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European Journal of Pharmaceutics and Biopharmaceutics 54 (2002) 89–93

European Journal of Pharmaceutics and Biopharmaceutics

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Note

Reversible aggregation of lysozyme in a biodegradable amphiphilic multiblock copolymer

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Received 29 August 2001; accepted in revised form 14 February 2002

Abstract

Lysozyme-loaded poly(ethylene glycol terephthalate)–poly(butylene terephthalate) (PEGT/PBT) films were prepared using a water-in-oil emulsification solvent evaporation method. Infrared spectroscopic analysis of the dried films indicated the presence of non-covalent lysozyme aggregates in the polymer matrix. The use of methanol to enhance the drying rate of the films increased the relative amount of aggregates. Surprisingly, quantitative in-vitro release of fully active, non-aggregated lysozyme was observed, indicating that lysozyme forms reversible aggregates during encapsulation in PEGT/PBT films.

Keywords: Poly(ethylene glycol terephthalate)–poly(butylene terephthalate); Polymer film; Lysozyme; Protein aggregation; Fourier transform infrared spectroscopy; Controlled drug delivery; Protein stability

1. Introduction

The controlled delivery of proteins from biodegradable polymeric matrices has become a major area of research in the pharmaceutical sciences. Initially, the focus was on microparticles composed of poly(lactic-co-glycolic acid), PLGA. However, obtaining suitable protein release kinetics and preservation of protein integrity are still a problem in the PLGA system [1]. At present, many other polymers are being investigated as a potential substitute for PLGA. Recently, zero-order controlled release of fully bioactive lysozyme from a poly(ether ester) consisting of copolymers of poly(ethylene glycol) terephthalate and poly(butylene terephthalate) (PEGT/PBT) was reported [2]. Similar to the common preparation method of PLGA microparticles [3], an emulsion procedure was used to encapsulate lysozyme inside the PEGT/PBT microparticles. It has been shown that emulsification may cause aggregation of lysozyme [4]. These aggregates were also co-encapsulated inside PLGA microparticles [5]. Furthermore, incomplete release and a decreased enzymatic activity was reported for lysozyme released from PLGA microparticles [6]. In contrast, the release of lysozyme from PEGT/PBT microparticles was complete and no loss in enzymatic activity was observed [2]. To investigate this apparent discrepancy, we analyzed dried lysozyme-loaded PEGT/PBT films by Fourier transform infrared spectroscopy (FTIR) and attempted to correlate the spectra of the encapsulated lysozyme to the release behavior and activity of the released lysozyme.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol)-terephthalate–poly(butylene terephthalate) (PEGT/PBT) co-polymers were obtained from IsoTis (Bilthoven, The Netherlands). The copolymers are indicated as aPEGTbPBTc, with a the PEG molecular weight, b the wt% PEG-terephthalate, and c the wt% PBT. Phosphate-buffered saline (PBS) was purchased from Life Technologies (Paisley, UK). Chloroform, obtained from Fluka Chemie (Buchs, Switzerland), and methanol from Merck (Darmstadt, Germany) were of analytical grade. Hen egg-white lysozyme (3× crystallized, dialyzed and lyophilized) was purchased from Sigma (St. Louis, MO, USA).

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0939-6411/02/$ - see front matter
PII: S0939-6411(02)00017-6

2.2. Preparation of (loaded) PEGT/PBT films

To prepare lysozyme-loaded films, a lysozyme solution (0.6 ml, 55 mg/ml) in PBS was emulsified in a polymer solution (1 g in 7 ml chloroform) using an ultra-turrax (Ika Labortechnik T25, Staufen, Germany). The resulting water-in-oil emulsion was cast onto a glass plate using a casting knife. After slow evaporation of the solvent, the films were removed from the glass plate and freeze-dried for at least 16 h. The dried films had a thickness of ~100 μm. Unloaded films were prepared similarly by casting a solution of the polymer in chloroform. To increase the solvent removal rate of some films, these were extracted in a methanol bath for 1 min (1000PEGT70PBT30) or 1 h (1000PEGT80PBT20) as soon as the film could be removed from the glass plate. Subsequently, the films were freeze-dried for 16 h.

2.3. In vitro lysozyme release

The lysozyme release from the loaded films was investigated by incubating pieces of the films (~1.8 cm^2) in 1.5 ml PBS at 37 °C under continuous shaking. Samples of the release medium were taken at various time points. The protein concentration of the buffer was determined using a microbichinchoninic acid (BCA) protein assay [7] (Pierce, Rockford, IL, USA). The buffer was refreshed after sampling.

2.4. Water uptake

Dry films (~1.8 cm^2) were weighed and immersed in PBS at 37 °C in a shaking bath. After ~3 days, the weight of the samples was determined after surface-water was removed by blotting the surface with a tissue. The water uptake (wt%) was calculated from the weight increase according to the following equation:

$$\text{Water uptake} = \frac{W_{\text{swollen}} - W_{\text{dry}}}{W_{dry}} \times 100\%$$

2.5. Lysozyme enzymatic activity

The specific enzymatic activity of lysozyme was determined by measuring the decrease in optical dispersion at 450 nm of a *Micrococcus luteus* suspension as described previously [4].

2.6. Circular dichroism (CD)

Far-UV (250–200 nm) CD spectra of released lysozyme solutions were recorded at ambient temperature in 1-mm quartz cells with a dual-beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems, Bogart, GA). The subtractive double-grating monochromator was equipped with a fixed disk, holographic gratings (2400 lines/mm, blaze wavelength 230 nm), and 1.24-mm slits. Each measurement was the average of at least five repeated scans (step resolution 1 nm, 1 s each step) from which the corresponding buffer spectrum was subtracted.

2.7. Gel electrophoresis

Non-reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 15% gel) was performed using a Bio-Rad Mini-Protean II apparatus (Segrate, Italy). To prevent dissociation of any non-covalently linked lysozyme, the SDS concentration in the sample buffer was ten times lower than customary (0.2% instead of 2%) and the samples were not boiled prior to application. Protein bands were stained using Coomassie Brilliant blue.

2.8. Infrared spectroscopy and structural analysis

Infrared spectroscopy of the PEGT/PBT films was performed in transmission mode on a BioRad FTS6000 FTIR spectrometer (Cambridge, MA, USA). Two thousand and forty-eight scans were averaged at 2 cm^{-1} resolution. Lysozyme solutions were measured in a transmission cell with a 6-μm spacer (Graseby SpecAc, Orpington, UK). Lysozyme aggregates were prepared by boiling a lysozyme solution for 15 min and subsequent lyophilization. Spectra of these aggregates were obtained in transmission mode using KBr pellets. The spectra of lysozyme aggregates obtained after emulsification were identical to those of heat-treated lysozyme aggregates [4].

Protein conformation was analyzed by the smoothed, inverted second-derivative in the Amide I region [8]. For protein solutions, the solvent background was interactively subtracted to obtain a flat baseline in the region 2300–1800 cm^{-1}. Any polymer contribution to the solid state spectra was interactively subtracted to obtain a flat baseline between 1710 and 1730 cm^{-1} [5].

3. Results and discussion

Fig. 1A shows typical transmission FTIR spectra of the PEGT/PBT films. The thickness of the films was such that in several spectral regions no light penetrated the film. This is observed in the spectra as flattened bands with an absorbance > 3.0. Apparently, the reference films were thinner, resulting in lower absorbance values in the region below 1500 cm^{-1}. The presence of protein in the lysozyme-loaded PEGT/PBT film was indicated by two bands at 1650 and 1550 cm^{-1} (Fig. 1B), which correspond the amide I and II band, respectively. Fig. 1B also shows that PEGT/PBT itself had a minor absorption band in the conformationally sensitive Amide I region, located around 1675 cm^{-1}. Fig. 1C contains the spectrum of a lysozyme-loaded PEGT/PBT film from which the spectrum of an unloaded film has been subtracted interactively to obtain a flat baseline between 1730 and 1710 cm^{-1}. Not all absorption bands from the PEGT/PBT could be corrected for, which means that a

small contribution of the PEGT/PBT may still be in present in the Amide I and II regions.

The secondary structure of lysozyme in the PEGT/PBT films was evaluated using the inverted second-derivative

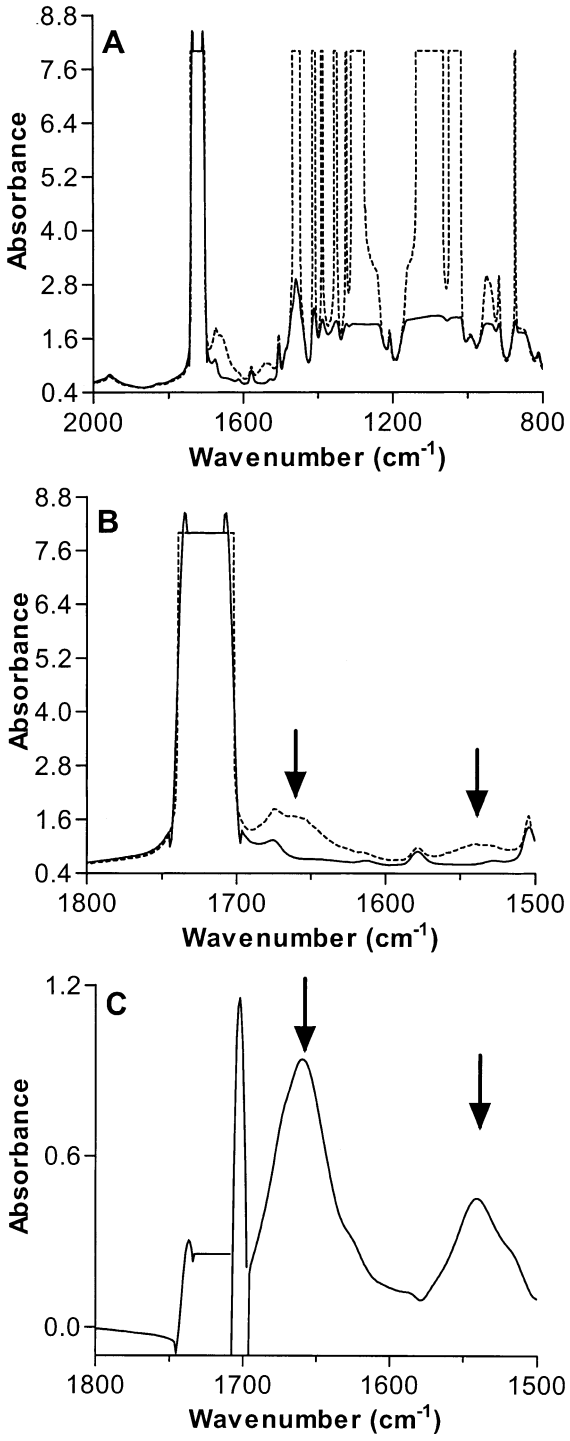

Fig. 1. FTIR spectra of lysozyme-loaded (dashed line) and unloaded (solid line) PEGT/PBT films. (A) Spectra of the fingerprint region; (B) enlargement of the spectral region containing protein-specific absorption bands, indicated by arrows; (C) partial spectrum of a lysozyme-loaded 1000PEGT70PBT30 film from which the polymer contribution has been subtracted, with protein bands indicated by arrows.

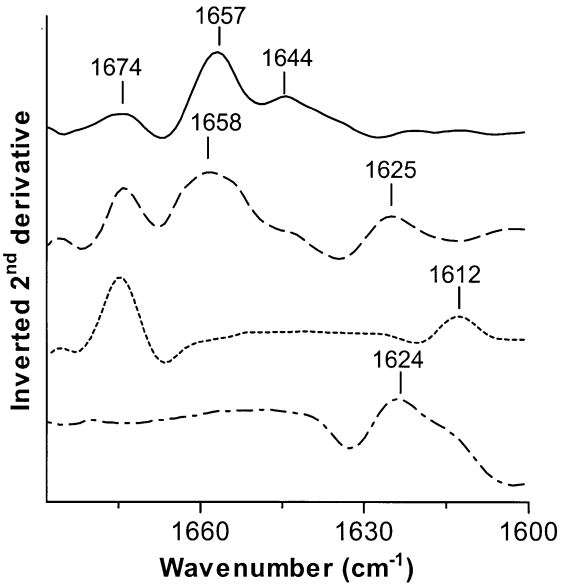

Fig. 2. Inverted second-derivative spectra in the Amide I region of lysozyme in solution (solid line), lysozyme in a 1000PEGT70PBT30 film (dashed line), unloaded 1000PEGT70PBT30 film (dotted line), lyophilized lysozyme aggregates (dash-dotted line).

spectra of the Amide I region (Fig. 2). The spectral region above 1690 cm^{-1} is not shown, since it contained strong side-lobes from the residual PEGT/PBT absorption band above 1700 cm^{-1}. The important absorption bands of lysozyme in solution were observed at 1674 cm^{-1} (turns), 1657 cm^{-1} (α-helix), and 1644 cm^{-1} (β-sheet). The same bands were observed for lysozyme in the PEGT/PBT film, but also an additional absorption band around 1625 cm^{-1}. The spectrum of unloaded PEGT/PBT (Fig. 2, dotted line) showed that this band was not introduced by insufficient or over-subtraction of the polymer spectrum. In contrast, the absorption band at 1675 cm^{-1} may have been affected by potential incorrect subtraction. An absorption band at ~1625 cm^{-1} in second-derivative spectra of proteins is typical for intermolecular β-sheets [9], which are found in non-covalently linked protein aggregates prepared by heat-treatment or emulsification (Fig. 2, dash-dotted line) [4]. Thus, the lysozyme in the PEGT/PBT probably consisted of at least two populations: one with a native-like structure, and one that was non-covalently aggregated. This suggests that lysozyme aggregates are co-encapsulated in PEGT/PBT films, similar to PLGA microparticles [5].

Methanol has been used previously in the preparation of PEGT/PBT microspheres to increase the rate of solvent extraction [10]. The use of methanol resulted in incomplete lysozyme release, and it was suggested that methanol caused protein denaturation [10]. FTIR analysis of lysozyme-loaded PEGT/PBT films treated with methanol (Fig. 3) indeed suggested an increase in protein denaturation. Note that the untreated 1000PEGT80PBT20 film (data not shown) gave similar results to the 1000PEGT70PBT30 film. The increase in aggregation is most obvious for the

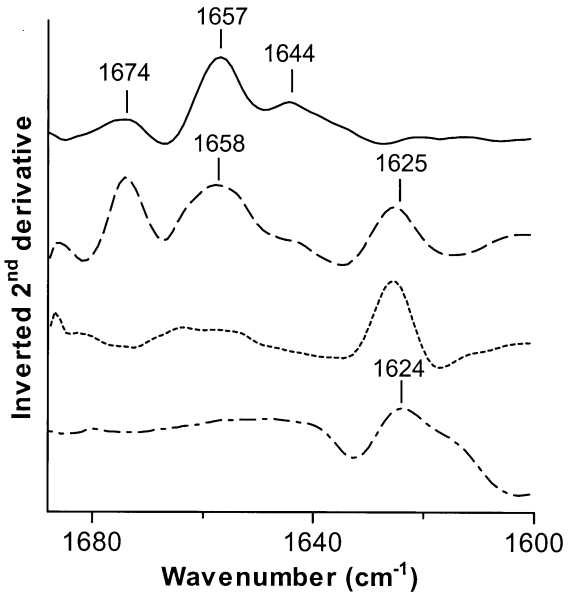

Fig. 3. Inverted second-derivative spectra in the Amide I region of lysozyme in solution (solid line), lysozyme in a 1000PEGT70PBT30 film treated with methanol for 1 min (dashed line), lysozyme in a 1000PEGT80PBT20 film treated with methanol for 1 h (dotted line), and lyophilized lysozyme aggregates (dash-dotted line).

1000PEGT80PBT20 film, which was treated with methanol for 1 h. Here, the 1625 cm^{-1} band typical for intermolecular β-sheet formation dominated the spectrum (Fig. 3). However, the aggregation is already enhanced after one minute of treatment with methanol. It was also observed that addition of methanol to lysozyme solutions or to binary mixtures of lysozyme-containing buffer and chloroform led to massive precipitation of the protein. FTIR spectra of the dried precipitate indicated that the precipitate consisted of non-covalently aggregated protein (data not shown). This indicates that methanol caused the enhanced aggregation of lysozyme observed in the films.

To investigate if the aggregation of the lysozyme in the

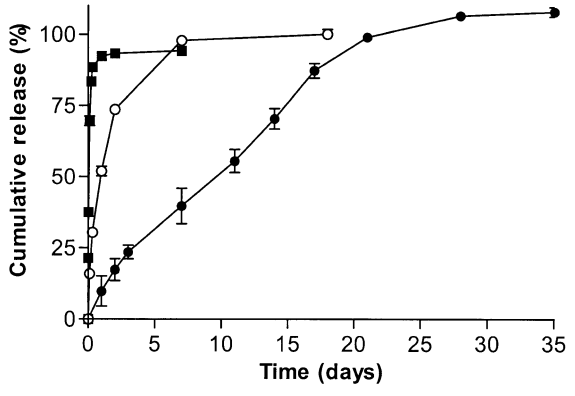

Fig. 4. Cumulative release ($n = 3$) of lysozyme from PEGT/PBT films: 1000PEGT70PBT30 (filled circles), 1000PEGT70PBT30 treated with methanol for 1 min (open circles), and 1000PEGT80PBT20 treated with methanol for 1 h (squares).

Table 1
Water uptake of methanol-treated and untreated PEGT/PBT films

Polymer composition	Preparation characteristic	Water uptake (wt%)
1000PEGT70PBT30	Untreated	75 ± 3
1000PEGT70PBT30	Methanol-treated for 1 min	97 ± 4
1000PEGT80PBT20	Untreated	108 ± 7
1000PEGT80PBT20	Methanol-treated for 1 h	134 ± 2

PEGT/PBT films resulted in an incomplete release, the release kinetics were studied (Fig. 4). The PEGT/PBT films used in this study were chosen for their known differences in release rate, yielding a release time-span of ca. 20 days for the 1000PEGT70PBT30 film (Fig. 4) and ca. 4 days for the 1000PEGT80PBT20 film (data not shown). The methanol-treated films showed a faster release rate than the untreated film (Fig. 4). This difference probably resulted from differences in water uptake. As shown in Table 1, the methanol-treated films had a higher water uptake than the untreated films. This suggests that more porous matrices were formed due to the methanol treatment, which is probably related to the increase in solvent removal rate [11]. Compared with the 1000PEGT70PBT30 films, the water uptake and the release rate of the methanol-treated and untreated 1000PEGT80PBT20 films were higher. This could be explained by the higher wt% of hydrophilic PEGT blocks in the copolymer [2].

Surprisingly, quantitative protein release was observed for all films. There are two possible explanations for the apparent discrepancy between the presence of lysozyme aggregates determined by FTIR and the complete release of lysozyme from the films: either the matrices are permeable for the lysozyme aggregates, or the aggregation is reversible. These hypotheses were checked by structural analysis on the released lysozyme. It has been noted earlier that non-covalently linked aggregates of lysozyme can be detected by SDS–PAGE as long as the samples are not boiled [12]. The SDS–PAGE analysis of the released lysozyme did not reveal the presence of any soluble lysozyme aggregates (results not shown). Moreover, the released lysozyme remained its full specific enzymatic activity, and the CD spectra of the released lysozyme were identical to those of native lysozyme in solution (data not shown). This suggested that in these films the aggregation of lysozyme, as detected with FTIR, was fully reversible. Surprisingly, lysozyme aggregates obtained after treating lysozyme solutions with methanol did not dissolve when buffer was added. This suggests that the polymer is involved in the refolding process of the lysozyme. Possibly, the polymer prevents very large aggregates to be formed, or alternatively, it serves as a template for refolding.

In this study, films were used as a model for lysozyme-loaded microspheres. However, the hardening process of microspheres and films is different. This might explain the complete lysozyme release shown here for methanol-treated films, as opposed to the incomplete lysozyme release of

methanol-treated PEGT/PBT microspheres reported earlier [10]. It is as yet unknown whether this reversibility of non-covalent aggregation is typical only for lysozyme. In cases where other proteins do not show such reversible aggregation, a suitable stabilization strategy needs to be developed [4].

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