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European Journal of Pharmaceutics and Biopharmaceutics 67 (2007) 589–596
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Effect of excipients on the encapsulation efficiency and release of human growth hormone from dextran microspheres

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Received 29 December 2006; accepted in revised form 12 April 2007
Available online 4 May 2007

Abstract

The possibility was investigated to modulate the encapsulation efficiency and release of human growth hormone (hGH) from hydroxyl ethyl methacrylated dextran (dex-HEMA) hydrogel microspheres by using excipients. Microspheres were prepared by polymerization of dex-HEMA in an aqueous two-phase system of this polymer and PEG with or without excipients (Tween 80, pluronic F68, sucrose, NaCl, urea or methionine). High hGH encapsulation efficiencies (50–70%) were obtained for microspheres prepared without excipients and with Tween 80, NaCl or methionine. Substantially lower encapsulation efficiencies (27% and 19%, respectively) were obtained for microspheres prepared in the presence of sucrose and urea, which was attributed to the more favoured partitioning of hGH over the PEG-phase due to higher hydrophobicity of the (partly) denatured hGH. Likely, differences in precipitate size of the encapsulated hGH resulted in different release profiles between microspheres prepared without excipients (biphasic release: 2 days delay time followed by 6 days release) and the release profile for microspheres prepared with Tween 80, pluronic F68, sucrose, NaCl and urea (release over a period of 6–8 days (without a delay time)). Microspheres prepared with methionine showed a concentration-dependent delay time varying from 0 to 2 days followed by almost zero-order release over 6 days, attributed to the effect of methionine on the polymerization of dex-HEMA. Especially, Tween 80 and methionine are attractive excipients since hGH was encapsulated in high yield (60–70%) and the protein was released from the microspheres mainly in its monomeric form without a delay time and with an almost zero-order release over 6–8 days.

Keywords: dex-HEMA microspheres; hGH; Excipients; Hydrogel; Protein release; Microspheres

1. Introduction

Due to the enormous advances in biotechnology many therapeutic proteins have become available. However, generally speaking (potential) therapeutic proteins are rapidly eliminated *in vivo* and have a poor physical and chemical stability. Therefore, improved methods are needed to ensure that proteins access their target at the right concentration and for a prolonged period of time. Consequently, considerable effort has been put in the development of polymer based biodegradable systems for the controlled release of proteins [1,2]. It is one of the primary considerations in the development process of protein formulations to maintain the concentration of the protein within the therapeutic window for a time period sufficient to achieve the desired therapeutic effect. Therefore, it is important to control the release kinetics of delivery system with a wide applicability.

Microspheres based on crosslinked dextran (dex-HEMA= hydroxylethyl methacrylate derivatized dextran) are attractive systems for the controlled release of proteins [3,4]. The formation of these microspheres is based on

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doi:10.1016/j.ejpb.2007.04.011

phase-separation between aqueous solutions of poly(ethylene glycol) (as the continuous phase) and dex-HEMA (as the discontinuous phase). Upon addition of an initiator system (KPS = potassium peroxodisulfate and TEMED = *N*,*N*,*N*′,*N*′-tetramethylethylenediamine) to the water-in-water emulsion, the dextran-bound methacrylate groups are polymerized which ultimately results in the formation of microspheres consisting of crosslinked dextran. It was shown that dex-HEMA microspheres are biodegradable and have a good biocompatibility [5]. The release is controlled by degradation of the matrix and diffusion of the protein through the matrix [4,6]. Under physiological conditions (pH 7 and 37 °C), hydrolysis of the carbonate ester linking the dextran to pHEMA results in a reduction of crosslink density and an increasing mesh size of the network over time. When the pore size in the matrix is larger than the hydrodynamic diameter of the protein, the protein will be released. Previous studies have shown that the release can be tailored by varying parameters such as water content and degree of methacrylate substitution (DS = number of HEMA groups per 100 glucose units) of the microspheres and the physical state of the protein [4,6].

In this study, the possibility was investigated to modulate the encapsulation efficiency and the release kinetics of a model protein (human growth hormone, hGH) from dex-HEMA microspheres using excipients (Tween 80, pluronic F68, sucrose, NaCl, urea and methionine). These excipients were dissolved in the aqueous two phase system prior to initiation of the polymerization. Sucrose and NaCl are known precipitating agents [7–10] and likely induce the precipitation or aggregation of the protein in the PEG and dex-HEMA phase, which in turn will likely affect both the encapsulation efficiency and the release kinetics. Tween 80 and pluronic F68 are known solubilizers of hGH and prevent its aggregation [12–15]. Urea is also often used to dissolve protein aggregates or proteins from inclusion bodies [15–17]. A previous study has shown that methionine residues in proteins can be oxidized by potassium peroxidisulfate (KPS) [18]. Therefore, methionine is added as anti-oxidant. However, as a result of this reaction, the KPS concentration available for initiation of the polymerization of dex-HEMA is reduced. It was shown by Chung et al. that a reduced KPS concentration results in an increased mesh size of the hydrogel network [19]. Therefore, it was anticipated that the release of hGH could be affected by methionine. Finally, the effect of the KPS concentration on the encapsulation efficiency and release of hGH was also established.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol) (PEG) 10 kg/mol and potassium peroxodisulfate (KPS) were obtained from Merck, Darmstadt, Germany. Dextran 40 kg/mol, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), pluronic F68, urea, sucrose, Tween 80, methionine and NaCl were purchased from Fluka (Buchs, Switzerland). Hydroxyethyl methacrylate derivatized dextran (dex-HEMA) with a degree of HEMA substitution 16 (DS 16; 16 HEMA groups per 100 dextran monomer units) was obtained from Polymer Service Centre Groningen (Groningen, the Netherlands) and synthesised and characterized according to Van Dijk-Wolthuis et al. [20]. Modified recombinant human growth hormone 190 (rhGH-190, Somategen®) as an aqueous formulation (concentration 3.2 mg/ml in a 7 mM phosphate buffer, pH 7, containing 10 mM glycine/mannitol) was kindly donated by Biotechna Sicor Inc. (Irvine, CA, USA).

2.2. Methods

2.2.1. Microsphere preparation

The different batches of dextran microspheres were prepared by the water-in-water emulsion technique as described elsewhere [3]. Briefly, 3.25 g of a 40% w/w PEG-solution in 25 mM phosphate buffer, pH 7.0, was transferred into a 15 ml tube. Then, 1.69 g of a 3.6% w/w dex-HEMA solution in 25 mM phosphate buffer, pH 7.0, containing 0.3% w/w hGH was added. The mixture was vortexed for 1 min and subsequently, 100 μl of a TEMED solution (20% (v/v), pH neutralized with 4 M hydrochloric acid) was added, followed by vortexing for 20 s. Then, 180 μl KPS (concentration 50 mg/ml) was added and the emulsion was shaken gently. The resulting emulsion was allowed to polymerize for 1 h at room temperature. The polymerized microspheres were washed 5 times with 5 ml of 25 mM phosphate buffer, pH 7.0. Microspheres were also prepared in the presence of excipients by dissolving appropriate amounts in the buffer or protein solution used for the preparation of the dex-HEMA and PEG solutions (Table 1).

The microspheres were investigated by optical microscopy (Microscope with D12 digital camera, Olympus Optical Co. Shibuya, Tokyo, Japan) and their average volumetric particle diameter and size distributions were measured with a particle size analyzer (Malvern Mastersizer 2000). The water content and methacrylate conversion of the microspheres were determined according to the methods as previously described by Stenekes et al. [21,22].

2.2.2. In vitro release of hGH

Directly after washing, the microspheres were resuspended in 5 ml of 25 mM phosphate buffer, pH 7.4, and incubated in a water bath at 37 °C. Periodically, the microsphere suspensions were centrifuged for 7 min at 3200*g*, 3 ml supernatant was removed and replaced by 3 ml of 25 mM phosphate buffer, pH 7.4. The hGH concentration and the percentage monomer in the samples were determined with size exclusion chromatography (SEC) as described in Section 2.2.5. The amount of protein, which was left in the microspheres after 12 days, was determined by nitrogen analysis (see Section 2.2.6). The amount of protein entrapped in the microspheres was calculated by

Table 1
Characteristics of hGH loaded microspheres

Formulations	Particle size (μm)	Water content (%)	MA conversion (%)	Encapsulation efficiency (%)	Recovery (%)	Fraction protein released as monomer (%)
KPS[a]						
−4.4 mmol/l (=Standard formulation)	10	62	93	58	88	79
−1.6 mmol/l	ND	57	88	64	89	83
Methionine						
−0.1%	9	63	94	59	89	81
−0.5%	ND	59	91	68	91	81
−1%	9	61	86	63	94	84
Tween 80 0.1%	12	61	95	58	91	84
pluronic F68 0.5%	14	54	91	47	91	73
Sucrose 25%	14	53	94	27	88	60
Urea 8 M	10	60	96	19	91	45
2 M NaCl	10	63	95	56	92	74

Note. MA = methylacrylate, ND = not determined.
For particle size, water content, MA conversion the values are the average of 3 independent measurements that deviated less than 5%. For encapsulation efficiency, total release, percentage monomer, the values are the average of 2 independent measurements that deviated less than 5%.
[a] The KPS concentrations correspond to the KPS concentrations in the dex-HEMA phase prior to emulsification [22].

adding up the amount of protein released after 12 days and the amount of protein that remained in the microspheres after 12 days. The encapsulation efficiency was obtained from the amount of protein entrapped in the microspheres divided by the amount of protein added for preparation of the microspheres. For all release curves reported, the amount of protein released after 12 days has been normalized to the released amount of protein to allow comparison of release profiles.

2.2.3. Size exclusion chromatography

The HPLC system consisted of a HPLC pump series 200, a vacuum degasser series 200, an autosampler series 200, a column LC 101 oven, and a UV detector series 200 (all Perkin-Elmer Instruments, Norwalk, USA), a thermostated (35 °C) Thosohaas TSKgel G300SWXL column and a Thosohaas TSKgel SWXL guard column (Montgomeryville, PA, USA) and a UV detector series 200 (Perkin-Elmer Instruments, Norwalk, USA). The flow rate was 0.7 ml/min and an aqueous solution containing phosphate buffer (64 mM, pH 7.0) and 3% (v/v) isopropanol was used as the mobile phase. The chromatograms were recorded and analyzed with the data acquisition system Totalchrom (Perkin-Elmer Instruments, Norwalk, USA).

2.2.4. Nitrogen analysis

To determine the amount of protein that was left in the microspheres after a release period of 12 days, nitrogen analyses were done by pyro-chemiluminescent nitrogen assay using a nitrogen analyzer. Placebo microspheres were used to substract background signal. The nitrogen analyzer consisted of an autosampler (ELS 2100) and the samples were injected into a nickel boat, which was transported into the furnace tube TN 30000, and a cold trap TX/TS module and a UV detector (all thermo Euroglass Corp., Delft, Holland). The injected volume was 500 μl. The argon flow was set at 340 ml/min, the oxygen flow was set at 15 ml/min and the ozonator at 110 ml/min. The temperature of furnace 1 and 2 was set at 1000 °C, while the temperature of the NOx scrubber was set at 320 °C. A calibration curve was obtained by injecting was 500 μl of 0.1–10 μg/ml glycine. The hGH-190 concentration was calculated by assuming that this protein contains 16.7% w/w nitrogen [23]. The emission of the NO_2-radical produced by reaction of NO (formed after pyrolysis of the samples) with ozone was measured and analyzed with the data acquisition system of Thermo Euroglass DIN 38409 (Thermo Euroglass Corp., Delft, Holland).

2.2.5. Rheological properties of macroscopic hydrogels prepared in the presence of excipients

dex-HEMA macrogels were prepared in the presence of the different excipients and their rheological properties were measured with dynamic mechanical analysis (DMA). Hydrogels were prepared by dissolving 450 mg dex-HEMA (DS 16) in 840 μl of the selected solution, resulting in a concentration of 0.1% Tween 80, 0.5% pluronic F68 or 25% sucrose, 5.7 M urea or 2 M NaCl, respectively, in the macrogels. Macrogels were also prepared with different concentrations of KPS (4.4 mmol/l, 1.6 mmol/l). After dissolving dex-HEMA the solutions were vigorously vortexed and centrifuged for 5 min at 13,000*g* to remove air bubbles from the solution. Then, 75μl of a 7.6% (v/v) TEMED (adjusted to pH 7.4 with 4 N HCl) solution was added. The solutions were cooled to 4 °C to slow down the polymerization and to increase the handling time during hydrogel preparation. A KPS solution (135 μl, 14.4 mg/ml) was mixed with the dex-HEMA solution, which was subsequently quickly transferred into a mould and allowed to polymerize for 1 h at room temperature to obtain hydrogel cylinders with a diameter of about

5 mm and a height of 6 mm. The mould was precoated with a PEG solution to facilitate removal of the gels.

The rheological properties of the obtained hydrogels were determined by dynamic mechanical analysis (DMA) as described previously [24]. Briefly, the DMA measurements were carried out with a DMA 2920 Dynamic Mechanical Analyzer (TA Instruments, New Castle, England) equipped with a compression clamp (upper plate 6 mm, lower plate 45 mm) installed with sandpaper glued on both plates. The experiments were performed by imposing various static forces between 0.1 and 8.0 N, depending on the gel, with amplitude of 10 mm and a frequency of 1 Hz. The modulus was determined for each static force. The compression modulus was obtained by plotting the modulus versus the static force and extrapolation to a static force of zero. For each formulation, at least three gels were measured. The experiments were performed at room temperature.

2.2.6. Precipitation of hGH in dex-HEMA solution

Seven ml of the stock solution of hGH was diluted with 14 ml of 25 mM phosphate buffer, pH 7.0. This protein solution was concentrated over a Centricon® filter YM-10 with cut-off 10 kDa (Millipore, Bedford, USA) by centrifugation for 30 min at 4600*g*. The concentration of the hGH solution was determined with SEC (Section 2.2.5). The concentrated hGH solution was split into two samples of 500 µl. To one of these samples 0.2 g dex-HEMA was added to obtain a 30% w/w dex-HEMA (w/w) solution. To the other sample, first NaCl (0.06 g) was added to obtain a concentration of 2 M. Thereafter, dex-HEMA (0.2 g) was added to obtain a 30% w/w dex-HEMA (w/w) solution. After incubation of 1 h at room temperature, the samples were centrifuged (3200*g* for 30 min). The supernatant was removed, diluted 50 times with 25 mM phosphate buffer, pH 7.0, and the concentration of protein was determined with SEC (see Section 2.2.5). The loss of protein by precipitation was calculated by dividing the amount of protein in the supernatant by the added amount of protein. The precipitate collected in the pellet was investigated with a light microscope.

3. Results and discussion

3.1. Microsphere formation

Table 1 summarizes the characteristics of the hGH loaded microspheres prepared with the various excipients. The volume mean diameter of microspheres ranged between 9 and 14 µm microspheres. The excipients had only marginal effects on the water content and methacrylate conversion of the microspheres (Table 1). The water content of the microspheres ranged from 53% to 63%, whereas the methacrylate conversion ranged from 86% to 96%, which is in good agreement with data reported previously [22]. For microspheres prepared with 1% methionine or 1.6 mmol/l KPS (note that for the standard formulation 4.4 mmol/l KPS was used), slightly lower methacrylate conversions were found (86% and 88%, respectively).

The compression moduli of the macroscopic hydrogels prepared with the different excipients are presented in Fig. 1. This figure shows that the moduli of the hydrogels prepared with NaCl, sucrose, pluronic F68, Tween 80 and urea were the same as the compression modulus of hydrogels of the standard formulation. This indicates that the presence of these excipients during polymerization did not affect the network structure. For hydrogels prepared with methionine, added as a protein anti-oxidant [18], a concentration-dependent decrease in elastic modulus was found ($P < 0.05$). Since KPS oxidizes methionine, its concentration for initiation of the polymerization of the HEMA groups decreases. Fig. 1 also shows that the compression modulus of hydrogels prepared with 1.6 mmol/ml is lower than the compression modulus of the standard formulation prepared with 4.4 mmol/l KPS. Thus, the lower modulus found for gels prepared in the presence of methionine can be very well explained by a lower KPS concentration available for initiation of the polymerization of the HEMA groups and in line with the drop in methacrylate conversion (1% methionine; Table 1). In a previous study, it was demonstrated that the compression moduli of hydrogels and microspheres correspond very well [25]. Therefore, the effects of excipients on the macrogels are supposed to be representative for the effects on the microspheres.

3.2. Precipitation of hGH

HGH readily precipitated when 30% (w/w) dex-HEMA was added to a solution of 21 mg/ml hGH in 25 mM, pH 7.4, phosphate buffer. Centrifugation of the samples and determination of the amount of hGH in the supernatant revealed that around 50% of the hGH was precipitated.

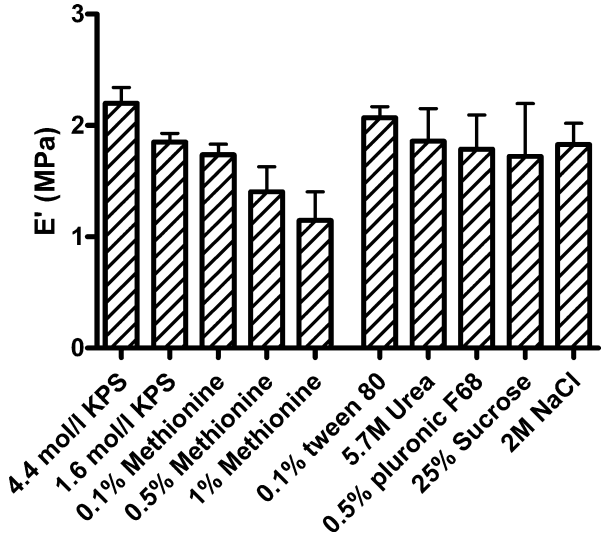

Fig. 1. Compression moduli (E′) of macroscopic hydrogels prepared with different excipients, measured by DMA (average ± s.d., $n = 5$).

From the encapsulation efficiency of hGH and the water content of the microspheres (Table 1) it is calculated that the dex-HEMA and protein concentration in the dex-HEMA phase during microspheres preparation were much higher (40% w/w and 52 mg/ml, respectively) than the protein and dex-HEMA concentration tested in this precipitation experiment. It is known that protein precipitation increases with an increasing protein concentration or an increasing concentration of precipitant [11,26]. Therefore, it is very likely that in the emulsified dex-HEMA droplets hGH precipitates are formed and that after polymerization, these precipitates are encapsulated in the microspheres.

The effect of NaCl on the precipitation of hGH in a 30% w/w dex-HEMA solution was also investigated. First a solution of hGH (21 mg/ml) in 2 M NaCl was prepared. In this solution no visible precipitation of hGH was observed. However, hGH readily precipitated when dex-HEMA (30% w/w) was dissolved in this solution; only 30% hGH of the initial amount of hGH was recovered in the supernatant, indicating that 70% was precipitated. Light microscopy analysis shows that the precipitates formed in the presence of NaCl (~1 μm) were about 10 times smaller than those formed in absence of NaCl (~10 μm) (Fig. 2). In general, the presence of NaCl stimulates preferential dehydration of proteins. Na^+ and Cl^--ions are excluded from the protein surface and therefore encourage the reduction of solvent exposed area [7–10]. Dextran is able to reversibly precipitate proteins in aqueous solution [26]. Its mechanism is explained by the excluded volume theory [11,27,28]. In principle, at high concentrations of dex-HEMA or NaCl, a further reduction of the exposed area is favoured by increasing the precipitate size. However, in situations of supersaturation (such as in the presence of both dex-HEMA and NaCl) precipitation by nucleation is favoured over precipitate growth processes, which will result in smaller particles [29]. This implies that after polymerization, the microspheres prepared with NaCl contain smaller precipitates than the microspheres prepared without NaCl.

3.3. Encapsulation efficiency

Table 1 shows that the encapsulation efficiency of hGH in the microspheres of the standard formulation, and in the microspheres prepared with Tween 80, pluronic F68, NaCl or methionine, ranged from 47% to 68%; the encapsulation efficiency of hGH in microspheres prepared with sucrose and urea was substantially lower (27% and 19%, respectively). The encapsulation efficiency of hGH depends on the amount of hGH in the dex-HEMA phase prior to polymerization which in turn is determined by the hGH partitioning over the dex-HEMA and the PEG phase. Partitioning of a dissolved protein in an aqueous PEG/dextran two-phase system depends on its net charge, its hydrophobicity, its solubility in both phases as well as on the type and concentration of ions present in the system [30–34]. In

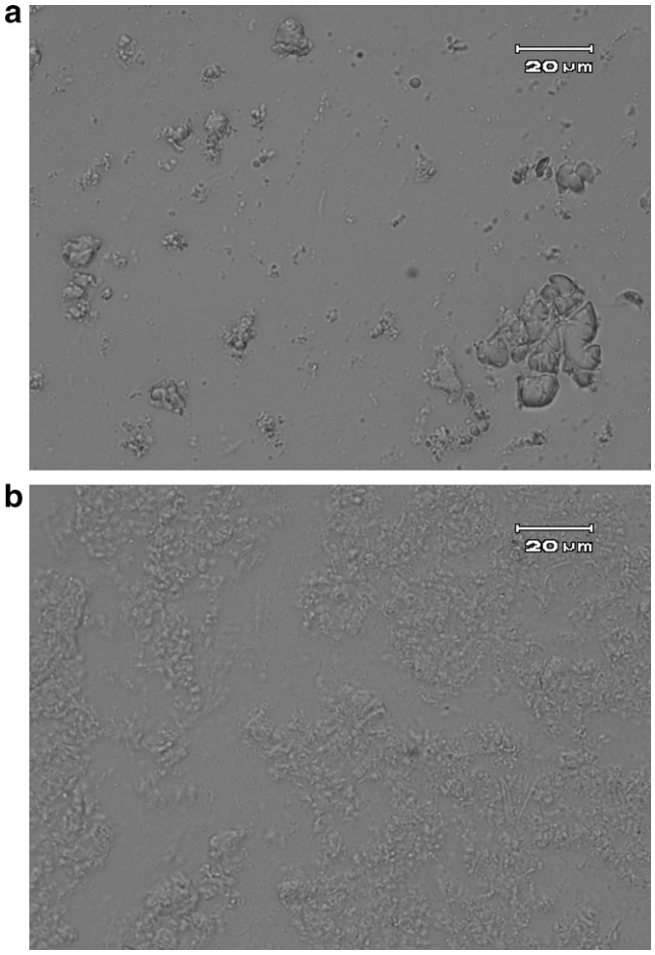

Fig. 2. (a) Microscopic image of hGH precipitate obtained in a 30% (w/w) dex-HEMA solution (magnification: 40×). (b) Microscopic image of hGH precipitate obtained in a 30% (w/w) dex-HEMA solution also containing 2 M NaCl (magnification: 40×).

Section 3.2 it was demonstrated that hGH precipitates in the dex-HEMA phase and consequently, the protein encapsulation efficiency is also dependent on the amount of protein that precipitates in the dex-HEMA phase. As the encapsulation efficiency of hGH in microspheres prepared with Tween 80 (58%), pluronic F68 (47%), NaCl (56%) or methionine (59–68%) is similar to the encapsulation efficiency of hGH in the microspheres from the standard formulation (58%), apparently, these excipients do not affect the protein solubility and therefore its partitioning.

Denaturation of the protein results in exposure of the relatively hydrophobic core of the protein to the solvent. In general, an increased hydrophobicity of proteins results in an increased partitioning over the PEG phase [30,34], and consequently in a decreased concentration in the dex-HEMA phase, which explains the observed lower encapsulation efficiency of hGH in dex-HEMA microspheres prepared with urea as excipient.

The reason for the low encapsulation efficiency of hGH in dex-HEMA microspheres prepared in the presence of sucrose is unclear. One might speculate that sucrose induces conformational changes of hGH and as a consequence, the

hydrophobicity of hGH changes resulting in a lower encapsulation efficiency.

3.4. Release of hGH from dex-HEMA microspheres

In Fig. 3, the normalized release curves of hGH from dex-HEMA microspheres prepared with and without excipients are shown. Basically, two different types of release curves are observed. In the first type of release profiles, hGH was released over a period of 6 days (for microspheres prepared with NaCl, sucrose, pluronic F68, Tween 80) or 8 days (microspheres prepared with urea). There was no burst release observed, except for microspheres prepared with pluronic F68, which showed a burst of around 15% of the loaded dose. Microspheres prepared with the standard method showed a different release pattern. During the first two days ('delay time') hardly any hGH was released and thereafter, hGH was released with almost zero-order release during the following 6 days. For all formulations tested, the protein recovery (total amount of protein released after 12 days) was 90% (Table 1).

Previous studies have shown that delay time in the release of proteins from dex-HEMA microspheres increases with a decreasing mesh size of the microspheres matrix, lower protein loading or by the presence of clusters of proteins in the microspheres [4,6]. Table 1 shows that microspheres prepared with Tween 80, pluronic F68, NaCl, sucrose and urea have the same characteristics in terms of equilibrium water content and methacrylate conversion as the microspheres of the standard formulation. Moreover, Fig. 1 demonstrates that the compression modulus of the hydrogels is not affected by the presence of these excipients during polymerization. Based on previous work by Stenekes et al. [25] it can be concluded that the different microspheres have the same mesh sizes. Moreover, the microspheres prepared with and without excipients have around the same encapsulation efficiency (50–70%) and recovery (90%), except for microspheres prepared with urea or sucrose, and hGH is mainly released as monomer (70–80%, Table 1). Consequently, the observed differences in release curves might be attributed to the presence of protein clusters in the microspheres. It is indeed shown that hGH precipitates in dex-HEMA (Section 3.2) and that likely these aggregates are entrapped in the microspheres after polymerization. The results in Section 3.2 also suggest that in microspheres prepared in the presence of NaCl smaller precipitates of hGH were encapsulated than in microspheres prepared in absence of excipients. In a previous study, it was indeed found that with increasing protein-cluster size, the release delay time was prolonged [6] which was explained by the restricted mobility of proteins inside a cluster. Nevertheless, the effects of cluster size on the release delay time were small, since in this model it was assumed that clusters of proteins consist of a group of monomeric proteins that were neither chemically nor physically linked and of which the protein molecules can move independently from each other once a sufficient number of crosslinks were hydrolyzed. In the present study it was shown that precipitates were encapsulated in the microspheres. This implies that once sufficient crosslinks are hydrolyzed to release a protein molecule, first the precipitate needs to dissolve before the dissolved protein molecules can be released. Likely, protein dissolution is a slow process in a matrix with a high concentration of dextran, and consequently, the delay time is increased due to the slow dissolution of the protein precipitate. To further explain this, a schematic presentation of the release of proteins from dex-HEMA microspheres containing large and small precipitates is given in Fig. 4. In Fig. 4a the release of protein from microspheres in which large precipitates are entrapped is indicated. At a certain time t, a certain number of crosslinks are hydrolyzed and there is an open network connection between the protein precipitate and

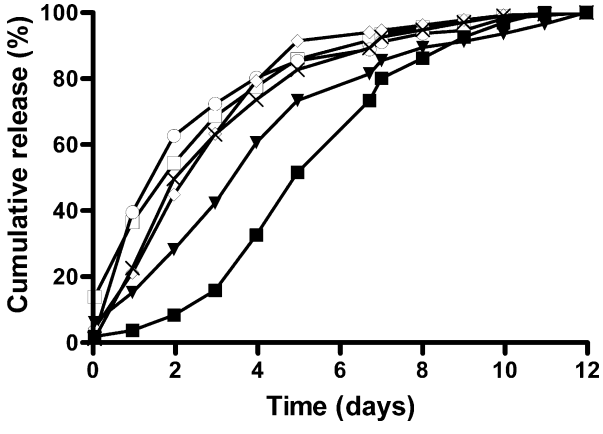

Fig. 3. Cumulative release of hGH from microspheres of the standard formulation (prepared with KPS 4.4 mmol/l) (■), urea 8 M (▼), Tween 80 0.1% (×), NaCl 2 M (◇), pluronic F68 0.5% (□), sucrose 25% (○). The values are the average of two independent measurements and deviated less than 5%.

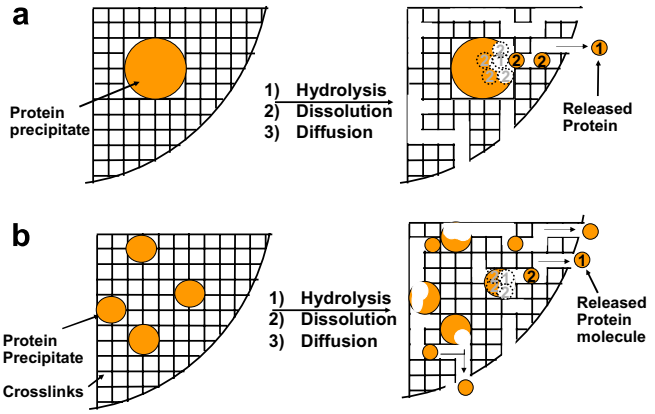

Fig. 4. Schematic representation of the release of protein molecules from a microsphere containing large protein precipitates (a), and small protein precipitates (b). (Note that each compartment (□) represents a number of crosslinks that need to be hydrolyzed before a protein can enter this compartment [6]. The numbers indicate the (original) position of the protein molecule inside the precipitate.)

the surface of the microsphere. However, to release all protein molecules from the precipitate, first the protein at position 1 needs to be dissolved and released, then protein 2, etc. In Fig. 4b the release of protein from microspheres in which small precipitates are entrapped is shown. At time t, the same number of crosslinks is hydrolyzed as in Fig. 4a, however, there are more open network connections between protein precipitates and the surface of the microsphere. Moreover, as the surface-to-volume ratio of a small precipitate is higher than that of a large precipitate and as a result more protein molecules are released in a certain time frame from microspheres with a small precipitate than from microspheres containing large precipitates.

The slightly longer release time observed for microspheres prepared in the presence of urea as compared to the other microspheres (Fig. 3) is explained by the higher fraction dimer and multimers in the released amount of protein (73% as dimer or multimer; Table 1), of which the released was delayed in time (data not shown). Removal of urea (due to washing of the microspheres) can result in incomplete refolding hGH or in the formation of dimers and multimers. Due to their larger size and lower diffusivity protein dimers (and multimers) release slower from the dex-HEMA matrices than monomeric protein molecules which explains the longer release time of hGH from the microspheres prepared with urea as excipient. The reason for larger fraction of dimers obtained in the release samples of microspheres prepared with sucrose is unclear. As indicated in Section 3.4, one might speculate that sucrose induces conformational changes of hGH and as a consequence, after release more dimers are formed in the release buffer, after release from the microspheres.

In Figs. 5a and b the release profiles of hGH from dex-HEMA microspheres prepared with different methionine and KPS concentrations are shown. All microspheres except the ones prepared with 1% methionine showed a biphasic release profile of hGH of which the delay time depended on the KPS or the methionine concentration. The release profiles of microspheres prepared under standard conditions or with 0.1% methionine showed a delay time of 2 days. A delay time of one day was found for microspheres prepared with 1.6 mol/l KPS and 0.5% methionine (Fig. 5a and b). For microspheres prepared with 1% methionine, no delay time was observed. Table 1 indicates that the methacrylate conversion and water content of microspheres prepared with and without methionine were similar whereas Fig. 1 shows that the compression modulus of dex-HEMA gels decreases with an increasing methionine concentration. A previous study has shown that a lower compression modulus indicates a lower crosslink density and therefore a larger mesh size [25]. This explains the shorter delay time for microspheres prepared with increasing methionine concentrations; a lower number of crosslinks need to be hydrolyzed to release a protein from the microspheres.

Table 1 shows that with increasing methionine concentration, the methacrylate conversion remained high

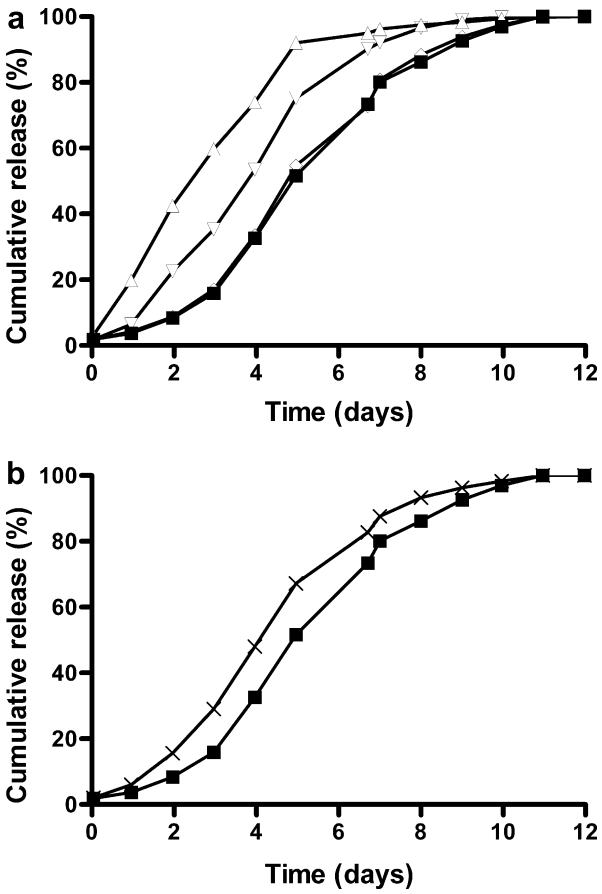

Fig. 5. (a) Cumulative release of hGH from microspheres prepared with 0% (■), 0.1% (◇), 0.5% (▽) and 1% (△) methionine. The reported values are the average of two independent measurements that deviated less than 5%. (b) Cumulative release of hGH from microspheres prepared with a KPS concentration of 4.4 mol/l (■) and 1.6 mol/l (×) KPS. The reported values are the average of two independent measurements that deviated less than 5%.

(>90%, 0.5% methionine) whereas Figs. 1 and 5a show that both the compression modulus of the hydrogel network and the release delay time decreased. In contrast, a decreasing KPS concentration immediately results in a decreased methacrylate conversion (Table 1). Previous studies have shown that residual monomers are generally toxic [35,36] and it is therefore preferred that the methacrylate conversion is high. Taken together, it can be concluded that tailoring the delay time of the release of hGH without affecting the methacrylate conversion can be established by increasing the methionine concentration.

4. Conclusions

This study demonstrates that the encapsulation efficiency and the release of hGH from dex-HEMA microspheres can be modulated by the nature and concentration of the excipients. Especially, Tween 80 and methionine are potential candidates to tailor/optimize the release of hGH as their encapsulation efficiency is high, the methacrylate conversion

is almost quantitative and the protein is essentially released in its monomeric form.

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