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Research Paper

New Insight into the Role of Polyethylene Glycol Acting as Protein Release Modifier in Lipidic Implants

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Purpose. It has recently been shown that the addition of polyethylene glycol 6000 (PEG) to lipidic implants fundamentally affects the resulting protein release kinetics and moreover, the underlying mass transport mechanisms (Herrmann, Winter, Mohl, F. Siepmann, & J. Siepmann, *J. Control. Release*, 2007). However, it is yet unclear in which way PEG acts. It was the aim of this study to elucidate the effect of PEG in a mechanistic manner.
Materials and Methods. rh-interferon α-2a (IFN-α)-loaded, tristearin-based implants containing various amounts of PEG were prepared by compression. Protein and PEG release was monitored in phosphate buffer pH 4.0 and pH 7.4. IFN-α solubility and stability were assessed by reverse phase and size exclusion HPLC, SDS PAGE, fluorescence and FTIR.
Results. Importantly, in presence of PEG IFN-α was drastically precipitated at pH 7.4. In contrast, at pH 4.0 up to a PEG concentration of 20% no precipitation occurred. These fundamental effects of PEG on protein solubility were reflected in the release kinetics of IFN-α from the tristearin implants: At pH 7.4 the protein release rates remained nearly constant over prolonged periods of time, whereas at pH 4.0 high initial bursts and continuously decreasing release rates were observed. Interestingly, it could be shown that IFN-α release was governed by pure diffusion at pH 4.0, irrespective of the PEG content of the matrices. In contrast, at pH 7.4 both—the limited solubility of the protein as well as diffusion through tortuous liquid-filled pores—are dominating.
Conclusions. For the first time it is shown that the release of pharmaceutical proteins can be controlled by an *in-situ* precipitation within inert matrices.

KEY WORDS: lipid; polyethylene glycol; protein; release mechanism; solubility.

INTRODUCTION

Sustained release systems for pharmaceutical proteins are highly desirable since they offer the possibility to deliver this type of bioactive agents to their target sites, to reduce their administration frequency and to enhance their *in vivo* stability (2). Generally, these systems are based on the embedding of the protein into a degradable or non-degradable matrix. In the case of degradable devices, protein release is usually controlled by drug diffusion and/or matrix erosion. In addition, matrix swelling and osmotic effects may play a role (3–5). In contrast to these rather complex, overlapping processes protein release from non-degradable matrix systems is often governed by simple diffusion through water-filled pores. In this case, protein release is primarily sustained by the restricted diameter and generally significant tortuosity of the pores (6,7). To assure complete drug release from this type of system, the formation of a completely interconnected channel network is essential, because the matrix former itself is mostly impermeable for the protein (8). Since non-degradable devices remain in the body or have to be removed surgically at the end of the release period, research on the development of controlled release systems for proteins has been focused on degradable polymers, in particular on polyesters of lactic and/or glycolic acid (5). However, the use of these materials as matrix formers comes along with several substantial sources for protein degradation (9–12).

To overcome these restrictions, naturally occurring lipidic materials (e.g., fatty acids, glycerides and waxes) have been evaluated as alternative matrix formers (13–18). Recently, our group proposed a triglyceride-based delivery system for the continuous release of rh-interferon α-2a (IFN-α) over 1 month (18). Importantly, this system provides practically complete protein release and moreover, IFN-α is released almost exclusively in its monomeric form. The protein release patterns can effectively be adjusted by the addition of polyethylene glycol 6000 (PEG) to the lipidic formulation (1,18).

The effects of polyethylene glycols on drug release from controlled delivery systems are usually ascribed to their role as pore formers. The addition of porogens facilitates the

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creation of interconnected pore networks and, thus, leads to increasing total amounts of drug released. However, the amplified pore creation reduces the geometrical hindrance of the pore networks, what accounts for increased burst effects and elevated release rates (14,19,20). For poly(lactic-co-glycolic acid) (PLGA)-based controlled release systems the accelerated pore formation increases the diffusion of acid species generated during polymer degradation. Consequently, the acidification within the devices becomes less pronounced and the tendency of protein aggregation is minimized (21). Furthermore, positive effects of PEG 400 addition have been shown when preparing PLGA microparticles by solvent extraction/evaporation methods. The major cause of protein denaturation occurring during this type of preparation techniques is the exposure of the macromolecules to the aqueous-organic interfaces. As also PEG 400 adsorbs to these interfaces, it competes with the proteins and, thus, protects them from degradation (22,23).

However, in a previous study on the effects of PEG on the release of IFN-α from lipidic implants we obtained results that are not in agreement with these well established theories (1). Tristearin-based implants exhibit no erosion or swelling during *in vitro* release and, thus, an adequate mathematical solution of Fick's second law of diffusion was fitted to the experimentally determined protein release kinetics. It was shown that IFN-α release from PEG-free implants is primarily governed by pure diffusion with constant diffusivities. In contrast, the addition of 5–20% PEG to the formulations significantly altered the underlying mass transport mechanisms. Even an addition of only 5% PEG resulted in systematic deviations between the experimental results and the purely diffusion-based mathematical model. One possible explanation for this phenomena might be a time-dependent increase in protein mobility due to an increase in matrix porosity. The continuous leaching of the protein and hydrophilic excipients [hydroxypropyl-β-cyclodextrin (HP-β-CD) and PEG] out of the implants increases their porosity and, thus, drug mobility. However, since the release of HP-β-CD and PEG was controlled by pure diffusion [irrespective of the initial PEG content of the matrix (1)], the relevance of such a scenario in this type of lipidic impants can be considered as marginal. Therefore, it was the aim of the present study to disclose the mechanism by which PEG affects the release of rh-interferon α-2a (IFN-α) from tristearin-based implants.

MATERIAL AND METHODS

Rh-interferon α-2a (IFN-α, Mw=19,237 Da) was provided as a gift by Roche Diagnostics (Penzberg, Germany). IFN-α was formulated in a 25 mM acetate buffer pH 5.0, containing 120 mM sodium chloride with a protein concentration of 1.7 or 4.9 mg/ml. IFN-α was co-lyophilized with hydroxypropyl-β-cyclodextrin (HP-β-CD; Merck, Darmstadt, Germany) at a ratio of 1:3. To increase protein stability the pH of the stock solution was adjusted to 4.2 (with acetic acid) before lyophilisation. Tristearin (Dynasan 118; Sasol, Witten, Germany), polyethylene glycol 6000 (PEG; Clariant, Gendorf, Germany) and all other materials (from VWR International, Darmstadt, Germany) were of high purity grade.

Preparation of Implants

Protein-loaded tristearin implants were prepared as described previously (1,18). Briefly, IFN-α/HP-β-CD lyophilisates were blended with different amounts of PEG and tristearin. The percentage of IFN-α/HP-β-CD lyophilisate was kept constant at 10% (w/w) that correspond to actual drug loading of 2.5%, optional the tristearin was replaced by 10, 15 or 20% PEG. The powder blends were filled into the cylindrical matrix of a compaction tool (diameter: 5 mm) and compressed with a force of 9.8 kN for 30 s using a 5 t hydraulic press (Maassen, Eningen, Germany). The average mass and height of the implants were 50 mg and 2.3 mm, respectively.

In Vitro Release Studies

The protein-loaded implants were placed into TopPac vials (cycloolefin copolymer vials; Schott, Mainz, Germany) filled with 2.0 ml isotonic phosphate buffer (1,44 g/l $Na_2HPO_4*2H_2O$, 0.2 g/l KH_2PO_4, 8 g/l NaCl, 0,2 g/l KCl, pH 7.4 or pH 4.0, PBS), containing 0.05% (w/v) sodium azide. The vials were incubated in a horizontal shaker at 40 rpm and 37°C (Certomat IS; B. Braun Biotech International, Goettingen, Germany). At predetermined time points, the release medium was completely exchanged. This frequent buffer exchange as well as the absence of acidic/basic degradation or release products ensured a virtually constant pH in the release media throughout the experiments. For example, the pH of aqueous media did not change during a 4 week incubation of the described implant systems (24).

IFN-α and PEG concentrations in the samples were determined as described elsewhere (1). Briefly, the protein concentration was measured by size-exclusion chromatography (SEC) using a TSKgel (G3000SWXL, 7.8 mm×30.0 mm column; Tosoh Biosep, Stuttgart, Germany). The mobile phase consisted of 120 mM disodium hydrogen phosphate dihydrate, 20 mM sodium dihydrogen phosphate and 4 g/l sodium chloride (adjusted to pH 5.0 with hydrochloric acid), the flow rate was 0.5 ml/min, and IFN-α was detected UV spectrophotometrically (λ=215 nm, UV 1,000; Thermo Electron Cooperation, Dreieich, Germany). PEG was determined by vigorously mixing (21°C, 1,400 rpm, 30 min, Eppendorf Thermomixer; Eppendorf, Hamburg, Germany) 150 µl of the sample with 500 µl ammonium ferrothiocyanate reagent (16.2 g/l anhydrous ferric chloride, 30.4 g/l ammonium thiocyanate) and 500 µl chloroform. Subsequently, the aqueous phase was removed and the absorbance of the chloroform phase measured at 510 nm (UV 1; Thermo Spectronic, Dreieich, Germany).

Solubility Studies

Solutions of PEG [(2–40% (w/v) in PBS pH 4.0 or 7.4] and IFN-α bulk solutions (initial concentration of 4.9 mg/ml pH 4.0 or 7.4) were mixed in ratio of 1:1 and equilibrated for 2 h at 37°C, 40 rpm (Certomt IS). Subsequently the protein precipitates were separated by centrifugation at 5,000 rpm (5°C, 5 min, 4K15 laboratory centrifuge; Sigma, Osterode, Germany) and the IFN-α concentrations in the supernatants were determined. Thus solubility is referred to the solute

concentration of the supernatant in equilibrium with the precipitated/crystalline phase. Protein concentration was determined with reversed phase HPLC, which was performed using a Jupiter 5u C18 300 Å 250×4.60 mm column (Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of a 49:51 (v/v) acetonitrile/ultra purified water mixture which was acidified with 0.1% (v/v) trifluoroacetic acid. The flow rate was adjusted to 1 ml/min; UV detection (UV 1,000; Thermo Electron Cooperation, Dreieich, Germany) was performed at 215 nm wavelength. The effect of pH on the precipitation of IFN-α was investigated at a final PEG concentration of 5%PEG. The pH of the PEG and protein solution was adjusted with 0.1 N NaOH or 0.1 N HCl prior to mixing, respectively.

Protein Stability

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography (SE-HPLC)

Since high amounts of PEG interfere with SDS-PAGE as well as with the size exclusion chromatography the supernatant was removed after protein precipitation and the obtained precipitates were dissolved in PBS buffer. SE-HPLC was performed as explained above. SDS PAGE was conducted under non-reducing conditions using an XCell II Mini cell system (Novex, San Diego, CA). IFN-α bulk solutions and reconstituted precipitates were diluted in a pH 6.8 tris-buffer, containing 2% SDS and 2% glycerine. Samples were denatured at 90°C for 30 min and subsequently 20 µl were loaded into the gel wells (NUPAGE Novex 10% Bis Pre-Cast Gel 1.0 mm; Invitrogen, Groningen, The Netherlands). Electrophoresis was performed in a constant current mode of 30 mA in a tris-glycine/SDS running buffer (MES running buffer; Invitrogen, Groningen, The Netherlands). Gel staining and drying was accomplished with a silver staining kit (SilverXpress) and a drying system (DryEase), both provided from Invitrogen, Groningen, Netherlands.

FTIR Spectroscopy

IFN-α was precipitated in the presence of 5–20% PEG as described. After centrifugation the supernatant was removed and the obtained protein pellet was re-dissolved. The obtained solutions, as well as IFN-α bulk solutions were filled in a calcium fluoride flow through cell (Aquaspec AS 1110 M, Bruker Optik, Ettlingen, Germany) with 6.5 µm path length. FTIR spectra were recorded with the Confocheck system on a Tensor 27 (Bruker Optik, Ettlingen, Germany) equipped with a nitrogen-cooled photovoltaic MCT detector at a resolution of 4 cm^{-1}. At least two measurements of 120 scans were performed. The temperature of the cell was maintained at 25°C via a cryostat (DC 30-K20; Thermo Haake, Dreieich, Germany). The obtained spectra were background subtracted and vector normalized from 1,480 to 1,720 cm^{-1} (amid I) for relative comparison. Finally, the second derivative spectra were generated via the spectrometer software (OPUS, Bruker Optik, Ettlingen, Germany).

Fluorescence Spectroscopy

Fluorescence studies were carried out on Varian fluorescence spectrometer Cary eclipse at 20°C. IFN-α bulk solutions (4.9 mg/ml, pH 7.4) were precipitated with 5–20% PEG and re-dissolved in excess of buffer to final protein concentration of 0.05 mg/ml the obtained spectra were compared to that of native IFN-α (also 0.05 mg/ml in PBS buffer). The excitation wavelength was fixed at 295 nm, and fluorescence emissions scans were collected from 300 to 450 nm using a scan speed of 30 nm/min at a excitation and emission slit width of 5 nm. Scans were performed in triplicate and the average spectra of each sample is shown.

"Macropore Release Model"

In order to get deeper insight into the underlying release mechanisms a "macropore release model" device was developed, which is schematically illustrated in Fig. 1. The device was filled with protein, HP-β-CD and PEG pellets prepared by compression. The pellets comprised IFN-α lyophilisate, IFN-α lyophilisate:PEG blends, as well as IFN-α lyophilisate:HP-β-CD blends, respectively. The lyophilisate:PEG ratios were the same as for the implant formulations, e.g. 1:1 for devices containing 10% PEG, 10% lyophilised protein; and 1:2 for implants containing 10% lyophilised protein and 20% PEG. The pellets were placed in a small volume container, connected with an open capillary of around 10 mm length and 2 mm diameter. Subsequently, the devices were filled with isotonic phosphate buffer (pH 7.4, 0.05% (w/v) sodium azide) and fixed at the bottom of a sterile polypropylene tube (Greiner bio-one, Frickenhausen, Germany). Afterwards, the polypropylene tube itself was filled with 3 ml acceptor medium (isotonic phosphate buffer, pH 7.4, 0.05% (w/v) sodium azide) and incubated at 37°C and 40 rpm (Certomat IS). At predetermined time points, the acceptor medium was completely replaced and the protein concentrations in the samples were determined by RP-HPLC as described above.

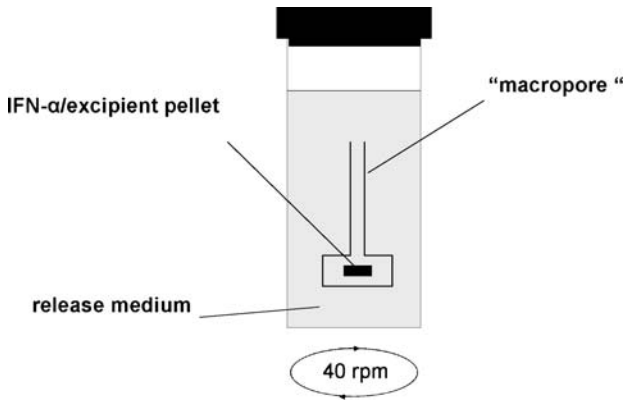

Fig. 1 Schematic presentation of the experimental setup of the "macropore release model." The idea is to simulate the conditions for protein diffusion within a "macropore" in the lipidic matrices. Protein, protein/PEG or protein/HP-β-CD pellets were placed into a cylindrical chamber with only one opening into a cylindrical capillary. This device was placed into a plastic flask filled with phosphate buffer pH 7.4. The flask was agitated at 40 rpm and kept at 37°C.

Mathematical Modeling of Protein and PEG Release

IFN-α and PEG release from the investigated lipidic implants into the release media was quantitatively described using an analytical solution of Fick's second law of diffusion. The model considers protein/PEG diffusion in axial as well as in radial direction in cylindrical matrices:

$$\frac{\partial c}{\partial t} = \frac{1}{r} \cdot \left\{ \frac{\partial}{\partial r}\left(r \cdot D \cdot \frac{\partial c}{\partial r} \right) + \frac{\partial}{\partial \theta}\left(\frac{D}{r} \cdot \frac{\partial c}{\partial \theta} \right) + \frac{\partial}{\partial z}\left(r \cdot D \cdot \frac{\partial c}{\partial z} \right) \right\} \tag{1}$$

where c is the concentration of IFN-α/PEG; t represents time; r, z denote the radial and axial coordinates and θ the angle perpendicular to the r–z-plane; D is the apparent diffusion coefficient of the protein/PEG within the lipidic implant.

The theory takes into account that perfect sink conditions are maintained throughout the experiments and considers the rotational symmetry around the z-axis (no concentration gradients in θ direction) as well as homogeneous IFN-α/PEG distributions within the implants at t=0 (before exposure to the release media). Based on these initial and boundary conditions, Eq. 1 can be solved using infinite series of exponential functions, leading to (25):

$$\frac{M_t}{M_\infty} = 1 - \frac{32}{\pi^2} \cdot \sum_{n=1}^{\infty} \frac{1}{q_n^2} \cdot \exp\left(-\frac{q_n^2}{R_2} \cdot D \cdot t \right) \times \sum_{p=0}^{\infty} \frac{1}{(2 \cdot p + 1)^2} \cdot \exp\left(-\frac{(2 \cdot p + 1)^2 \cdot \pi^2}{H^2} \cdot D \cdot t \right) \tag{2}$$

where M_t and M_∞ represent the absolute cumulative amounts of protein/PEG released at time t and infinite time, respectively; q_n are the roots of the Bessel function of the first kind of zero order [$J_0(q_n)=0$]; R and H denote the radius and height of the cylinder. If IFN-α/PEG release levelled off below 100%, the experimentally determined plateau values (amounts of mobile protein/PEG) were considered as 100% reference values for protein/PEG diffusion.

RESULTS

The release patterns of IFN-α from tristearin implants containing various amounts of PEG are illustrated in Fig. 2. Protein release from PEG-free implants levelled off at 31% after 7 days, whereas complete IFN-α release was observed from devices containing more than 10% PEG. As explained in a pervious study (1) this can be ascribed by an increase in the inner and outer porosity of the lipidic matrices with increasing PEG loadings. The addition of 10% PEG to the lipid formulation ensures the creation of a highly interconnected pore network that allows the macromolecule to diffuse out. In contrast, at lower PEG loadings not all of the protein has direct access to water-filled pores and is thus mechanically trapped within the impermeable lipid matrix.

Importantly, PEG-containing implants exhibited a lower burst effect than PEG-free systems. For example, matrices containing 0% PEG released 18.3% (± 1.7%) of the incorporated IFN-α within the first 10 h, whereas implants comprising 10% PEG released only 11.9% (± 2.2%) within the same time period. In order to better understand the underlying protein release mechanisms, an adequate mathematical model was fitted to the experimentally determined IFN-α release kinetics (1). Interestingly, protein release from PEG-free devices was controlled by pure diffusion with constant diffusion coefficients, whereas also other phenomena must be involved in all PEG-containing implants. Both effects of PEG—the reduced burst release as well as the significant deviations from purely diffusion controlled protein release—are astonishing, considering the well known role of PEG as a pore former in controlled release matrix systems.

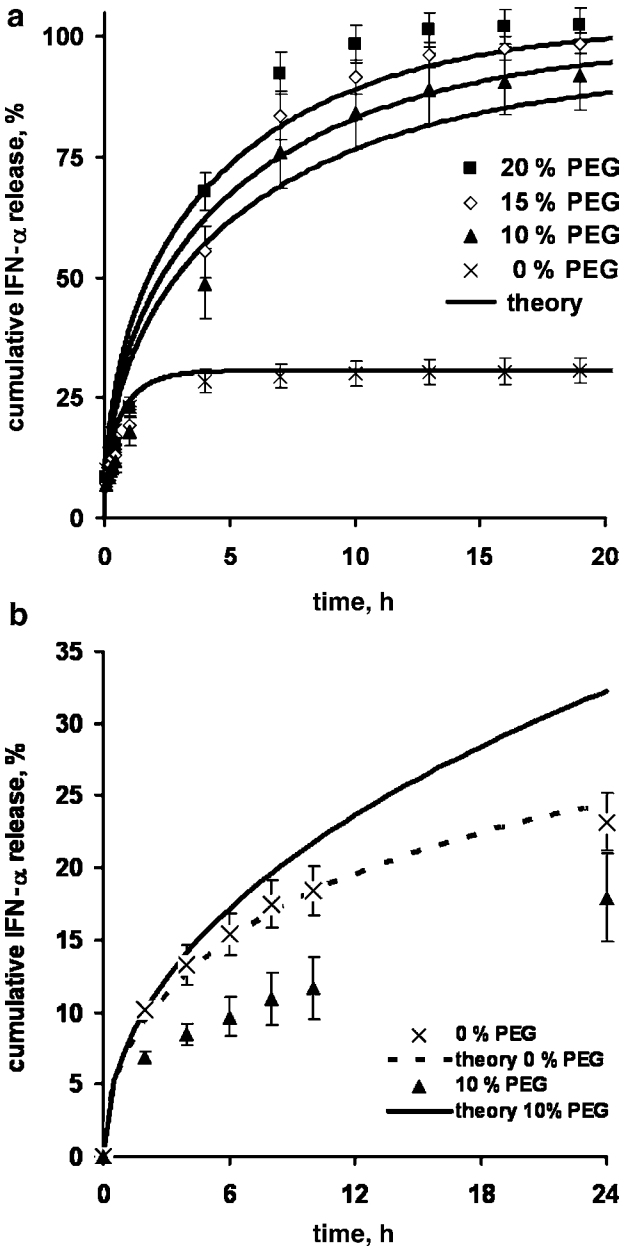

Fig. 2 (**a**) Effects of the addition of different amounts of PEG (indicated in the figure) on IFN-α release from tristearin-based cylindrical implants (containing 10% IFN-α/HP-β-CD) in phosphate buffer pH 7.4. (**b**) shows the protein release from implants containing 0 or 10% PEG within the first 24 h, respectively. [*symbols*: experimental values (average +/− SD; *n*=3), *solid curves*: theory (Eq. 2)] [the experimental results and theoretical calculations for implants containing 0 and 10% PEG are reproduced from reference (1)].

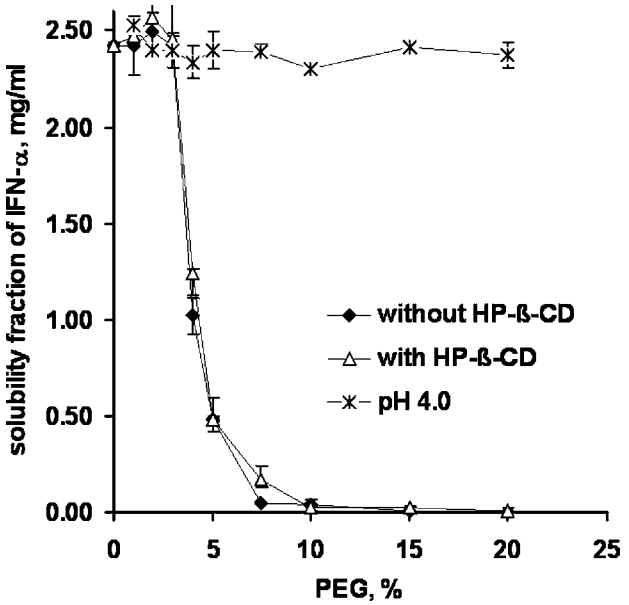

Fig. 3 Effects of the PEG concentration on the soluble fraction of IFN-α in phosphate buffer pH 7.4 at 37°C in the presence and absence of HP-β-CD as well as at pH 4.0, as indicated (average +/− SD; *n*=3).

On the other hand, the ability of PEG to reversibly precipitate/crystallize proteins has been widely used for protein separation and purification (26,27) but so far it has not been evaluated whether this precipitation might also occur during release within controlled release systems. Therefore, we hypothesized that the observed phenomena might be ascribed to an altered solubility or even to a reversible precipitation of IFN-α within PEG-containing matrices.

Solubility Studies

In order to evaluate the ability of PEG to precipitate IFN-α, the solubility of the protein was experimentally determined in differently concentrated aqueous PEG solutions. As illustrated in Fig. 3, the apparent solubility of IFN-α is strongly affected by the presence of PEG. At PEG concentrations above 3%, the protein spontaneously precipitates in phosphate buffer pH 7.4 and the solubility fraction decreases non-linearly with increasing PEG concentration. Importantly, already 10% PEG resulted in an almost negligible protein solubility (0.02 mg/ml). In order to evaluate potential effects of the presence of the co-lyophilisation agent hydroxypropyl-β-cyclodextrin (HP-β-CD) on the precipitation of IFN-α in phosphate buffer pH 7.4 containing different amounts of PEG, the soluble protein was also determined in the presence of HP-β-CD. Clearly, the presence/absence of this co-lyophilisation agent did not alter the PEG impact on precipitation (Fig. 3). Generally, the precipitation of proteins by PEG has been explained on the basis of volume exclusion effects. Mainly due to sterical hindrance proteins are excluded from the solvent space that is occupied by the linear PEG chains. Hence the protein is concentrated until the solubility is exceeded and precipitation and/or crystallization occurs (27–30).

Figure 4 shows a microscopic picture of the obtained solid material upon precipitation. The ordered, needle like structures indicate a crystallization of IFN-α in the presence of PEG. This is an important information considering the protein stability, since the stability of the protein in the crystalline form might be higher than that of the corresponding soluble amorphous material (31). It is further apparent from Fig. 4

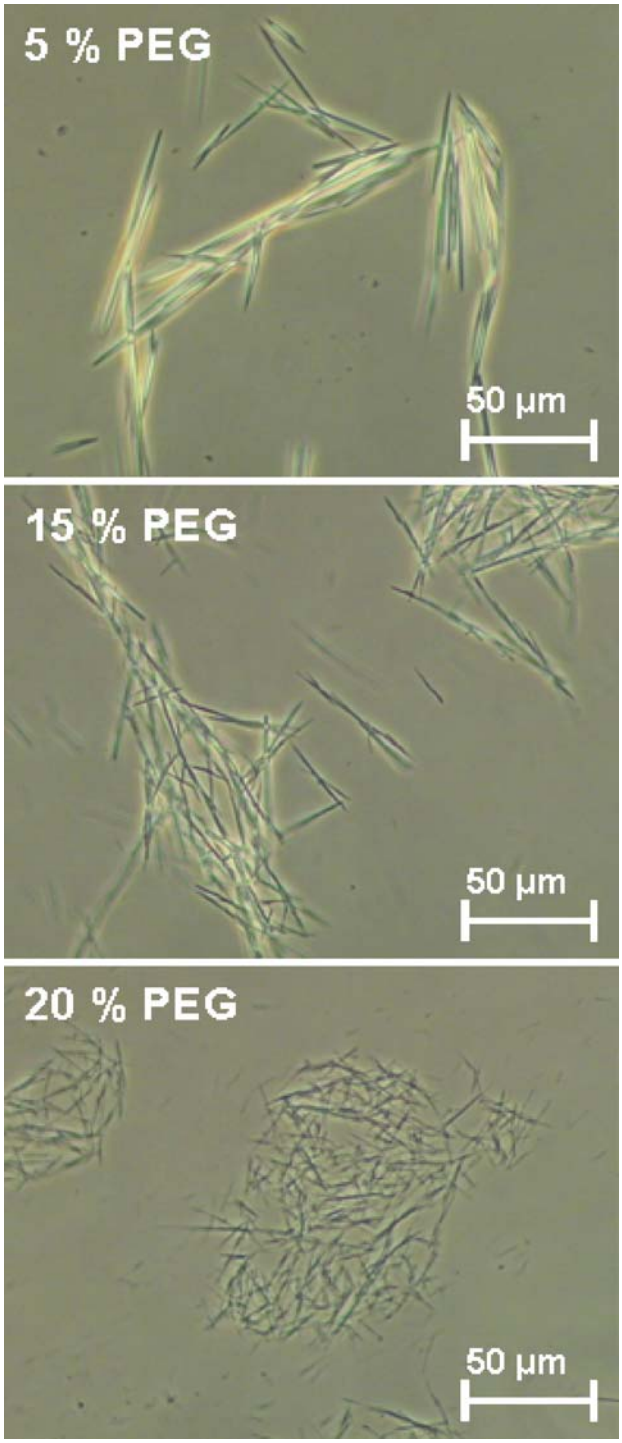

Fig. 4 Morphology of the IFN-α precipitates obtained during the protein solubility studies in phosphate buffer pH 7.4 (37°C), containing different amounts of PEG (indicated in the figures).

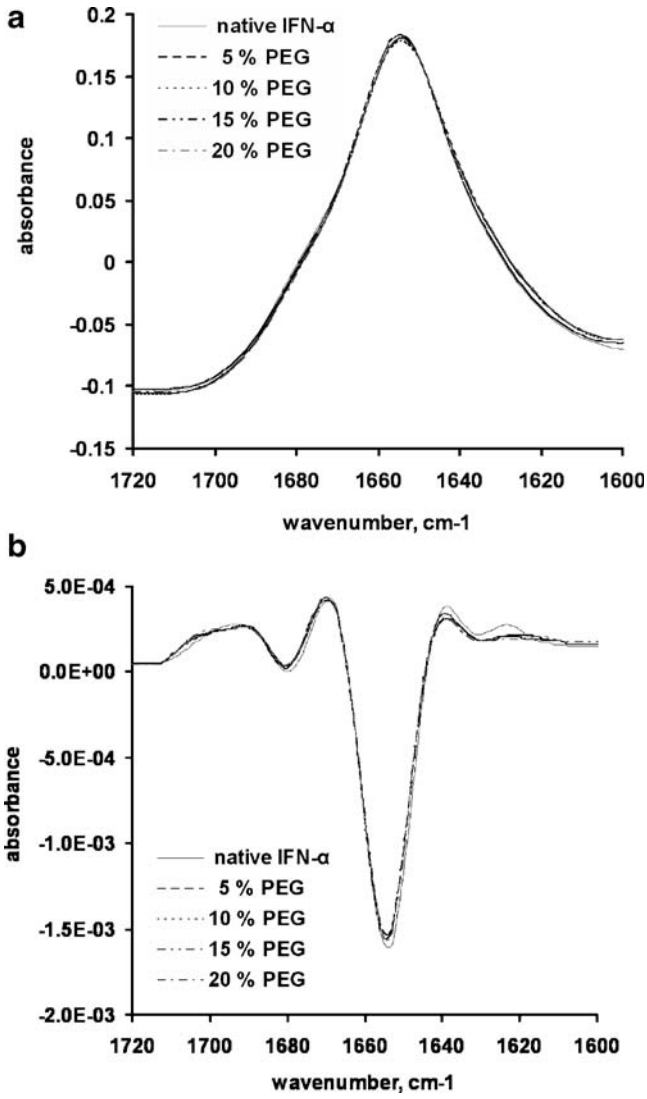

Fig. 5 Effects of precipitation and re-dissolution of IFN-α in phosphate buffer pH 7.4 on the secondary protein structure: (**a**) Background corrected and vector normalized amid I FTIR spectra; (**b**) Second-derivative amid I FTIR spectra. The PEG concentrations at which IFN-α precipitation was performed are indicated in figures.

that increasing PEG concentrations lead to smaller needles. By increasing the nucleation with increasing precipitant concentrations the amount of protein available per crystal nucleus is reduced leading to smaller crystals (32).

Precipitation/crystallization might offer the benefit of protein stabilization during the fabrication process of controlled release devices. For example, the formation of an insoluble complex of rhGH and zinc enables the encapsulation and slow release of unaltered rhGH from PLGA microspheres (33–35). Also van de Wetering used the precipitation of rhGH by zinc or by PEG to protect the protein from reaction with gel precursors during the preparation of chemical crosslinked hydrogels (36). Furthermore the reversible precipitation of α-chymotryphsin and lactate dehydrogenase in the presence of poloxamer 407 has been used to increase the protein loading of *in-situ* forming poloxamer gels (37).

However, in the present study IFN-α, PEG and the lipid were simply mixed as solid powders and subsequently compressed to implants. Therefore a potential protein precipitation only occurs *in-situ* during release and to our knowledge this effect has so far not been considered in a mechanistic manner. Since the release of PEG itself is delayed in the first 5–7 days (1), the dissolution of PEG can be expected to lead to initially highly concentrated PEG solutions within the narrow pores of the lipidic implants (e.g. after drying of incubated implants loaded with 10% PEG a pore volume of about 10 µl was determined using a helium pycnometer). Taking into account the dramatic effect of PEG on the solubility of IFN-α, reduced protein solubility or even a reversible precipitation of IFN-α within PEG-containing matrices is highly likely. Consequently, only a minor, non-precipitated fraction of the incorporated protein is available for diffusion out of the matrix. Thus, at an early stage of release the protein release from PEG-comprising implants is hindered. Furthermore, the creation of water-filled pores might be reduced due to the un-dissolved protein. Both effects explain the reduced burst of protein release from PEG-containing implants in comparison to PEG-free implants.

In the case of purely diffusion controlled drug release, the time-dependent prolongation of the diffusion ways results in monotonically decreasing release rates. This is true for all PEG-free implants. In contrast, in PEG-containing devices the solubility of IFN-α within the water-filled pores increases continuously due to the leaching of PEG out of the system. Thus, the amount of protein available for diffusion increases with time. Consequently, the increasing length of the diffusion pathways is partly compensated, resulting in about constant IFN-α release rates over prolonged periods of time. For these reasons systematic deviations between the presented mathematical model (considering free protein solubility and constant diffusion coefficients) and the experimentally determined IFN-α release kinetics from PEG-containing implants were observed. Furthermore, the leaching of PEG promotes the formation of an interconnected pore network at a later stage of release (1), facilitating complete protein recovery.

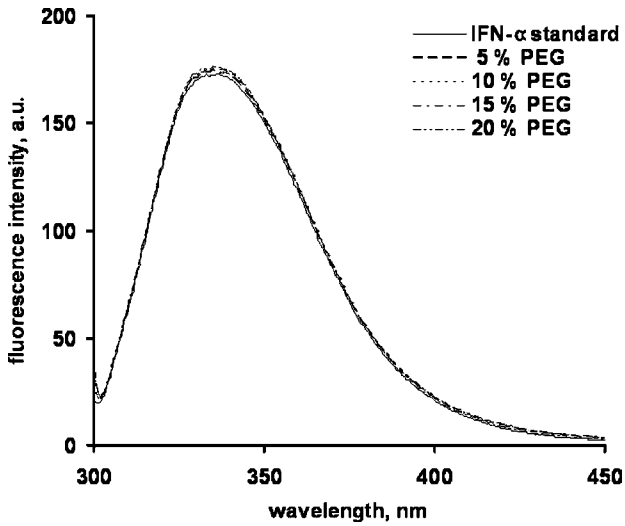

Fig. 6 Trp. fluorescence emission spectra of IFN-α 20°C and after precipitation and redissolution with various amount of PEG (as indicated in the figure).

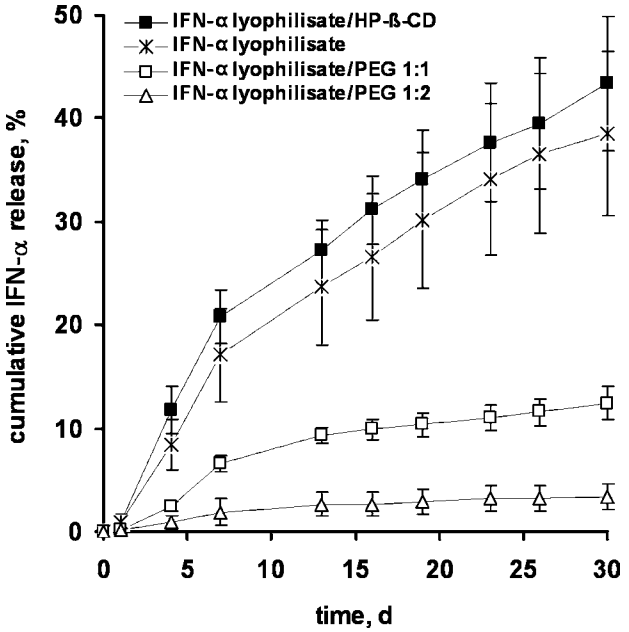

Fig. 7 IFN-α release from pellets consisting of IFN-α lyophilisates, IFN-α lyophilisate: PEG blends and IFN-α lyophilisate:HP-β-CD blends into phosphate buffer pH 7.4 measured using the "macropore release model" (schematically illustrated in Fig. 1) (average +/− SD; *n*=3).

Effects of IFN-α Precipitation and Re-Dissolution on Protein Stability

Protein denaturation and/or aggregation within controlled release systems have been identified as major reasons for incomplete protein release (11,12). Therefore, it was necessary to evaluate the effects of IFN-α precipitation and re-dissolution on protein stability. Dispersions of protein precipitates were diluted with buffer to PEG concentrations below 0.2%. The precipitates instantaneously dissolved in an excess of buffer and subsequent RP-HPLC revealed a virtually complete protein recovery, irrespective of the PEG concentration used for precipitation (4–20%). Moreover, no chemical degradation such as oxidation occurred, since the amount of oxidized IFN-α detected by RP-HPLC in standard solutions as well as in the solutions of re-constituted IFN-α was less than 2%.

Furthermore, size exclusion chromatography and SDS gel electrophoresis were used to detect protein aggregation and fragmentation. These analyses indicate that there were no significant differences in protein quality before and after precipitation and re-dissolution.

For the investigation of the effects of precipitation and re-dissolution on the secondary structure of IFN-α, the FTIR spectra in the amid I region of re-constituted precipitates were compared to that of native IFN-α. As it can be seen in Fig. 5, the spectra of native IFN-α revealed an intense peak at 1,654 cm^{-1}, the typical feature of an alpha helices protein (38). Importantly, the vector normalized FTIR spectra as well as the corresponding second derivatives of re-constituted protein precipitates were almost congruent with that of native IFN-α, irrespective of the PEG concentration used for precipitation. Therefore, the secondary structure of IFN-α remained unaffected upon precipitation and re-dissolution.

Figure 6 shows the Trp fluorescence emission scans of IFN-α in PBS puffer at a pH of 7.4. The observed λ_{max} of Trp emission at 336 nm indicates that the two Trps of IFN-α were partially buried in the hydrophobic core of the protein. Due to protein unfolding hydrophobic amino residues would be more solvent exposed (39,40). In our case the thermal induced unfolding of IFN-α resulted in a shift of λ_{max} to 340 nm (39). Importantly, λ_{max} of IFN-α after precipitation and re-dissolution was determined at 336 nm and the obtained fluorescence scans were almost identical with those of native IFN-α.

"Macropore Release Model"

To determine whether the reduced protein solubility or even a reversible precipitation within the lipidic matrix could play a crucial role in the control of IFN-α release, a model device simulating the conditions in the water-filled pores within the implant was developed. The idea was to assay the relevance of protein dissolution *versus* protein diffusion in the presence of PEG by mimicking the inner content of a pore filled with IFN-α and the water-soluble excipients. For that purpose a small volume container connected with a capillary ("macropore") was intended to imitate a drug reservoir with an associated pore within the controlled release system. The container was filled with compressed lyophilised protein pellets or with compressed lyophilised protein/PEG pellets. In the latter case, lyophilisate/PEG ratios of 1:1 or 1:2 (corresponding to implants containing 10 or 20% PEG, respectively) were studied. In addition, pellets containing HP-β-CD instead of PEG were placed into the "macropore release model" (in contrast to PEG, HP-β-CD does not cause protein precipitation).

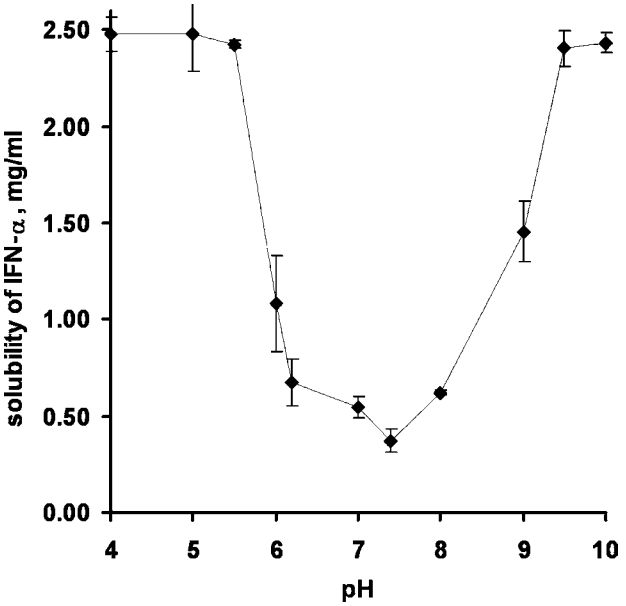

Fig. 8 Effects of the pH of the phosphate buffer solution on the apparent solubility of IFN-α at 37°C (in the presence of 5% PEG) (average +/− SD; *n*=3).

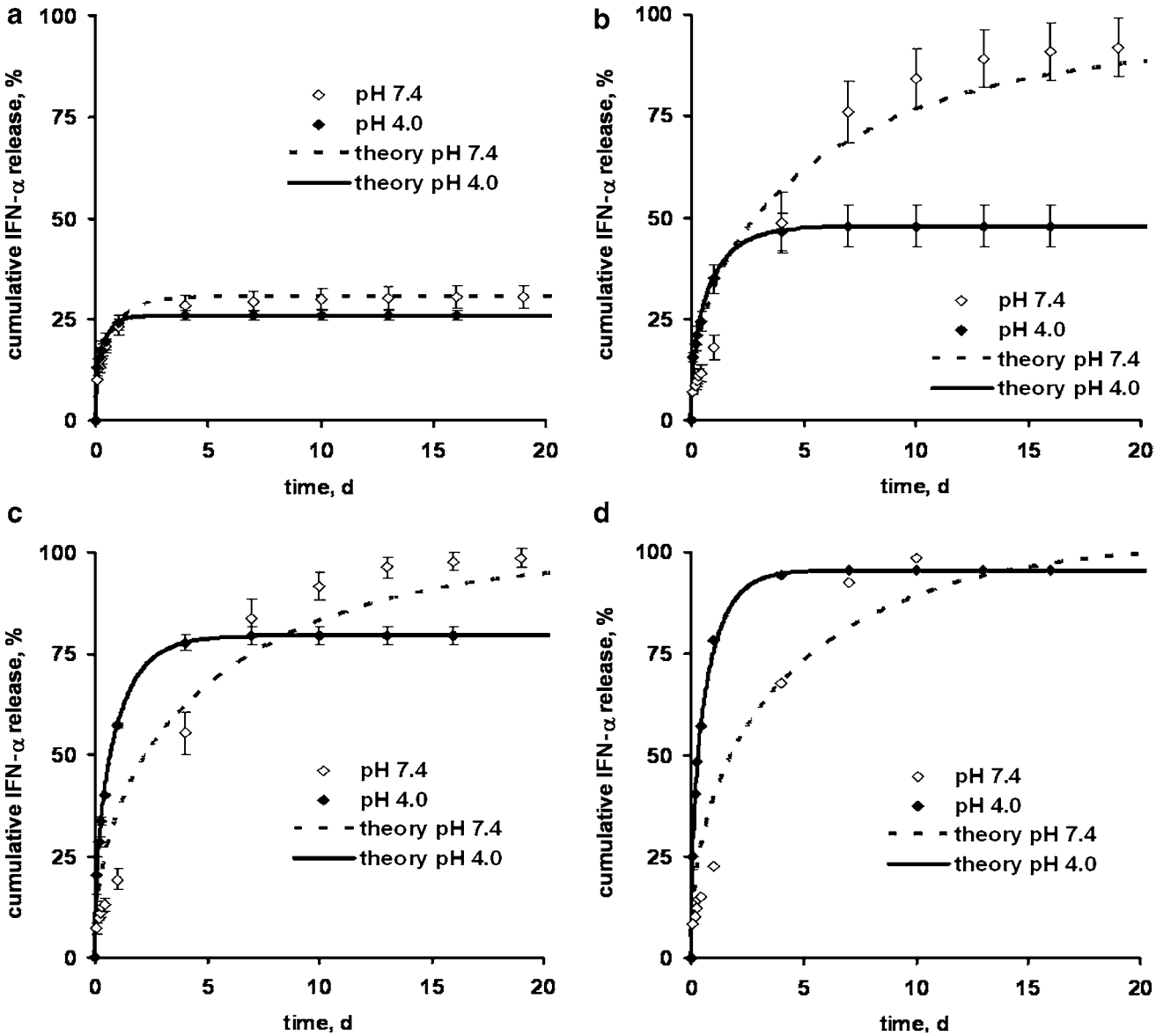

Fig. 9 Effects of the pH of the phosphate buffer solution on IFN-α release from tristearin-based implants. Matrices were loaded with 10% IFN-α/HP-β-CD lyophilisate and 0% PEG (**a**), 10% PEG (**b**), 15% PEG (**c**) and 20% PEG (**d**). The release was studied either in phosphate buffer pH 7.4 [*open symbols*: experimental values (average +/− SD; *n*=3), *dashed curves*: theory (Eq. 2)] or in phosphate buffer pH 4.0 [*closed symbols*: experimental values (average +/− SD; *n*=3), *solid curves*: theory (Eq. 2)] [the results obtained in phosphate buffer pH 7.4 are reproduced from reference (1)].

Figure 7 shows that the release of IFN-α from PEG-free devices is almost ten times faster than protein delivery from systems containing pellets with a lyophilisate/PEG ratio of 1:2. Interestingly, the pellets did not disintegrate and remained visible during the entire release periods when PEG was present. This indicates that within the "macropores" highly concentrated PEG solutions are created and, hence, the dissolution of the protein/excipient pellets is hindered. Therefore, the dissolution step can be considered as one of the major rate limiting factors for protein release in the presence of PEG.

Effects of the pH of the Release Medium on Protein Release

Further insight into the significance of reduced protein solubility and reversible precipitation within PEG-containing tristearin implants was obtained by studying the pH dependency of protein solubility and release. As it can be seen in Fig. 8, the solubility of IFN-α in phosphate buffer containing 5% PEG strongly depended on the pH: A minimum was observed in the pH range 6–9. At lower or higher pH values, no protein precipitation was notified under the given experimental conditions. Therefore a pH change of the release buffer offers the possibility to "switch off" the *in-situ* precipitation. Since several authors reported good IFN-α stability at low pH values (41,42), pH 4.0 was chosen to further investigate the effects of PEG on protein solubility and even PEG concentration up to 20% did not induce a protein precipitation (Fig. 3).

Figure 9 compares the release kinetics of IFN-α from tristearin-based implants into phosphate buffer pH 7.4 and

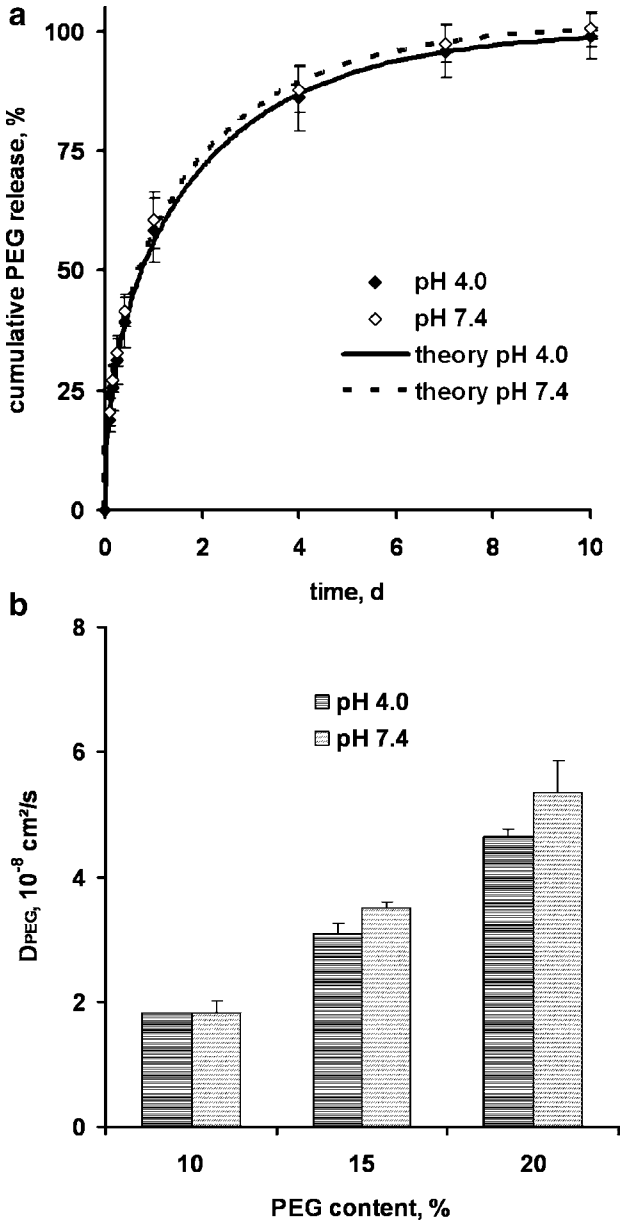

Fig. 10 Importance of the pH of the phosphate buffer on: (**a**) PEG release from tristearin-implants (loaded with 10% IFN-α/HP-β-CD lyophilisate and 10% PEG). The release was studied at pH 7.4 or 4.0, as indicated [*symbols*: experimental results (average +/− SD; n=3), *curves*: theory (Eq. 2)]. (**b**) The apparent diffusion coefficient of PEG in the lipidic implants [determined by the fittings shown in **a**] [the results obtained at pH 7.4 are reproduced from reference (1)].

pH 4.0. In the case of PEG-free implants, the pH had no effect on the resulting *in-vitro* release kinetics (Fig. 9a). Protein release leveled off at around 30% after 4 days, irrespective of the pH of the medium. In contrast, the pH was found to fundamentally affect the IFN-α release patterns from lipidic implants containing PEG. As shown in Fig. 9b–d, the lack of protein precipitation at pH 4.0 resulted in significantly accelerated protein release rates with a high burst effect. For example, at pH 4.0 implants loaded with 20% PEG exhibited a high initial release of 78.3% (± 0.43) protein released after 24 h and the release was already complete after only 4 days. In contrast, sustained IFN-α release was observed from the same type of implant in phosphate buffer pH 7.4. Importantly, not only the slope of the release curves, but also their shapes were affected by the pH of the release medium: all release rates monotonically decreased at pH 4.0, whereas at pH 7.4 they remained about constant during 7 days. This is an interesting observation, because it indicates differences in the underlying protein release mechanisms. To get deeper insight into the involved mass transport phenomena, an analytical solution of Fick's second law of diffusion considering radial as well as axial mass transfer in cylinders with constant diffusion coefficients (Eq. 2) was fitted to the experimentally determined release rates. This model takes into account the homogeneous distribution of the protein within the implants before exposure to the release media (at t=0), as well as the sink conditions maintained throughout the experiments. Importantly, the theory is based on the assumption that the initial concentration of the protein is below its solubility (monolithic solution). As it can be seen in Fig. 9, good agreement between theory and experiment was obtained in all cases at pH 4.0, irrespective of the initial PEG loading of the implant (R^2>0.99). Thus, protein release from the investigated implants is predominantly controlled by pure diffusion at pH 4.0 with constant diffusivities. In contrast, at pH 7.4 the presence of PEG within the lipidic systems resulted in systematic deviations between the applied mathematical theory and the experimentally measured protein release kinetics.

At pH 7.4 the addition of 10% PEG (or more) to a lipidic matrix allowed complete protein recovery due to the formation of completely interconnected pore networks (1). In contrast at pH 4.0 IFN-α release leveled off at 48% after 4 days. One explanation for this observation might by a pH dependence of the kinetics of pore creation. Since the formation of water-filled pores occurs upon the dissolution and leaching out of the incorporated hydrophilic excipients PEG and HP-β-CD the release rates of both were determined simultaneously with protein release. As it can be seen in Fig. 10a, the pH of the release medium did not affect the release of PEG from the tristearin-based implants. Exemplarily, the results obtained with devices (initially) containing 10% PEG are shown. The tendencies with 15 and 20% PEG-loaded implants were similar (data not shown). Fitting an adequate solution of Fick's law of diffusion (Eq. 2) to the experimentally determined PEG release kinetics, good agreement between theory and experiment was obtained in all cases (two examples are shown in Fig. 10a). This clearly indicates that PEG release was primarily controlled by pure diffusion with constant diffusivities, irrespective of the pH of the release medium. Based on these calculations, the apparent diffusion coefficients of PEG within the lipidic implants could be determined (Fig. 10b). The diffusivity of PEG significantly increased with increasing initial PEG loading, which can be attributed to increasing porosities of the implants (1). Interestingly, the pH of the phosphate buffer (pH 7.4 or 4.0) was only of minor importance for the mobility of PEG within the tristearin-based implants. Similar results were obtained when studying the pH dependence of the release of the co-lyophilisation agent HP-β-CD (data not shown). Thus, potential differences in the creation of water-filled pores due to different velocities of PEG leaching out of the

implants at different pH values of the bulk fluid can be excluded as reason for the observed significant effects of the pH on protein release. An alternative explanation for the incomplete release even from PEG-loaded matrices at pH 4.0 might be protein aggregation/denaturation within the matrix (10–12). At pH 7.4 the solubility of IFN-α is reduced in the presence of PEG, thus only low protein concentrations within the implant pores are generated. In contrast, at pH 4.0 the water imbedding and the subsequent dissolution of IFN-α results in highly concentrated protein solutions, which favors intermolecular interactions and, thus, increased aggregation (40).

Consequently, the presented sustained release matrix composition offers two mayor benefits via its *in-situ* precipitation mechanism. First the reversible precipitation of IFN-α in PEG containing lipidic implants facilitates the sustained protein release with nearly constant release rates and a low burst effect. Moreover, the precipitation also ensures low protein concentrations within the implant pores and therefore the tendency of protein aggregation is reduced.

CONCLUSIONS

In this work we have shown that the role of PEG as protein precipitant is crucial for the full understanding of IFN-α release from PEG-containing matrices. While IFN-α release from PEG-free matrices can be described well with an analytical solution of Ficks second law of diffusion considering constant diffusivities and instantaneous protein dissolution upon water penetration, systematic deviations between the applied theory and the experimentally determined protein release kinetics were observed when PEG was added. Our hypothesis to explain the phenomena by the reduced protein solubility and a reversible IFN-α precipitation during release in PEG-containing matrices was confirmed by two additional types of experiments: a "macropore release model" setup revealed that the dissolution of IFN-α in the presence of PEG is a limiting factor for protein release. Furthermore, our assumption was backed by the pH dependence of the IFN-α solubility in the presence of PEG that is reflected in the protein release kinetics.

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