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Microencapsulation of rh-erythropoietin, using biodegradable poly(D,L-lactide-co-glycolide): protein stability and the effects of stabilizing excipients

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Abstract

Parenteral delivery systems allowing controlled drug release over one month are of particular interest for proteins and peptides. We investigated the microencapsulation of recombinant human erythropoietin (EPO), a stimulating factor of red blood cell production, into poly(D,L-lactide-co-glycolide) (PLG) microspheres, using a water-in-oil-in-water (W/O/W) double emulsion technique. The integrity and stability of EPO during microencapsulation and storage was characterized. Effects of various excipients on in vitro release properties and formation of EPO aggregates were investigated. The formation of EPO aggregates in the W/O/W double emulsion technique was mainly influenced by the first homogenizing step, when preparing the water-in-oil (W/O) emulsion, whereas the subsequent processing steps, including drying, proved to be noncritical. A rotor/stator homogenizer generated ca. 5% covalently bound EPO aggregates, ultrasonication and vortexing slightly increased aggregate-formation, as demonstrated by size-exclusion chromatography and native-polyacrylamide gel electrophoresis (PAGE). Using excipients, such as hydroxypropyl-β-cyclodextrin, L-arginine, or bovine serum albumin (BSA), a distinct reduction of the formation of EPO aggregates could be achieved. The discontinuous in vitro release behavior from PLG microspheres was not significantly modified by these additives, influencing predominantly the initial drug release phase. During the in vitro release, an accumulation of EPO aggregates in the residual microparticles was detected, which could not be suppressed by excipients. An accelerated stability test demonstrated no change in drug content, release behavior and aggregate profile over 56 days at -20, 8°C or room temperature.

Keywords: Erythropoietin; Excipients; Microspheres; Parenteral depot-systems; Poly(D,L-lactide-co-glycolide); Protein stability

1. Introduction

Recombinant human erythropoietin (EPO), a 30.4 kDa glycoprotein consisting of 165 amino acids, is the main regulator and growth factor of red blood cell production [1]. EPO is clinically used for chronic therapy of renal anemia and related conditions [2]. EPO produced in chinese hamster ovary cells, contains a high proportion of carbohydrates (ca. 40%). Tetradentate oligosaccharides, including sialic acid as component and intact disulfide bonds are necessary for its biological activity [3]. The susceptibility of EPO to proteolysis in the gastro-intestinal tract and the rapid clearance from blood after parenteral administration argue for the use of a controlled delivery system to overcome these biopharmaceutical problems [4].

While microparticles from biodegradable polyesters have been successfully used to control the release of peptides over a period of 1 month [5,6], only few examples of therapeutically relevant proteins are found in the literature, e.g. interleukin-2 (IL-2) [7] and IFN-β [8]. Compared with peptides like leuprorelin [6],

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proteins have a substantially higher molecular weight and are more susceptible to chemical and physical degradation [9]. The low protein drug loading, the high molecular weight and the insolubility of proteins in matrices of PLG renders the control of a continuous protein release profile difficult, because pore diffusion and PLG erosion have to be carefully synchronized [10,11]. Attempts to increase the protein release rate via the pore-diffusion mechanism have included modifications of the microencapsulation process [12], or augmentation of the formation of porous channels by incorporating hydrophilic macromolecules, such as serum albumin [7].

Additionally, many proteins show a pronounced tendency to self-association, forming high molecular weight aggregates of non-covalent or covalent nature [13]. Protein aggregates must be avoided, because this change in the protein structure is frequently associated with a loss of biological activity and the risk of antibody formation after repeated parenteral applications. Moreover, protein release will be reduced by the higher molecular weights of these aggregates. EPO is quite sensitive to dimerization, and elevated temperatures, as well as redox-reactions, will ultimately lead to insoluble, covalently bound aggregates [14]. Aggregate formation may be induced in the microparticle preparation process [15,16] or during microsphere hydration when insufficient water is available to dissolve the protein [17,18].

We report here on an investigation of the microencapsulation of EPO into PLG microparticles using a water-in-oil-in-water (W/O/W) double emulsion technique. Particular emphasis was put on the characterization of the aggregates induced by the process steps and on EPO stability. We modified the process conditions and studied the addition of various protein stabilizing excipients, such as carbohydrates, polyols and amino acids, to minimize aggregate formation of EPO.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-co-glycolide) (PLG) with a monomer ratio of lactide to glycolide of 50:50 (M_w = 40.5 kDa, $M_w/M_n = 2.4$) was purchased from Boehringer Ingelheim. Polyvinyl alcohol (88% hydrolyzed, M_w = 130 kDa), PEG 1.5, 10 and 35 kDa and mannitol were obtained from Hoechst, Frankfurt. Bovine serum albumin (BSA, fraction V), guanidine-HCl, trehalose and citric acid were purchased from E. Merck, Darmstadt. 2-Hydroxypropyl-β-cyclodextrin (CD) was a gift from Roquette, Neu-Isenburg. L-arginine-HCl was obtained from Serva. Poly-L-arginine (PA) was purchased from Sigma. Pluronic F127 was obtained from BASF, Dextran 20 000 from Fluka and Dextran 40 000 (PL40VC) from Pfeifer and Langen. All the other reagents used were of analytical quality. The antibodies for the enzyme linked immuno-sorbent assay (ELISA) (antimouse IgG and anti-EPO antibody) and EPO conjugated peroxidase were obtained from Boehringer Mannheim.

2.2. Preparation of EPO microparticles

The microparticles were prepared by a double emulsion method previously reported [10] with some modifications. Briefly, PLG 50:50 (700 mg) was dissolved in 2.3 ml of dichloromethane (DCM). A concentrated EPO solution (3.5 mg) in 0.8 ml distilled water was added and the water-in-oil (W/O) emulsion generated using a high speed homogenizer Utraturrax T18-10 (Janke and Kunkel, Staufen, Germany) operating at 20 000 rpm for 2 × 30 s (RT). The W/O emulsion was immediately added to 300 ml of an aqueous PVA solution (0.1% w/w), further homgenized for 30 s using an Ultraturrax T 25 (8000 rpm) to form transiently a W/O/W emulsion. After stirring for 2–3 h with a mechanical stirrer, the microparticles were collected by filtration, washed with distilled water and lyophilized. The microspheres were stored at 4–8°C in a desiccator.

2.3. Determination of microparticle size

Approximately 10 mg microparticles were redispersed in 2–3 ml distilled water containing 0.1% Tween® 20 for several min using an ultrasonic bath (Branson) and an aliquot of the microsphere suspension was added into the small volume recirculation unit, operating at 60 ml/s. The microparticle size was determined by laser diffractometry using a Malvern Mastersizer X (Germany). Particle size is expressed as weighted mean of the volume distribution D [4,3].

2.4. Scanning electron microscopy

Microsphere morphology and surface characteristics were observed using a Hitachi S 510 scanning electron microscope (Hitachi, Tokyo). The samples were mounted on aluminum holders and sputter-coated three times for 2 min with gold in an argon atmosphere.

2.5. Quantitation of EPO in PLG microspheres

Microparticles (ca. 20 mg) were weighed and mixed with 0.3 ml DCM in an Eppendorf® cup (1.5 ml). After dissolution of the polymer 0.7 ml acetone were added, mixed for 2 min on a Eppendorf® shaker and centrifuged (5 min, 14 000 rpm, Eppendorf® 5415C). The supernatant was removed and 0.9 ml of a 3:1 acetone/methylene chloride mixture was added. This washing

procedure was repeated three times. After lyophilization the solid residue, containing EPO was dissolved in 1 ml 6 M guanidine-HCl in phosphate buffered saline (PBS). After centrifugation (5 min, 14 000 rpm) EPO concentration was determined spectro photometrically at 280 nm (Shimadzu UV-160) using a calibration curve. Each measurement was carried out in triplicate.

2.6. Size exclusion chromatography to quantitate EPO in the release buffer

EPO as well as aggregates were determined quantitatively using size exclusion chromatography, in analogy to [14]. After extraction from microspheres as described above, EPO was analysed using a TSK G3000 SWXL (Tosohaas) gel column (7.8 × 300 mm, 5 μm particle diameter). The high performance liquid chromatography (HPLC) equipment consisted of a high pressure pump (L-6000), an autosampler (AS 2000A) and a fluorescence detector (F-1000), operating at an excitation wavelength of 280 nm and an emission wavelength of 340 nm, all from Hitachi-Merck (Darmstadt, Germany). The flow rate was 0.7 ml/min using a mobile phase of phosphate buffer with 0.3 M NaCl. For quantitation, the Millenium software (Millipore–Waters) was used. The peak-areas of EPO were calibrated using standards of known concentrations. For estimating the molecular weight M_w, BSA (67 kDa), aldolase (158 kDa) and ferritin (440 kDa) were used as molecular weight standards.

2.7. SDS- and native-PAGE to determine EPO-aggregates in microspheres

Vertical sodium dodecyl sulphate and native polyacrylamide gel electrophoretic analysis (SDS-PAGE) of microencapsulated EPO was performed as detailed in [19]. Briefly, extracted EPO was dissolved in SDS-sample buffer and subjected to electrophoresis on a 12.5 or 15% native or SDS-polyacrylamide gel. Alternatively, EPO microparticles were dissolved in DMSO and the solution directly loaded in the sample wells. Proteins were visualized by Coomassie blue staining. For quantification of the protein bands, a densitometer (Desaga, Germany) was used. The optical densities were used to calculate relative ratios between EPO monomers and aggregates. The LMW kit (Pharmacia) was used to estimate the molecular weights.

2.8. In vitro release studies of EPO from PLG microspheres

Microparticles (40 mg) were placed into vials in 4 ml 10 mM PBS pH 7.4 (120 mM NaCl, 3 mM KCl, 0.01% Tween® 20). The vials were shaken on a rotating metal block at 37°C and 30 rpm. At selected time intervals, samples were collected by centrifugation (4000 rpm) and the EPO concentration in the supernatant was determined by SEC or ELISA. The in vitro release buffer was replaced completely by fresh buffer solution.

2.9. Enzyme linked immuno-sorbent assay for EPO

To determine the immunoreactivity of EPO released from PLG microspheres under in vitro conditions, a competitive ELISA was used [19]. Briefly, polystyrene microtiter plates were coated with a polyclonal anti-mouse IgG antibody, directed against the Fc part of IgG. After washing, a mouse anti-EPO antibodiy was added. Thereafter, a mixture of test solutions (standard and unknown samples) and EPO conjugated to peroxidase (EPO-POD) was serially diluted across the 96 well microtiter plate. After washing, bound enzyme conjugate was detected by adding a chromogen solution containing 2,2 - azino - di(3 - ethyl - benzthiazoline sulphonate) (ABTS, Boehringer Mannheim). The colour development was determined in a microplate reader at 405/490 nm. EPO concentrations were calculated using a calibration curve.

2.10. Stability testing of EPO microspheres

Approximately 100 mg of each formulation were weighed in 2 ml Eppendorf® vials for every sampling point and storage condition. At selected time intervals, samples were withdrawn and stored at −20°C prior to analysis by SDS-PAGE and SEC. For SDS-PAGE microparticles (10 mg) were weighed in 1.5 ml Eppendorf® vessels and 333 μl DMSO/DMF (30:70) were added to dissolve both EPO and the PLG. Of this solution, 30 μl, were loaded directly in the sample wells of a 15% SDS gel and developed as usual.

3. Results and discussion

Microencapsulation of EPO, using the W/O/W double emulsion process and commercially available biodegradable polyesters are of interest for parenteral delivery systems providing continuous plasma levels over 1 month. Continuous administration of EPO seems to offer advantages over pulsatile delivery in an experimental animal model, using implantable minipumps [20]. Microencapsulation of EPO using zein and PLG to obtain an orally active preparation has been recently described [21]. Zein is, however, not approved for parenteral administration. We, therefore, prepared EPO microspheres using PLG to investigate the feasibility of a parenteral delivery system.

The W/O/W double emulsion method was found to be suitable for the microencapsulation of EPO, yielding drug loading levels in the range 0.21–0.44% (w/w). The

Table 1
EPO microsphere characteristics

Batch	Additives	EPO content (%)	Aggregates[a] (%)	EC[b] (%)	Particle size[c] (μm)
1	—	0.32	5	62	23
2	—	0.36	3	71	25
3	—	0.34	5	65	24
4	10% CD	0.44 ± 0.01	<5	90	35
5	10% BSA	0.41 ± 0.04	$<1^2$	86	28
6	5% D40/1% PA	0.36 ± 0.02	3	70	31
7	4.3% PEO 1550	0.21 ± 0.05	15	43	25
8[d]	—	0.5	16^1	n.d.	27
9[e]	—	0.5	16^1	n.d.	31

[a] Total amount of aggregates determined by SDS-PAGE/[1]native PAGE (representative mean values)/[2]SDS-PAGE-Western blot.
[b] EC (encapsulation efficiency) = 100 × effective EPO loading/theoretical loading (%).
[c] Volume average particle size, D, [3,4] determined by laser diffractometry.
[d] Ultrasonication used for preparation of the W/O emulsion.
[e] Vortex mixer used for preparation of the W/O emulsion.

encapsulation efficiency, defined as the ratio of actual over theoretical EPO content of the microspheres, was found to be between 43 and 90%. The yield of isolated EPO microspheres was 60–80% for batch sizes in the range of 500–1000 mg. A scaling down of the W/O/W process was necessary to permit the investigation of this expensive protein. The weighted average of the particle volume distribution D [4,3] was found to be in the range of 23–31 μm, allowing subcutaneous or intramuscular injections. The results of the EPO microsphere characterization are summarized in Table 1, including batches containing excipients, which are thought to stabilize proteins. Scanning electron micrographs of these batches demonstrate regularly shaped spherical microparticles with smooth surfaces, as shown in Fig. 1.

The W/O/W double emulsion has been frequently used to encapsulate 'model' proteins, mainly BSA [10,12,23,24] or ovalbumin (OVA) [22]. With both proteins, no covalently bound aggregates were formed, as determined by SDS-PAGE for BSA [23,24] or OVA [22]. In contrast to these observations, we detected in EPO, extracted from PLG microspheres, the formation of higher M_w aggregates by SDS-PAGE in the range of 5–10% (w/w) of the encapsulated protein as shown in Table 1. Alonso et al. [25] noted the formation of aggregates in tetanus toxoid microspheres as a consequence of the lyophilisation procedure. Therefore, the manufacturing method, schematically illustrated in Fig. 2, was studied to identify those process steps, which induced the formation of covalent EPO aggregates. Fig. 3 summarizes the mass balance of monomeric EPO and the relative amount of covalent EPO aggregates as determined by SDS-PAGE at various stages of the microencapsulation process. The first process step,

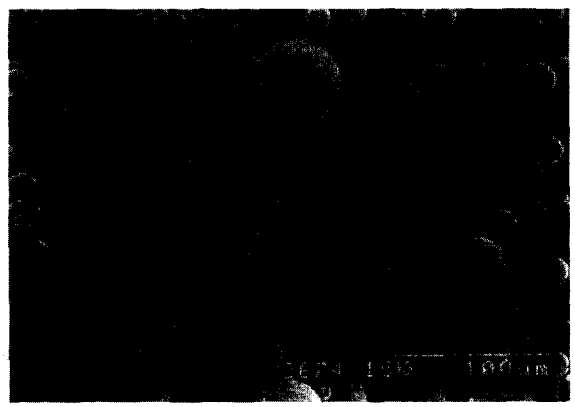

Fig. 1. Scanning electron micrograph of EPO microspheres with theoretical 0.5% drug loading (no additives).

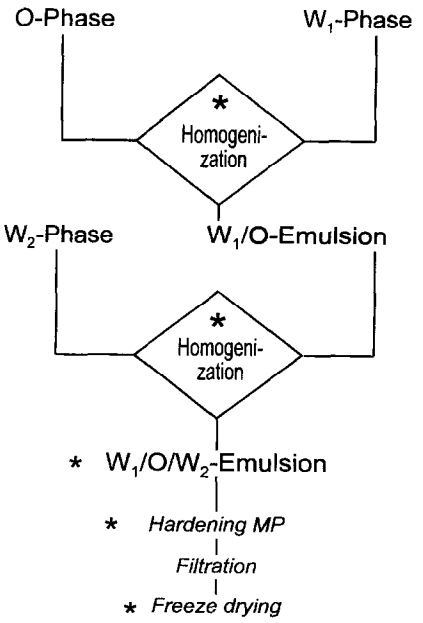

Fig. 2. Schematic diagram representing the preparation of EPO microspheres using the W/O/W double emulsion method. Critical process steps are indicated by *.

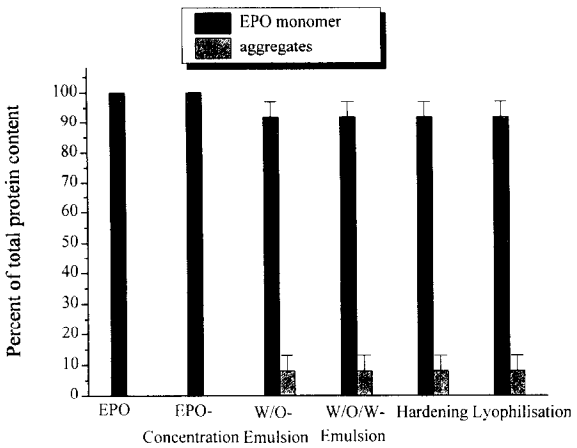

Fig. 3. EPO-monomer/aggregate ratio after every manufacturing step for EPO PLGA microparticles (without additives, 15% SDS gel, samples directly dissolved in DMSO).

namely the formation of the W/O emulsion was mainly responsible for EPO aggregation. All the following steps showed no further increase in the amount of aggregates. The stability of protein based pharmaceuticals has been reviewed recently [25], demonstrating several mechanisms for protein aggregation. Interactions of EPO with the interfaces between water and the lipophilic O-phase, or air/water/O-phase interactions may contribute to EPO aggregate formation due to unfolding and denaturation of the protein, similar to porcine growth hormone [26] or insulin [27]. The formation of covalently bound aggregates is thought to involve a reduction of the Cys7-Cys161 disulfide bridge, followed by a random intermolecular reoxidation [14].

Since the interface between air/water or air/water/organic phase is critically influenced by the droplet size of the W/O emulsion, we studied the effect of different homogenizers on the formation of EPO aggregates. Homogenizers, based on a rotor/stator design, have frequently been used for the W/O/W microencapsulation process [6,10,12,22,23]. We studied additionally ultrasound and vortex mixing for the preparation of the W/O emulsion. All subsequent steps were carried out as described in the standard preparation method. The EPO microspheres had similar characteristics concerning drug loading and particle size (Table 1). The ratio of EPO aggregates increased significantly when ultrasonication or vortex mixing was used for the preparation of the W/O emulsion, as shown in Fig. 4. Variation of the homogenizing conditions like shortening the homogenization time from 60 to 30 s did not affect the aggregate formation significantly. While the importance of the internal structure and drug distribution on the release properties of biodegradable microspheres has been recognized [6,10,12,28–30], the effect of homogenization on protein integrity has also to be considered. It appears, that EPO is more sensitive to aggregation processes, than model proteins such as OVA and BSA.

By optimizing the process conditions of the W/O/W technique, a reduction of the covalent EPO aggregate formation was possible. A complete suppression of aggregates could, however, not be achieved. We, therefore, studied the influence of various excipients, described in the literature as protein stabilizers [9,25]. In the absence of an unifying theory allowing predictions of protein stability after addition of various excipients, such as surfactants, carbohydrates, etc. it is necessary to determine experimentally the effect of these stabilizers on the specific protein under investigation. The excipients were added to the internal water phase in the concentration indicated in Table 2 and the microspheres were prepared in the usual way. The concentration of the stabilizer is calculated on a weight basis of total solids. The effect of the stabilizer and several binary mixtures on aggregate formation was estimated by SDS-PAGE.

In Fig. 5, a typical example of a 15% SDS-PAGE gel is shown. The influence of CD and PEG on the formation of aggregates is demonstrated in lanes 2–4, while lanes 5 and 6 shows EPO microparticles without stabilizers. Moreover, the initial release rate of EPO from these microspheres over 24 h was determined under in vitro conditions. A significant reduction of aggregates $< 1\%$, designated by two arrows, and an initial protein release rate $< 10\%$ within 24 h were our objectives. While poly(oxyethylene), abbreviated PEO, led to an increase in EPO aggregates, almost independent of the molecular weight, the initial drug release is in the desired range, probably due to a plasticizing effect. Addition of pluronic F127 and PEO generated deformed and relatively soft EPO microparticles. Carbohydrates, such as sucrose, trehalose or mannitol have been used alone or in combination with PEO to stabilize sensitive proteins during freeze-drying [31]. In contrast to lactate dehydrogenase, EPO does not benefit from these excipients. On the other hand, hydroxypropyl-β-cyclodextrin, abbreviated CD, caused a significant reduction of covalent aggregate formation during microparticle preparation. Similar effects were

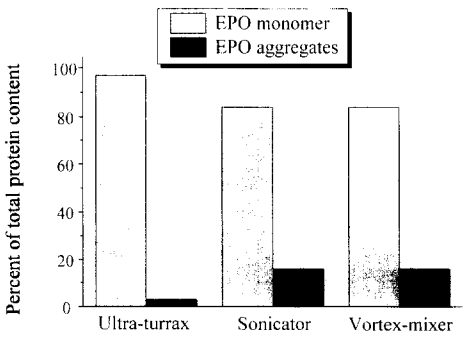

Fig. 4. Aggregate-formation in dependence of the homogenization unit. Results are based on native gel investigations.

Table 2
Effect of various protein stabilizing excipients on the formation of EPO aggregates and initial in vitro drug release

Stabilizer	Content (%)	Aggregation[b]	Initial EPO release[c] (%)
BSA	5	↓↓	40
BSA	10	↓↓	40
CD	5	↓↓	30
CD	10	↓↓	40
CD	15	↓↓	40
CD/PEO 10 000	5/5	↓	7
CD/PluF127	5/5	↓	8
CD/Trehalose	5/5	↓↓	30
PEO 10 000/PluF127	5/5	—	5
Trehalose	0.7	—	12
Trehalose	1.4	—	15
Trehalose	5–10	—	16
Mannitol	0.5	—	18
Mannitol	5	—	22
Dextran 20 000	10	—	n.d.
Dextran 40 000	5	↓	10
D40/Poly-(L)-arginine	5/1	↓	n.d.
Arginine	0.2	↓	15
Arginine	4.8	↓↓	18
PEO 1550	0.43	—	12
PEO 1550	4.3	↑	1.3
PEO 10 000	10	↑	0.6
PEO 35 000	10	↑	n.d.
Pluronic F127	10	↓	20
Citrate, pH 6.4 (mM)	30	—	n.d.

[a] Stabilizer content relative to total solids in weight percent.
[b] Effect of stabilizer on covalent EPO aggregates compared with microspheres without additives.
[c] Cumulative in vitro EPO release from stabilizer containing PLG microspheres during day 1.

also noted by Brewster et al. [32] for the prevention of aggregates in the case of somatotropin and IL-2. The exact mechanism by which CD stabilizes sensitive proteins is not known with certainty, but interactions of aromatic amino acids, e.g. phenylalanine with the hydrophobic cavity of CD seems to stabilize the protein conformation against denaturation [33]. Positive effects were also seen with BSA as well as, with L-arginine. A possible explanation for the stabilizing effects of these excipients could be due to ionic interactions with the EPO in the case of L-arginine or surface active properties of BSA, stabilizing EPO in the W/O emulsion. It should be noted, however, that the reduction of EPO aggregate formation by excipients was associated with increased initial drug release rates from EPO microspheres.

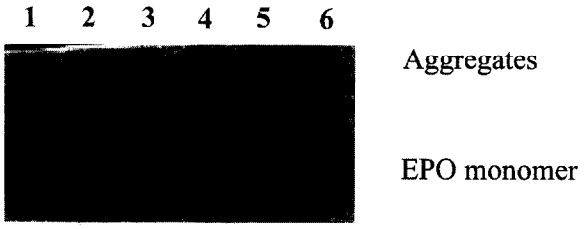

Fig. 5. SDS-PAGE protein bands extracted from PLGA microparticles with various additives: lane 1, EPO 10 μg (standard); lane 2, 10% CD; lane 3, 5% CD; lane 4, 5% PEO 1.550; lane 5 and 6, no additives.

To investigate the stabilizing effects of the most promising excipients in more detail, larger batches of EPO microspheres were produced, as described in Table 1 (batch 4–8). The most impressive reduction in covalent aggregates was noted for 10% CD and 10% BSA, whereas 5% PEO had negative consequences for EPO aggregates and encapsulation efficiency. The in vitro release of EPO from PLG microspheres containing stabilizing excipients yielded almost identical results for all batches tested. During the first day, between 5 and 40% of the encapsulated EPO is released into the surrounding aqueous medium. Thereafter, no further release could be detected up to 60 days. The stabilizers investigated had only an effect on the initial drug release phase, as shown in Fig. 6. Control experiments with EPO solution and EPO in the presence of placebo microspheres suggest, that neither EPO stability in the in vitro release medium, nor EPO adsorption to PLGA microspheres were responsible for this unexpected release behavior.

Therefore, a mass balance experiment was carried out determining the ratio of monomeric and aggregated EPO remaining in the PLGA microspheres as a function of time. As shown in Fig. 7, the amount of

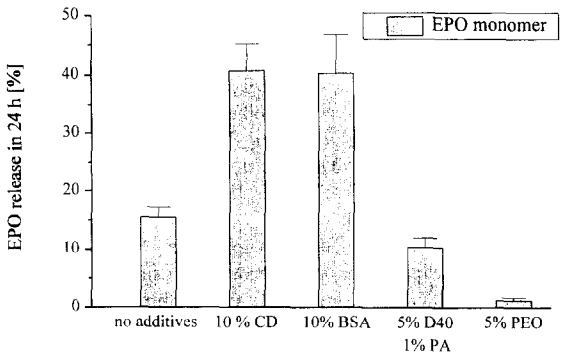

Fig. 6. Influence of additives on the initial EPO release (protein release within the first 24 h) of PLGA microparticles.

monomeric EPO available for passive diffusion decreases rapidly after 12 days, reaching 50% on day 32. These findings suggest, that EPO is undergoing a moisture-induced aggregation, similar to other proteins, such as insulin [27]. For IL-2, very low in vitro release rates were observed, even at a drug loading of 12% [7] indicating that aggregation may have affected the concentration gradient of EPO. Similar results were obtained for bovine insulin in PLG microspheres, where degradation of insulin caused by acidic conditions inside the microspheres, yielded only 1% release during 7 days under in vitro conditions [34]. An increased in vitro release of EPO during the polymer erosion phase (ca. day 15–25) was not observed. On the contrary, a distinct increase in the amount of EPO aggregates was observed in the period from day 12 to 20. Even after 32 days incubation, nearly 60% of EPO was not covalently aggregated. Due to analytical problems, associated with the extraction of EPO from PLG microspheres, no information could be obtained on the content of non-covalently bound EPO aggregates. Therefore, one possible explanation for the discontinuous in vitro release profile of EPO might be the formation of high molecular weight, water insoluble non-covalently bound EPO aggregates.

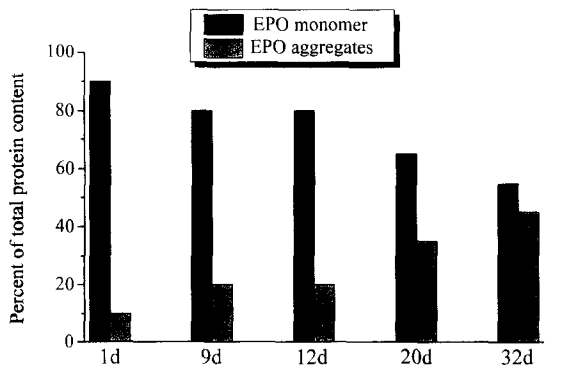

Fig. 7. Ratio of EPO monomer to covalent aggregates in EPO microspheres (without additives) as a function of on the in vitro release time.

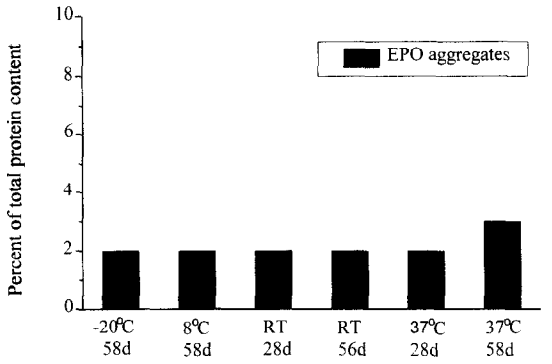

Fig. 8. The influence of storage conditions to the formation of EPO aggregates in EPO PLGA microparticles (theoretical drug loading: 0.5%, EPO microparticles containing 10% CD).

The formation of covalently bound EPO aggregates might also occur under storage conditions, unaffected by the hydration of EPO microspheres. We studied EPO microspheres, containing 10% CD in an accelerated stability test over a period of 56 days. As shown in Fig. 8, the amount of aggregates did not increase significantly over this period at −20, 8°C or room temperature. At 37°C a small increase from 2 to 3% was noted, indicating that the formation of EPO-aggregates at 37°C under in vitro release conditions was not a consequence of the elevated temperature, but was rather caused by other factors, such as moisture induced aggregation and/or pH induced aggregation as discussed above. The shelf-life of EPO microspheres, containing 10% (w/w) of CD as stabilizer, seems not to be prohibitive for further investigations.

4. Conclusions

EPO microspheres can be manufactured in the desired size-range of 20–50 μm using a W/O/W technique and commercially available PLG. The homogenization of the W/O emulsion seems to be critical with respect to formation of covalently bound aggregates. The amount of aggregates is dependent on the homogenization method and seems to be less pronounced with high speed homogenizers, compared with ultrasonication or vortex mixing. By adding stabilizing excipients, such as CD, BSA or L-arginine, EPO microspheres can be obtained with a very low content of covalently bound aggregates. The yield and encapsulation efficiency is not compromised. Unfortunately, the in vitro release profile of EPO from PLG microspheres is characterized by an initial drug burst in the range of 15–40% within the first 24 h of the in vitro release experiment. Subsequently, very little EPO is released, although 60% of EPO can be extracted from hydrated PLGA microspheres after 32 days in monomeric form. Moisture and/or pH induced aggregation of EPO might be re-

sponsible for the formation of non-covalently bound (physical) high M_w aggregates. This aggregation phenomenon cannot be eliminated with stabilizers, probably due to their rapid diffusion from the microspheres.

Our study highlights the fact, that the microencapsulation of therapeutically relevant proteins, such as EPO is more complex than previously thought. Biodegradable polymers with intrinsic protein stabilizing and improved diffusion properties would be advantageous to allow the formulation of parenteral protein delivery systems based on biodegradable microspheres with a controlled release profile over 1 month.

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