Little Chalfont, England). Carboxymethyl cellulose sodium salt (CMC, low viscosity 29-70cP) was purchased from Fisher Scientific (Loughborough, UK).

Concentration of Lysozyme Solution

PD-10 desalting columns (Amersham Biosciences AB, Uppsala, Sweden) were used to exchange the buffer from ^{14}C-methylated lysozyme from 0.01 M sodium phosphate to 0.001 M sodium phosphate. The column was equilibrated with sodium phosphate buffer (0.001 M, 25 mL) and ^{14}C-methylated lysozyme (2 mL) was loaded onto the column and washed on with a further 0.5 mL of buffer (0.001 M). The eluate up to this point was discarded. Additional buffer (0.001 M, 3.5 mL) was used to elute the radiolabeled protein, which was collected into preweighed containers. The ^{14}C-methylated lysozyme, now in 0.001 M sodium phosphate, was frozen at –70°C before freeze drying overnight to concentrate the protein.

Preparation of Microspheres

For the preparation of ^{14}C-methylated lysozyme-loaded microspheres, an aqueous radiolabeled lysozyme solution (0.6 mL, 55 mg lysozyme [3 mg ^{14}C-methylated and 52 mg no-radiolabeled] per milliliter of PBS) was emulsified with a polymer solution (1 g of PEGT/PBT in 7 mL of $CHCl_3$) using an ultra turrax (IKA Labortechnik T25) for 30 s at 19 krpm. This water-in-oil emulsion was poured into 50 mL of PBS containing 4% (w/v) of polyvinyl alcohol. After 5 min stirring at 1000 rpm, 100 ml PBS was added. The resulting water-in-oil-in-water emulsion was stirred at constant speed for 2 h at room temperature. The microspheres were collected by centrifugation (200 × g) and washed three times with PBS and freeze-dried for 16 h.

To study the influence of the matrix composition on the lysozyme release behavior, three microsphere formulations were prepared containing 1000PEGT70PBT30, 1000PEGT60PBT40, and 600PEGT77PBT23 copolymers, respectively. To evaluate the effect of the radioactive label, a batch of 1000PEGT70PBT30 microspheres containing nonradiolabeled lysozyme only was prepared as a reference under similar process conditions.

Morphology and Size

A Philips XL 30 environmental scanning electron microscope was used to evaluate the morphology and the size of the microspheres. Samples were sputter-coated with a thin gold layer.

Lysozyme Content

The encapsulation efficiency was determined indirectly by measuring the amount of lysozyme that was not encapsulated during the microsphere preparation. The outer water phase of the water-in-oil-in-water emulsion was analyzed for the total protein concentration by micro bicinchoninic acid (BCA) protein assay, and for radioactivity content by liquid scintillation counting (LSC).

The loading of the microspheres was also determined directly by extraction of the radiolabeled protein from the microsphere formulations, consisting of microspheres suspended in 1% CMC solution in PBS. Aliquots of the dosing formulations were weighed and diluted with chloroform. Both the chloroform layer and the CMC layer were analyzed for radioactivity concentration.

Assuming a complete release of the encapsulated lysozyme (9), the loading could also be determined from the total amount of lysozyme released in the *in vitro* release experiments as described below.

In Vitro Release

The *in vitro* lysozyme release from the loaded PEGT/PBT microspheres was investigated by incubating 50 mg of microspheres in 1.5 mL of PBS. Vials were continuously agitated at 37°C. At various time points, the suspension was centrifuged, (110 × g) and 800 μL of the release medium was removed. After sampling, the volume of each sample was replenished with 800 μL of PBS, and the microspheres were resuspended and mixed continuously as before until the next sampling point. The sampled supernatant was analyzed for the total protein concentration by BCA, and for radioactivity content by LSC.

In Vivo Study

The lysozyme release from the PEGT/PBT microspheres was studied in Sprague–Dawley rats (male, 230–250 g). The animals were housed in groups of three in a well-ventilated environment under controlled and monitored temperature (21 ± 2°C) and humidity (55 ± 15%). Food and water were available *ad libitum*. Before injection, the microspheres were resuspended in 1% CMC solution in PBS. For each controlled release formulation, three rats were injected subcutaneously with approximately 150 mg of microspheres (ca. 15 μCi per kg body weight). Another three rats received free ^{14}C-methy-

lated lysozyme (15 μCi per kg body weight) dissolved in a 1% CMC in PBS solution. One rat from the group administered 1000 PEGT70PBT30 microspheres died after 1 h; however, this was not attributed to administration of ^{14}C-methylated lysozyme. After 28 days, the rats were killed.

In Vivo Release

The *in vivo* release of lysozyme from the PEGT/PBT microspheres was evaluated from the plasma concentrations. At preselected time intervals, serial blood samples were collected from the caudal vein (0.4 mL) of each animal, and a final terminal sample (8 mL) was collected by cardiac puncture under isoflurane anesthesia. Each blood sample was delivered into a heparinized container and centrifuged. Subsequently, the plasma was transferred to clean polypropylene tubes. The lysozyme plasma concentrations were determined by radioactivity content measurements using LSC.

To ensure that only bound ^{14}C label is detected during the *in vivo* release study, the proportion of radioactivity associated with the protein fraction was measured by trichloroacetic acid (TCA) precipitation. The remaining plasma was pooled by time point for each microsphere formulation. An aliquot was dispensed in a 15% aqueous solution of TCA to precipitate any of the ^{14}C-radiolabel remaining in association with the protein. After centrifugation, the radioactivity content of the supernatant, assumed to represent ^{14}C unassociated with protein, was determined by LSC. Subsequently, the proportion of the total radioactivity content associated with the protein fraction was calculated by difference.

After collection of the final sample, the rats were sacrificed and the dose sites (skin and underlying muscle) were excised from the carcasses to evaluate the remaining lysozyme at the injection sites. The explants were solubilized by digestion with a solution of NaOH and Triton X-405 in aqueous methanol. Subsequently, the radioactivity content of the digests was measured by LSC.

In Vitro/in Vivo Correlation

To compare the *in vitro* and *in vivo* release of ^{14}C-methylated lysozyme from the microsphere formulations the radioactivity concentration-time data were processed using the software WinNonlin Pro version 3.1 (Pharsight Corp., Mountain View, CA, USA).

RESULTS AND DISCUSSION

In Vitro Characteristics

Morphology and Size

The lysozyme-loaded PEGT/PBT microspheres prepared via a water-in-oil-in-water emulsion were spherical with relatively smooth surfaces (Fig. 1). Some small pores were visible at the surface (Fig. 1B). The internal structure of the microspheres was dense but also contained some small pores (Fig. 1C). The small pores may originate from the water droplets in the water-in-oil emulsion. The size of the microspheres ranged approximately from 40 to 120 μm (Fig. 1A), which was comparable to previous studies (10).

Lysozyme Content

The final content of lysozyme in the microspheres was determined by the efficiency of the encapsulation process. The encapsulation efficiency was calculated in several ways: from the amount of protein or radioactivity in the outer water phase after preparation, from extraction of the radioactivity from the microspheres, and from the total amount of protein or radioactivity released. Figure 2 shows the encapsulation efficiencies calculated by the various methods for the three different copolymer compositions. Although the values obtained depended on the calculation method, the trend in the encapsulation efficiencies determined by the various methods is comparable for all three microsphere formulations. The encapsulation efficiency based on the loading determined by extraction of radioactivity generally showed the lowest values, which is probably due to incomplete extraction (26). Although indirect, the encapsulation efficiency based on the amount of protein in the outer water phase is probably the most reliable as no processing steps are required. The difficulty of this method, however, is the small sample size used for analyses compared to the total volume of the outer water phase. Therefore, a small deviation in the protein concentration will result in a large deviation in the encapsulation efficiency. In addition, as only one microsphere batch per copolymer composition was prepared, the encapsulation efficiencies are based on a single measurement. The lower value of the BCA determined encapsulation efficiency from the protein concentration in the outer water phase was possibly

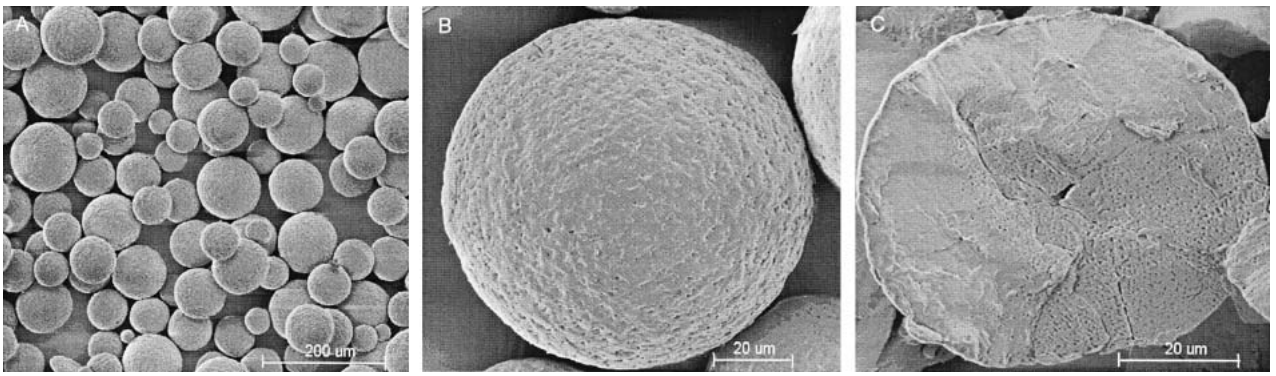

Fig. 1. Scanning electron micrographs of lysozyme-loaded 1000 PEGT:70 PBT:30 microspheres: surface (B), internal structure of fractured microsphere (C), and overview (A).

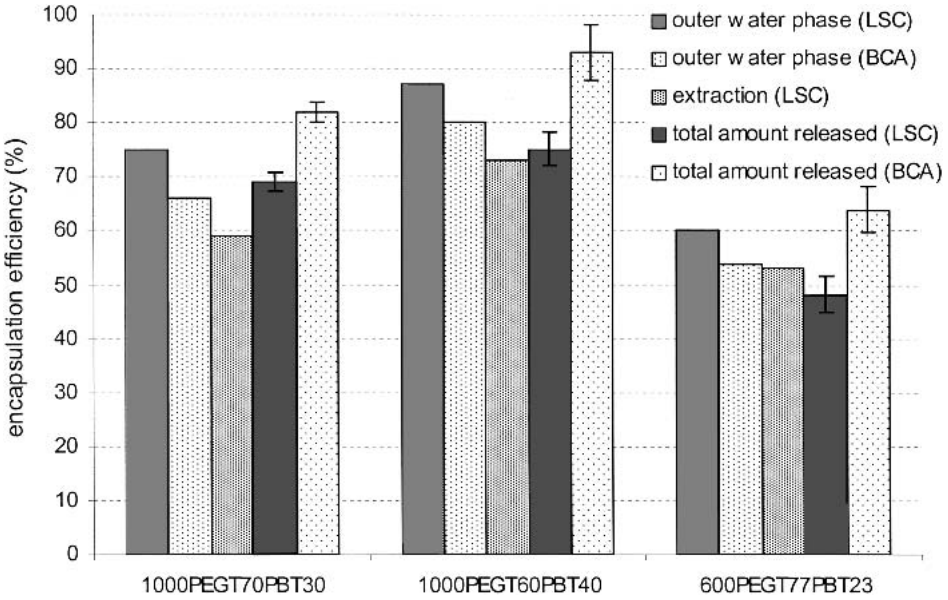

Fig. 2. Efficiency of encapsulation of ^{14}C-methylated lysozyme in PEGT/PBT microspheres determined from outer water phase by extraction and total amount of lysozyme released.

caused by the interference of polyvinyl alcohol in the BCA analyses. Previous studies showed a complete release of lysozyme from PEGT/PBT matrices (9). Therefore, the total amount of lysozyme released from microspheres can also be used as indication of the encapsulation efficiency. The higher value for the encapsulation efficiency determined from the total release by protein analyses compared to the encapsulation efficiency based on the radioactivity measured in the outer phase is probably caused by interference of polymer degradation products with the BCA reagent (in this case approximately 10%). The total amount released based on radioactivity is slightly lower than expected from the outer water phase measured for radioactivity. This might be instability of part of the ^{14}C-methylated lysozyme caused by the processing it has undergone (e.g. labeling and concentration). Subsequently, partly aggregated ^{14}C-methylated lysozyme might prevent a complete release from PEGT/PBT microspheres in contrast to native lysozyme.

In conclusion, the various different methods used for determination of the encapsulation efficiency result in calculated values with relatively small variations (± 10%). For the *in vitro* release, the encapsulation efficiency based on the radioactivity concentration in the outer water phase was used.

As described before, the encapsulation efficiency depended on the copolymer composition (10). This phenomenon was attributed to the swelling of the copolymer matrix. A higher swelling of the matrix resulted in a lower encapsulation of the protein in the matrix resulting from premature protein release during preparation. The relatively low encapsulation of the 600PEGT77PBT23 microspheres, however, was unexpected and did not correspond with previous results (10).

In Vitro Release

The *in vitro* release of (^{14}C-methylated) lysozyme from PEGT/PBT microspheres was evaluated in PBS. The cumulative release profiles were obtained from radioactivity measurements and corrected for the encapsulation efficiency based on the outer water phase (Fig. 3). As shown in previous publications, the protein release rate could be effectively tailored by varying the composition of the copolymer (9,10). Lysozyme release from 1000PEGT70PBT30 microspheres was complete within 14 days, whereas the 600PEGT77PBT23 microspheres continued releasing for 28 days. Previous studies on PEGT/PBT matrices showed similar release profiles of fully active lysozyme (9,10,13). Irrespective of the copolymer composition, an initial protein burst did not occur, indicating that the protein was effectively encapsulated in the microspheres. The release profile is attributed to a combination of diffusion of the lysozyme through the swollen matrix and degradation of the matrix (9). The diffusion coefficient appeared to be highly dependent on the swelling of the polymer matrix, which is mainly determined by the PEG segment length. At a constant PEG-segment length, the swelling can be modulated to a certain extent by varying the PEGT content. The volume swelling ratio's (Q) of the copolymers used in this study are 1.92, 1.73, and 1.66 for 1000PEGT70PBT30, 1000PEGT60PBT40, and 600PEGT77PBT23, respectively (9). The order in the swelling ratios of the copolymers corresponds with the order of the lysozyme release rates from these matrices (Fig. 3).

Total protein concentration measurements showed similar release profiles to those obtained with radioactivity measurements, although the total estimated release was always lower (by ca. 10%) for the radioactivity measurements. Therefore, it was concluded that the label remained associated with the protein after release from the microspheres. In addition, no significant differences were observed for the batches of 1000PEGT70PBT30 microspheres containing either radiolabeled lysozyme or nonradiolabeled lysozyme (data not shown), indicating that the label had no effect on the lysozyme release.

***In Vivo* study**

The *in vivo* release of ^{14}C-methylated lysozyme from PEGT/PBT microspheres was evaluated in rats. Plasma ra-

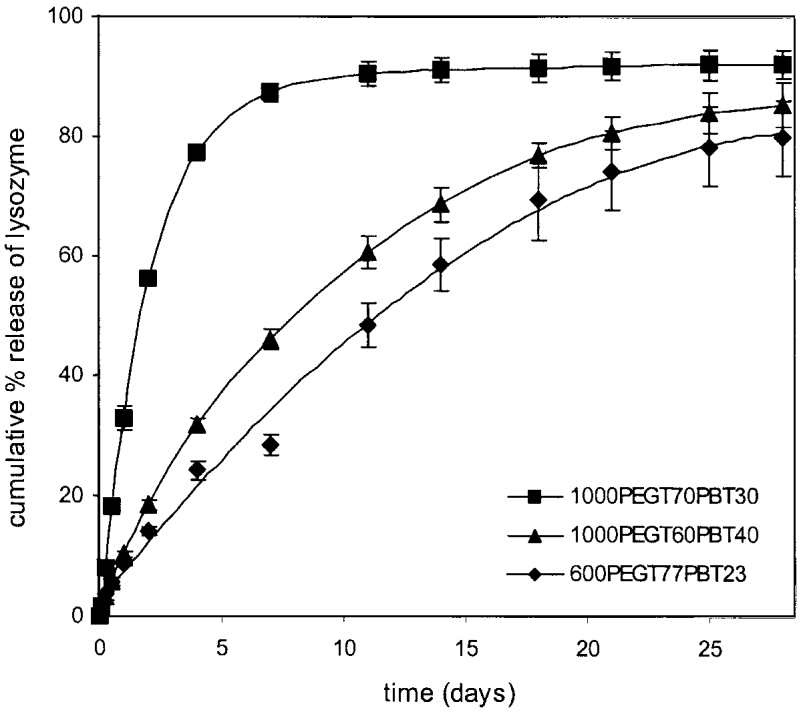

Fig. 3. Cumulative *in vitro* release of ^{14}C-methylated lysozyme from PEGT/PBT microspheres in PBS (n = 3; ± SD) based on radioactivity measurements.

dioactivity concentration–time profiles resulting from subcutaneous administration of free ^{14}C-methylated lysozyme and the three microsphere formulations are presented in Fig. 4. After the administration of free ^{14}C-methylated lysozyme, the plasma radioactivity concentrations declined in a biexponential manner and were below the limit of quantification beyond 14 days after dose. The mean plasma radioactivity concentration–time profiles after administration of the microsphere formulations indicated that passage of radioactivity into the systemic circulation was fastest for 1000PEGT70PBT30 microspheres and the slowest for 600PEGT77PBT23 microspheres. Furthermore, the mean plasma radioactivity concentrations were below the limit of quantification beyond 14 days after dose for the 1000PEGT70PBT30 microspheres, but were measurable up to the last sample point (28 days) for the 1000PEGT60PBT40 and 600PEGT77PBT23 microspheres. Table I shows the pharmacokinetic parameters as derived from the plasma levels. Independent of the microsphere formulation, the maximum plasma concentration was approximately 10-fold lower compared with the C_{max} of the free ^{14}C-methylated lysozyme injection. Although C_{max} for the free lysozyme formulation was achieved at the first sampling point (1 h), the maximum measured concentrations occurred at 24 h for the 1000PEGT70PBT30 and 1000PEGT60PBT40 formulations. For the 600PEGT77PBT23 microspheres, which showed the slowest release *in vitro*, C_{max} was not reached until 21 days after dose. However, it should be noted that for this formulation, large variation in plasma radioactivity concentrations was observed for individual animals, particularly at the later time points (coefficient of variation ranged from 17 to 132%). The latter might be explained by either variation in the total amount injected or by variation in the injection depth for this formulation (27).

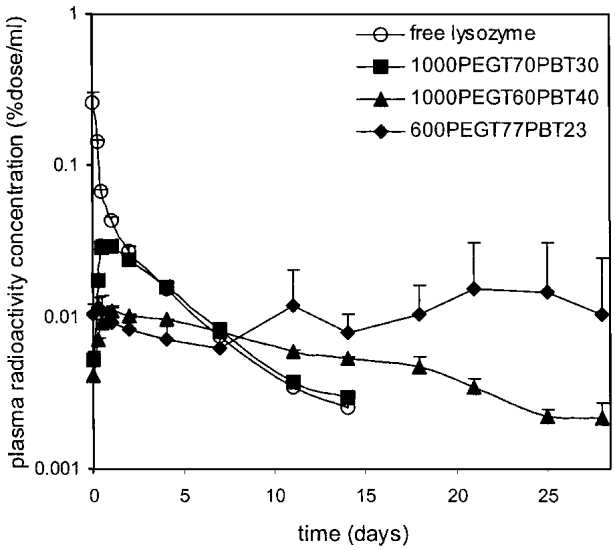

Fig. 4. Plasma radioactivity concentrations in rats after administration of single subcutaneous doses of free ^{14}C-methylated lysozyme and microspheres formulations (n = 2 1000PEGT70PBT30, n = 3; ± SD 1000PEGT60PBT40 and 600PEGT77PBT23).

The relative bioavailability, based on the area under the curve (AUC_{all}), was calculated relative to the subcutaneous injection of free lysozyme (Table I). The relative bioavailability for all microsphere formulations was higher than 64%. Relative bioavailabilities of proteins released from microspheres formulations based on poly(lactic-co-glycolic acid) were somewhat lower than we observed. For recombinant human growth hormone bioavailabilities of 33–55% relative to a subcutaneous injection of recombinant human growth hormone have been reported (28). The low relative bioavailability of 40.7% for bovine derived superoxide dismutase

Table I. Pharmacokinetic Parameters and Total Amount of Radioactivity at Dose Sites

Formulation	C_{max} (%dose/mL)	T_{max} (hs)	AUC_{all} (% Dose·h/mL)	Relative bioavailability (% of free lysozyme)	Radioactivity dose site (total % dose)
Free lysozyme	0.2607	1	6.1 ± 0.2	–	0.3 ± 0.1
1000PEGT70PBT30 microspheres[a]	0.0294	24	4.0	66	4.5
1000PEGT60PBT40 microspheres	0.0112	24	3.9 ± 0.1	64	9.5 ± 1.4
600PEGT77PBT23 microspheres	0.0156	504	7.1 ± 4.0	117	8.2 ± 3.2

[a] n = 2.

(bSOD) was attributed to instability of bSOD in the PLG microspheres and the higher susceptibility to enzymatic degradation in subcutaneous tissue of the gradually released bSOD (6). It should be noted that in our study radioactivity measurements were used, and thus quantifications are not affected by any reduction of protein activity through metabolism. Therefore, the high relative bioavailabilities observed for the PEGT/PBT microsphere formulations cannot be extrapolated to other (biologically active) proteins. In addition, it cannot be excluded that a sampling point within in first hour for the free lysozyme formulation might have increased the C_{max} value, which would result in lower relative bioavailabilities.

The high relative bioavailabilities for the microsphere formulations used in this study indicate that there was no prolonged retention of the protein at the injection site. This observation was confirmed by radioactivity measurements on the dose sites after sacrificing the animals (Table I). For all microsphere formulations, less than 10% of the total dose remained at the injection site after 4 weeks. However, the protein release from the 1000PEGT60PBT40 and 600PEGT77PBT23 microspheres was not complete within 4 weeks, which corresponds with the *in vitro* release profiles (Fig. 3).

To ensure that only bound ^{14}C label is detected during the *in vivo* release study, the plasma was dispensed in TCA to precipitate the ^{14}C-radiolabel remaining in association with the protein. The proportion of radioactivity associated with precipitable protein was greater than 77% for all time points. Therefore, it was concluded that the radioactivity measurements reflected the release of protein during the *in vivo* study. This study did not assess the integrity of the released lysozyme. *In vitro* studies, however, have shown a complete release of fully active lysozyme from PEGT/PBT matrices (9,13).

In Vitro/in Vivo Correlation

To compare the *in vitro* and *in vivo* release of ^{14}C-methylated lysozyme from the microsphere formulations, the mean radioactivity concentration-time data of the free ^{14}C-methylated lysozyme injection were modeled using WinNonlin Model 8 (2 compartment IV-Bolus, macro-constants, no lag time, first-order elimination) to provide the reference parameters for deconvolution analysis. The resultant cumulative input of ^{14}C-methylated lysozyme into the plasma after the administration of the microsphere formulations and the *in vitro* release of the microsphere formulations are presented in Fig. 5. Examination of the plots indicated that for the 1000PEGT70PBT30 (A) and 1000PEGT60PBT40 (B) microspheres, the *in vitro* and *in vivo* curves were very similar in shape. Greater variability was observed between *in vitro* and *in vivo* for the 600PEGT77PBT23 microspheres (C), the slowest release formulation. The latter might be due to variation in injection depth (27) for this formulation as discussed before.

Point-to-point correlation analysis for each formulation yielded *in vitro/in vivo* correlation coefficients of 0.995, 0.997, and 0.962 for the 1000PEGT70PBT30, 1000PEGT60PBT40, and 600PEGT77PBT23 microspheres, respectively. Independent of the release rate, the *in vitro* release and the *in vivo* release showed excellent congruence. In contrast to other studies (4,14,15,17), the *in vitro* release buffer PBS at 37°C mimicked the *in vivo* situation surprisingly well for our release system. The observed *in vivo* release very often differs from the *in vitro* release concerning shape and/or time-course (4,7,14–16). *In vivo* lysozyme release from PLGA microspheres described by Jiang *et al.* was mimicked the best by *in vitro* release in a glycine-HCl buffer (pH 2.5) whereas a slower and more incomplete release was observed in PBS (4). This was ascribed to the adsorption of lysozyme onto the hydrophobic PLG matrix in PBS. Because of the presence of hydrophilic PEG blocks in our polymer system, incomplete release caused by protein adsorption was not observed. The slow *in vitro* release compared with the *in vivo* release has been reported for many PLG-based release systems (7,14,15). In most of these cases, the release of the active compound is mainly determined by degradation of the polymer matrix. The *in vivo* degradation of PLG microspheres was found to be two times faster than the *in vitro* degradation because of the foreign body response (21,22). Therefore, for degradation controlled-release systems, the *in vivo* release will differ from the release profile obtained *in vitro*. On the contrary, for diffusion-controlled sustained-release systems, a proper *in vitro/in vivo* correlation has been described (29). The release mechanism for our PEGT/PBT system is based on diffusion of the protein through the water-swollen matrix. The diffusion of the lysozyme through the matrix is time-dependent because of degradation of the polymer matrix. As the molecular weight decreases, the mesh size in the polymer matrix increases, resulting in an increased diffusion rate (9). An important degradation mechanism of these PEGT/PBT copolymers is hydrolysis, which occurs both *in vitro* (PBS) and *in vivo*. Therefore, the perfect *in vitro/in vivo* correlation for the release of lysozyme from PEGT/PBT microspheres found in this study can be explained by the diffusion of lysozyme combined with the hydrolysis of the polymer matrix. Both swell-

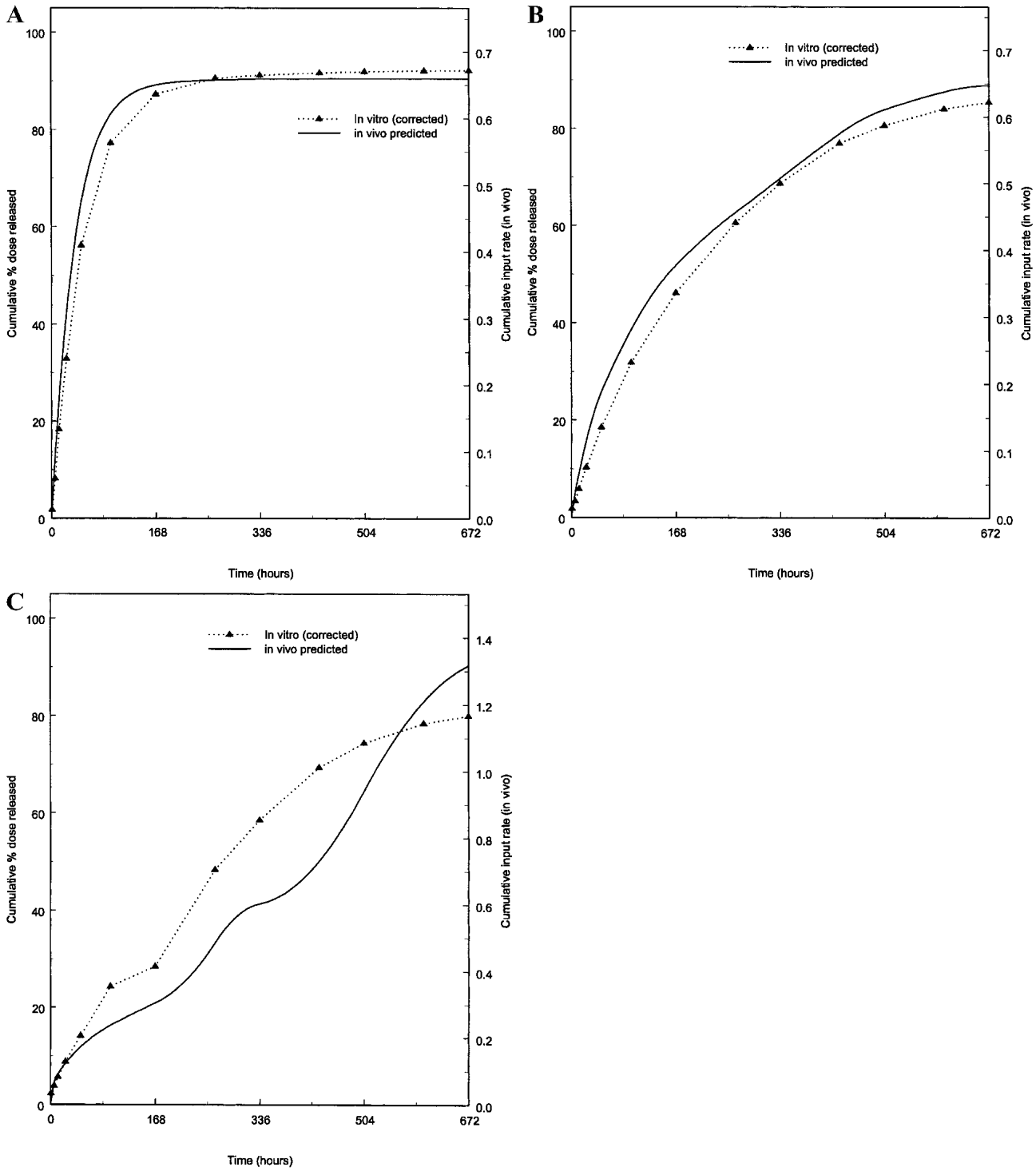

Fig. 5. *In vitro* cumulative release and *in vivo* cumulative input of ^{14}C-methylated lysozyme from microspheres of 1000PEGT70PBT30 (A), 1000PEGT60PBT40 (B), and 600PEGT77PBT23 (C) based on radioactivity measurements.

ing and degradation of the matrix are determined by the copolymer composition. Of the three compositions used in this study, the 600PEGT77PBT23 copolymer showed the lowest swelling due to the shortest PEG segments. Therefore, the degradation of this copolymer is more important for the lysozyme release, compared to the higher swelling compositions. In case the *in vivo* situation affects the degradation rate of the polymer matrix, this effect will be more pronounced for the 600 g/mole PEG segment-containing copolymer. This might also be an explanation for the somewhat greater variability observed between the *in vitro* and *in vivo* release for the 600PEGT77PBT23 microspheres.

The excellent congruence between the *in vitro* release in PBS and the *in vivo* release of lysozyme from PEGT/PBT microspheres, however, cannot be directly extrapolated to other proteins. *In vivo*, the protein-dependent metabolism

(17,18,20) and immune response (6,17,28,30) will affect the protein plasma levels. In addition, the size of the protein may determine the route of transport through the tissue into the blood stream (18,19). However, the *in vitro* release may be affected by the stability and solubility of the protein in the release buffer (3,4,16). Therefore, for every protein used in a controlled release formulation an *in vitro*/*in vivo* correlation should be established.

CONCLUSION

^{14}C-methylated lysozyme was successfully encapsulated in PEGT/PBT microspheres via a water-in-oil-in-water emulsion. The release rate of radiolabeled lysozyme from PEGT/PBT microspheres could be tailored by varying the copolymer composition. The *in vitro* release in PBS and *in vivo* release in rats showed an excellent congruence independent of the release rate. The *in vitro*/*in vivo* correlation coefficients were greater than 0.96. In addition, at sacrifice less then 10% of administered radioactivity remained at the dose site for the microsphere formulations, indicating that there was no notable retention of the protein at the injection site after 28 days.

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