European Journal of Pharmaceutics and Biopharmaceutics 73 (2009) 351–356

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Influence of degree of substitution of HES–HEMA on the release of incorporated drug models from corresponding hydrogels

Ariane D.A. Schwoerer [a], Steffen Harling [b], Kristin Scheibe [c], Henning Menzel [b], Rolf Daniels [d,*]

[a] Technical University of Braunschweig, Institute of Biochemistry and Biotechnology, Braunschweig, Germany
[b] Technical University of Braunschweig, Institute of Technical Chemistry, Braunschweig, Germany
[c] Technical University of Braunschweig, Institute of Pharmaceutical Technology, Braunschweig, Germany
[d] Eberhard Karls University of Tübingen, Department of Pharmaceutical Technology, Tübingen, Germany

ARTICLE INFO

Article history:
Received 25 July 2008
Accepted in revised form 10 August 2009
Available online 14 August 2009

Keywords:
Drug delivery system
Biodegradable hydrogels
Hydroxyethyl starch
Aqueous two-phase separation system
Proteins

ABSTRACT

Hydrogel microparticles were produced by a radical polymerization of hydroxyethyl methacrylate–hydroxyethyl starch (HES–HEMA) in an all aqueous two-phase system (ATPS). The microspheres show a monomodal size distribution and have the ability to entrap high amounts of water. The release of proteins or other testing substances from the HES–HEMA hydrogels can be controlled by the choice of the network density of the hydrogel by varying the degree of substitution (DS), the size of the entrapped substance, and by conditions enhancing the degradation of the hydrogel network.

1. Introduction

The progressive development in the field of biotechnology leads to a high number of interesting new products, especially in the range of therapeutically active proteins [1].

The clinical application of those proteins is still challenging, because of their unique properties, such as low stability, short circulation half-life and large molecular size [2].

Therefore, a successful application of proteins as active ingredients requires an efficient delivery system, which allows gentle entrapment into the drug delivery system (DDS) and controlled release of physiological amounts of proteins next to the target tissue over a certain period of time. Hydrogels can be considered as promising candidates for these demands [3]. Hydrogels resemble natural living tissue more than any other class of synthetic biomaterials. This is due to their high water contents and soft consistency, which is similar to natural tissue. Furthermore, the high water content of the materials contributes to their biocompatibility [4] and their ability for gentle entrapment of proteins.

The release of proteins from the hydrogel network can originate from two effects: degradation of the hydrogel and/or diffusion out of the network [5]. An approach to such a drug delivery system is the inclusion of proteins in a hydrogel matrix, based on cross-linked hydroxyethyl methacrylate–hydroxyethyl starch (HES–HEMA, Fig. 1).

Hydroxyethyl starch (HES), which is the starting material for the synthesis of HES–HEMA, is used in medicine for a long time as volume therapy, so that a good physiological compatibility of the hydrogels can be assumed [6].

Furthermore, this hydrogel matrix is biodegradable, so a continuous release of active ingredients in the course of the erosion of the hydrogel and by diffusion is possible. The inclusion of proteins in the hydrogel delivery system (HDS) is accomplished by an aqueous phase separation process, avoiding the use of any organic solvents, intensive shear stress, extreme temperatures and large pH shifts [2].

The basic principle of such systems has been extensively reported for the combination of PEG and hydroxyethyl methacrylate derivatives of dextran (DEX-HEMA). However, it is known that the chemical nature of the demixing polymers affects the partition coefficient of the protein, which shall be entrapped in the cross-linked hydrogel. Thus, there is in addition to the DEX-HEMA/PEG system, some need to find other biocompatible ATPS with different affinity for more or less polar therapeutic proteins. In this study, we substituted hydroxyethyl starch (HES) for DEX in order to have a less hydrophilic polymer.

The primary aim of this study was to assess the influence of the degree of substitution (DS) of the HEMA group and the molecular weight of the entrapped substances on the release kinetics of the

* Corresponding author. Eberhard Karls University of Tübingen, Institute for Pharmacy, Department of Pharmaceutical Technology, Auf der Morgenstelle 8, D-72076 Tübingen, Germany. Tel.: +49 7071 2972462; fax: +49 7071 295531.
E-mail address: rolf.daniels@uni-tuebingen.de (R. Daniels).

0939-6411/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2009.08.003

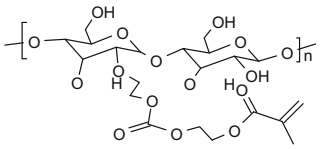

Fig. 1. Chemical structure of HES–HEMA.

HDS. Secondly, the influence of accelerated degradation of the hydrogel at alkaline pH on the release rate was evaluated for a better understanding of the involved release mechanisms.

2. Materials and methods

2.1. Materials

Polyethylene glycol (PEG) (Pluriol E 12,000) with an average molecular weight of 12,000 was obtained from BASF (Ludwigshafen, Germany).

Hydroxyethyl starch 130,000 was obtained from Fresenius Kabi (Bad Homburg, Germany). The HEMA derivative of hydroxyethyl starch (HES–HEMA) was synthesized and characterized according to Dijk-Wolthuis et al. [7] and Heim et al. [8].

Varying reaction conditions yielded HES–HEMA with different degrees of substitution (DS) in the range of 0.01–0.22. HES–HEMA derivatives with a DS beyond this range have been disclosed from this study due to inappropriate gelling properties (DS < 0.01) or insufficient aqueous solubility (DS > 0.22), respectively.

Fluorescence labelled lysozyme (f-lysozyme) was purchased from Active Motif Chromeon (Regensburg, Germany).

Fluorescein isothiocyanate-labelled dextran (FITC-dextran) with molecular weights of 20,000, 70,000 and 500,000 Da (FD 20, FD 70 and FD 500) was from Sigma–Aldrich (Munich, Germany).

Irgacure® 2959 was used as a photoinitiator and was a gift from Ciba Specialty Chemicals (Basel, Switzerland).

2.2. Preparation of microspheres

HES–HEMA microspheres were prepared by a water-in-water emulsion method (Fig. 2). The basic principle is the formation of an aqueous two-phase system (ATPS) upon the mixing of two aqueous polymer solutions, namely PEG 12,000 and HES–HEMA due to a miscibility gap [9]. The ability of PEG to induce aqueous phase separation was described for systems with HES and in much more detail for systems with dextran [12]. PEG molecular weight affects the concentration necessary to form an ATPS. PEG 12,000 was chosen [13] to reach a stable miscibility gap within a reasonable concentration range.

For the preparation of microspheres, PEG 12,000 and HES–HEMA were used at a ratio of 10:1 [9–11].

For this, 4.5 g of an aqueous 4% HES–HEMA solution were mixed with 4.5 g of a 0.001% solution of the model drug (f-lysozyme or FITC-dextran) and supplemented with 0.9 mg of the photoinitiator Irgacure® 2959.

The added quantity of the model drug was chosen in such a way that the dried microspheres were expected to have a theoretical load of 0.4%.

Subsequently, 6.0 g of a 30% PEG solution were added in order to initiate aqueous phase separation.

All substances were dissolved in a 20 mM pH 7.0 sodium-phosphate buffer solution.

After cooling at 0 °C over 10 min, the system was mixed by a Vortex-Genie mixer (Bender & Hobein AG, Zurich, Switzerland) at speed control 6 for 1 min.

The occurring water-in-water emulsion was exposed to UV light with a wavelength of 366 nm and an intensity of 3 mW/cm^2 for 30 min (Model Nu-8 KL, Benda, Wiesloch, Germany) [14].

The resulting suspension was transferred to a centrifuge tube and centrifuged at 5000 rpm for 5 min.

Washing of the microparticles involved resuspension in demineralised water, subsequent centrifugation and removal of the supernatant.

Washing steps were repeated five times.

After final washing, the microspheres were freeze-dried for storage.

Encapsulation efficiency was calculated from the cumulative loss of model drug in the supernatants and the total amount, which was introduced initially.

2.3. Preparation of hydrogel cylinders

HES–HEMA rods were prepared by photo cross-linking of a mixture containing HES–HEMA 10%, Irgacure 2959® 0.1%, protein/testing substances and sodium phosphate buffer in 150 µl vials. Cross-linking was initiated by exposing the unsealed vials to UV light (366 nm; 3 mW/cm^2) (Model Nu-8 KL, Benda, Wiesloch, Germany) for 30 min, positioning the vials as close as possible under the UV lamp. The cylinders could be removed with a scoop and had a diameter of about 6 mm, a length 7 mm and a weight of 150 mg (±2 mg). Drug load was with 0.3% with reference to the dried hydrogel cylinders (average weight 15 mg).

2.4. Characterization of the hydrogels

2.4.1. Scanning electron microscopy (SEM)

Freeze-dried samples of the microspheres were examined by scanning electron microscopy using a DSM 940 A (Carl Zeiss, Oberkochen, Germany) to image the surface of the microparticles. Pictures were taken with a camera (Contax M 167 MT, Yashica-Kyocera, Hamburg, Germany) and were digitalized using a frame grapper (Orion 5, E.L.I. sprl, Brussels, Belgium). Each sample was fixed on an aluminum pin using double sided adhesive tape (Tempfix) and then coated with a thin gold layer prior to examination using a Sputter Coater (model E 1500, Bio-Rad, Biorad, Munich, Germany). The samples were sputtered four times for 60 s and exposed to 20 mA current and 2.1 kV acceleration voltage at a vacuum of 0.02–0.03 mbar.

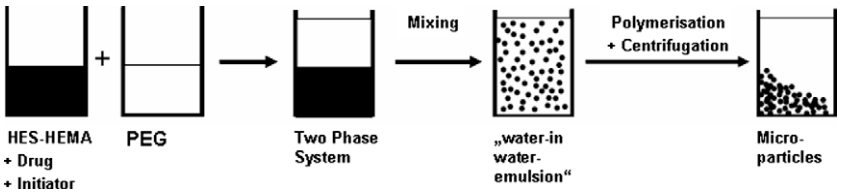

Fig. 2. Schematic representation of the manufacturing method for HES–HEMA microparticles.

2.4.2. Confocal microscopy

FITC-dextran-loaded samples were examined by confocal laser scanning microscopy (CLSM-510-META, Zeiss, Göttingen, Germany). Recording was performed with a pinhole size of 78 μm using an excitation wavelength of 488 nm, a 488/543 double dichroic mirror and a 505–530 band pass filter together with a 590 nm long pass filter.

2.4.3. Transmission electron microscopy

After shock-freezing in melting nitrogen (−210 °C), samples were freeze-fractured in a BAF 400 (Balzers, Wiesbaden, Germany) at −100 °C and etched for 60 s at −100 °C and 10^{-7} mbar. Shadowing was performed with platinum/carbon at 45°, and replicas were stabilized by vertical deposition of 20 nm pure carbon. After cleaning with a methanol–chloroform mixture (1:1), the replicas were viewed in a transmission electron microscope (LEO EM 922, Zeiss, Oberkochen, Germany), operated at 80 kV.

2.4.4. Particle size distribution

A laser diffraction particle size analyser (Beckman Coulter LS 13320 PIDS, Fullerton USA) has been employed to analyse the particle size and the size distribution of the drug-loaded hydrogel microparticles.

Desagglomeration of the microparticles was assured by first dispersing a sample of about 10 mg in 2 ml glycerol followed by sonication for 15 min before diluting the resulting suspension to the final concentration with water (approx. 125 ml).

Software assisted calculation of the particle size was based on Mie theory.

Each sample was measured in triplicate.

2.4.5. In vitro release studies

Freeze-dried microspheres (15 mg, accurately weighed) were dispersed in 1 ml medium (0.2 M pH 7 sodium phosphate buffer; 0.1 M pH 9.6 sodium carbonate buffer) and incubated in 2 ml micro tubes (Sarstedt, Nümbrecht, Germany) in a shaking water bath at 37 °C. pH 7 phosphate buffer was chosen to characterize the release under physiological condition, whereas pH 9.6 buffer was used as stress conditions to accelerate the hydrolytic cleavage of the hydrogel cross-links.

Each sample was incubated in triplicates.

Samples were collected periodically after centrifugation, while the suspensions were centrifuged for 1 min at 13,000 rpm, and 500 μl of the supernatant was removed and replaced by fresh solution.

The supernatants were analysed spectrophotometrically with a fluorescence micro-plate reader (Tecan Austria GmbH, Grödig, Austria) for f-lysozyme or FITC-dextran.

3. Results

3.1. Morphology and particle size distribution

An aqueous two-phase system (ATPS) is an aqueous, liquid–liquid, biphasic system, which is obtained by mixture of aqueous solution of two "incompatible" polymers. The existence of an ATPS allows the formation of a water-in-water emulsion by simply mixing the two immiscible aqueous polymer solutions. After emulsification, the polymer in the dispersed phase can be cross-linked to form hydrogel microparticles. As described in preliminary studies, HES–HEMA and PEG 12,000 were used as incompatible polymers, and cross-linking was achieved by radical polymerization of the hydroxyethyl methacrylated HES in the presence of a suitable photoiniator, e.g. Irgacure® 2959 [13].

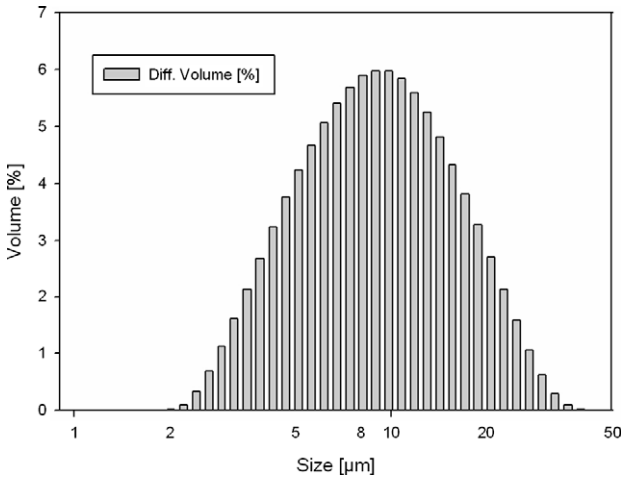

Fig. 3. Particle size distribution (Beckman Coulter LS 13320 PIDS).

HES–HEMA microspheres prepared from such an ATPS show a monomodal particle size distribution with an average size of about 10 μm (Fig. 3), a smooth surface and a homogenous loading (Fig. 4A and B). Microparticle yield was 120 mg (±15 mg) from 15 g batch size. Encapsulation efficiency was 68.2 ± 4.1% for FITC-dextran, and 50.0 ± 5.8% for f-lyzozyme, respectively. Initial water content after preparation was 77 ± 2%.

3.2. Release of fluorescence labelled lysozyme

The release of model drugs from HES–HEMA hydrogels is expected to be strongly dependent from the obtained network architecture, which can be affected by the degree of substitution with reactive HEMA groups and the geometry of the hydrogels. Thus, f-lyzozyme release from cylinders and microspheres prepared from HES–HEMA with different DS was studied.

Fig. 5 compares the release profiles from hydrogel microparticles (Fig. 5A) and hydrogel cylinders (Fig. 5B).

In both cases, release rate decreased with increasing DS. As expected, a higher DS leads to a denser polymer network after cross-linking. This reduces both, the release by diffusion and the release upon network degradation.

The individual release curves from hydrogels with a lower DS show a high variation. Presumably, this might be attributed to a less defined network structure due to a more random cross-linking of polymer chains as only a limited number of reaction partners are available.

All hydrogel microparticles show substantial burst release, whereas only hydrogel cylinders prepared from HES–HEMA with a DS < 0.1 demonstrate a significant initial release. Obviously, the smaller surface/volume ratio of the microparticles together with the formation of a sufficiently dense polymer network at higher DS allows to capture the protein completely within the hydrogel matrix. On the other hand, the observed release from the hydrogel cylinders is always faster than from the microparticles. This indicates that cross-linking, which is initiated by UV radiation is more effective if the layer thickness of the hydrogel is not too large.

3.3. Release of FITC-dextran

For a better understanding of the relation between DS and network density, microparticles from HES–HEMA with two different DS, namely 0.05 and 0.22, have been prepared. The resulting network density was assessed by incorporating differently sized

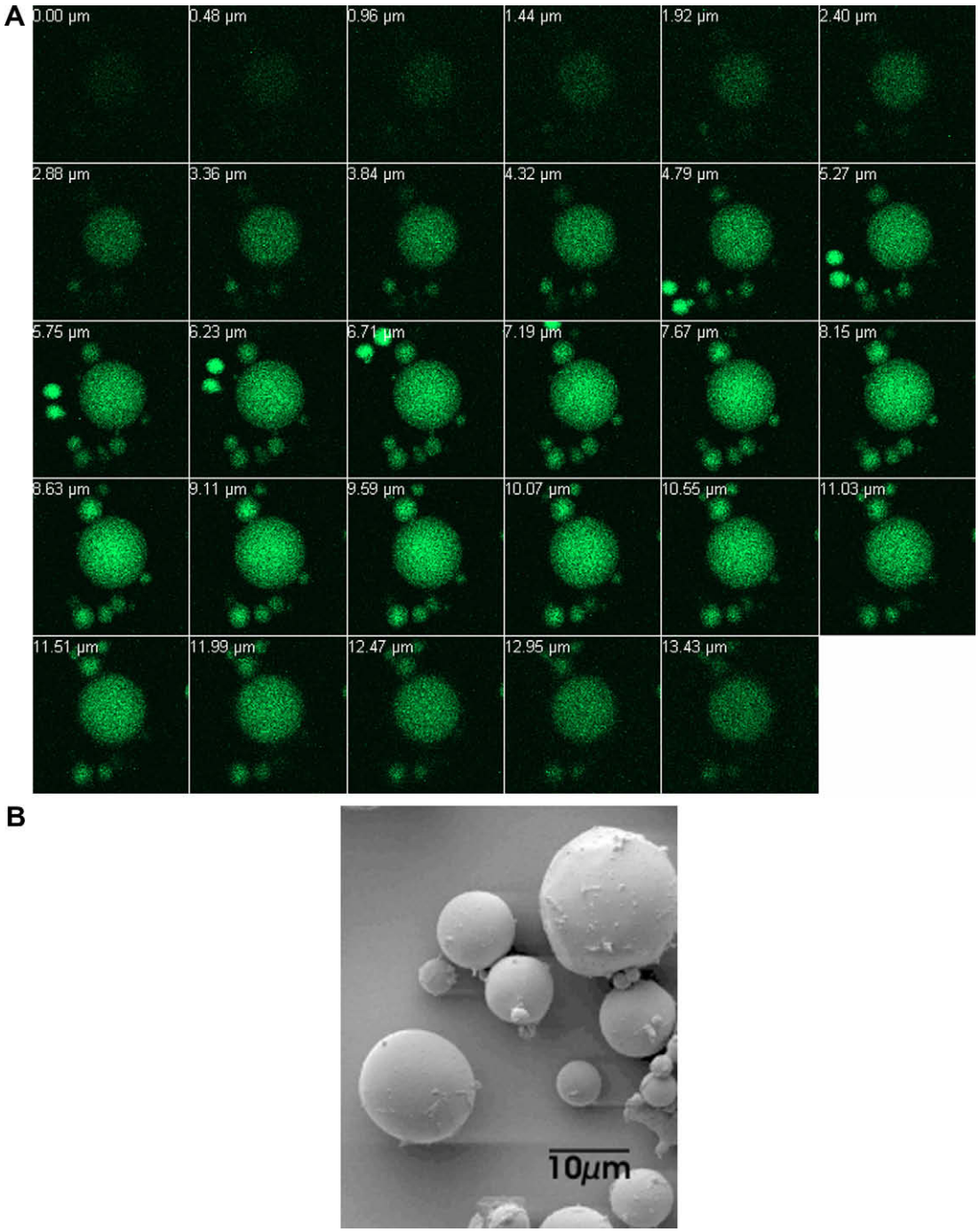

Fig. 4. (A) Confocal laser light microscope image of FITC-dextran-loaded microparticles, (B) REM image of freeze-dried microspheres. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

FITC-dextrans (molecular weight 20–500 kD) into the microparticles as molecular size probes.

Fig. 6 shows that the release of FITC-dextran was controlled by both FITC-dextran molecular weight and hydrogel structure. In all cases, biphasic release profiles can be observed. A much faster initial release rate was followed by sustained release, which could be detected for more than 90 days. The second phase was accompanied by substantial swelling of the microparticles. This could be measured first time 48 h after incubation at pH 7. Maximum particle size was seen after 19 days.

Especially, the initial release depends strongly on both, dextran size and DS of HES–HEMA.

FD 500 with a molecular weight of about 500 kDa (average hydrodynamic diameter of 17.8 nm calculated according to Vollmert [15]) showed an initial release of 1.7% from the hydrogel with DS 0.05, while about 15% of FD 70 (6.6 nm) and 32% of FD (3.6 nm) were released initially.

Increasing the DS to 0.22 has almost no impact on the release of FD 500, but reduces clearly the initial release of the smaller FITC-dextrans FD 70 and FD 20.

For all molecular weights, over a given time interval, a lower release percentage was observed with FD-loaded hydrogels prepared from HES–HEMA with a higher DS (0.22) compared with the same hydrogels prepared from HES–HEMA with lower DS (0.05).

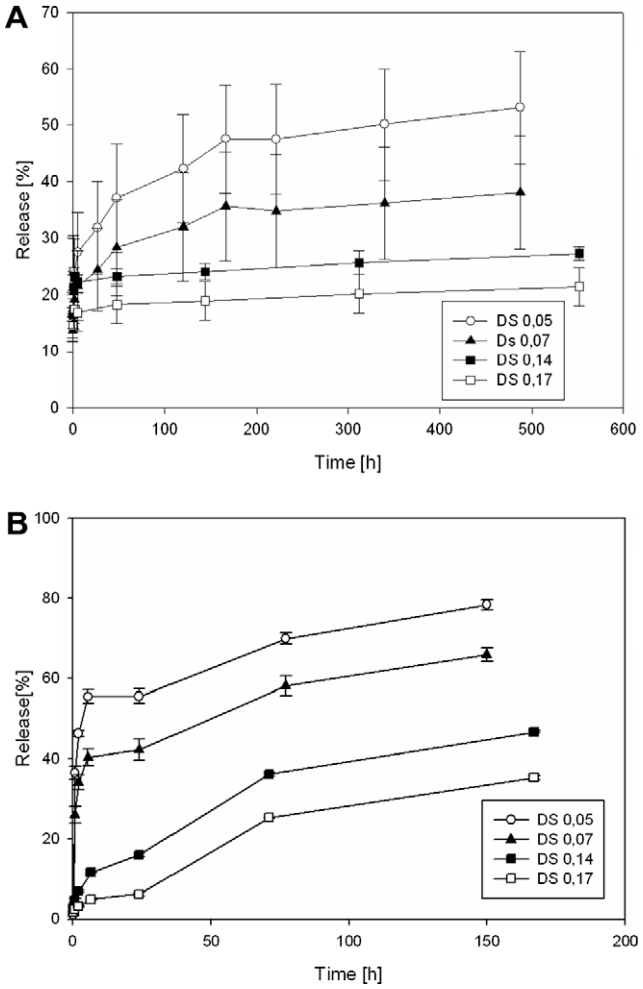

Fig. 5. Effect of DS of HES–HEMA on the release of fluorescence labelled lysozyme from (A) microspheres and (B) hydrogel cylinders.

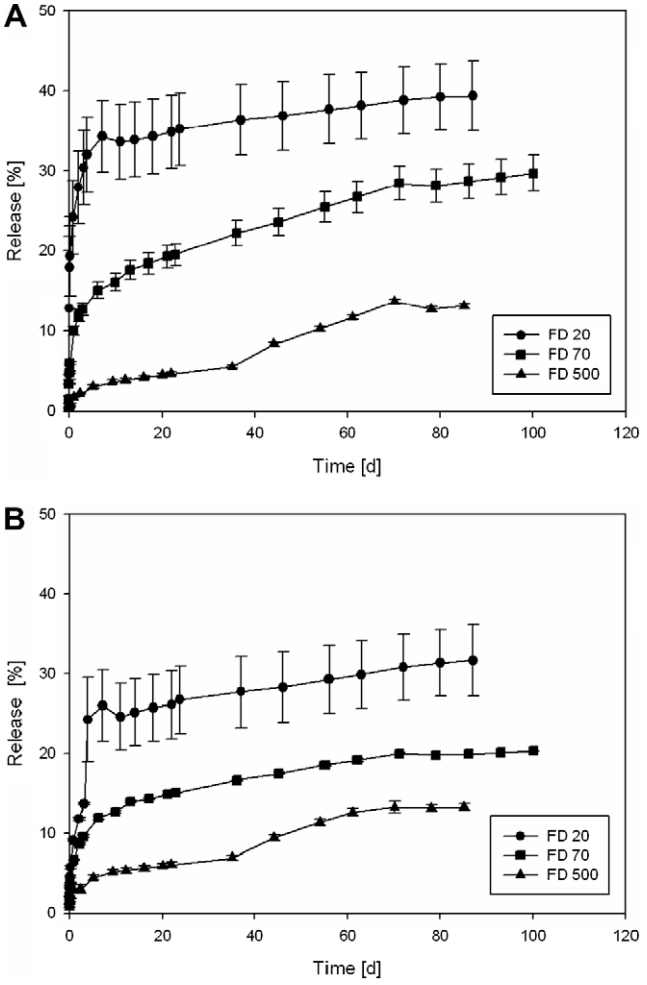

Fig. 6. Release of FITC-dextran (FD20 – 20 kDa, FD70 – 70 kDa, FD500 – 500 kDa) from microspheres (A) DS 0.05 and (B) DS 0.22.

Interestingly, the most prominent influence of the DS on the release profile was observed with FD 70. Here, not only the initial release is reduced, but also the more erosion controlled second phase is altered.

From these results, the conclusion can be drawn that the hydrogels DS 0.05 and 0.22 differ in the amount of pores in the range of 6.6 nm (~70 kDa) [16].

These findings are supported by TEM images of a swollen HES–HEMA hydrogel.

Fig. 7 shows that the hydrogel represents a sponge-like structure, which consists of a more compact matrix and water filled pores with a size of <100 nm. This characteristic structure of the hydrogel results presumably from a phase separation process during polymerization. This can be attributed to the lower aqueous solubility of HES–HEMA which, moreover, continuously decreases when the molecular weight rises during the cross-linking reaction. The characteristics of release accomplish as a reason of a less hindered diffusion from the pores and a decelerated release from areas of the hydrogels with higher network densities after degradation of the hydrogel structures.

In order to assess the influence of fortified hydrolysis of the hydrogel network, additional release studies were performed at pH 9.6. The initial release displayed again the same differences as described earlier for the release at pH 7.0. However, the second release phase was only little influenced by the DS of HES–HEMA and the molecular weight of FITC-dextran (Fig. 8). Release was in all cases completed in less than 200 h. Obviously, FITC-dextran release under these conditions is predominantly controlled by

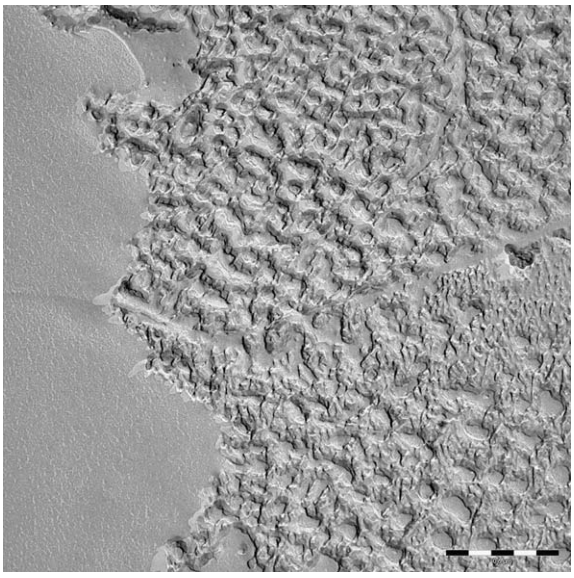

Fig. 7. TEM image of a freeze-fractured and replicated sample of a hydrated HES–HEMA hydrogel microparticle (bar = 0.5 μm).

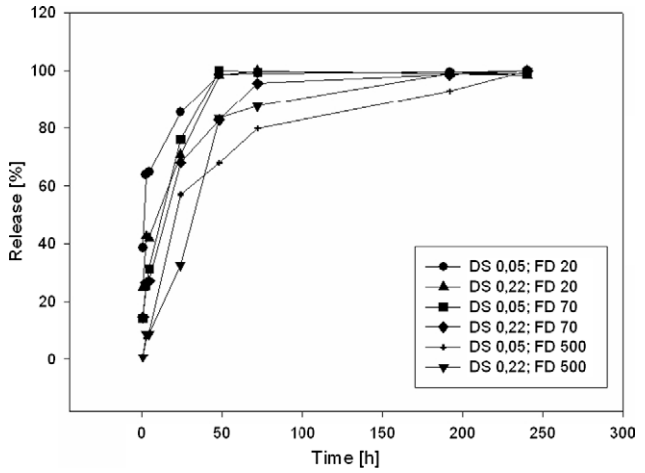

Fig. 8. Release of FD 20, FD 70 and FD 500 from HES–HEMA microparticles with DS 0.05 and 0.22 at pH 9.6.

hydrogel erosion. This further supports the hypothesis that the distribution of FITC-dextran within the water filled pores and a dense hydrogel matrix is the cause for the biphasic release, and that it depends on the size of the entrapped molecules.

4. Conclusion

The results presented in this paper demonstrate that the use of an ATPS consisting of PEG and photo cross-linkable HES–HEMA is well suited for the preparation of protein-loaded hydrogel microspheres with a monomodal particle size distribution. The release of active ingredients can be controlled by the choice of HES–HEMA derivatives of different DS. Contrary to Dex-HEMA hydrogels, HES–HEMA undergoes a phase separation during the cross-linking reaction. This can be attributed to the lower aqueous solubility of HES–HEMA, which, moreover, continuously decreases when the molecular weight rises during the cross-linking reaction.

Thus, HES–HEMA hydrogels behaved as a filter with the pore size of approximately 6.6 nm. The diffusion of FITC-dextran was dependent on the molecular weight. If the molecular size of FITC-dextran was greater than the gel pore size, its diffusion through HES–HEMA hydrogel was too much hindered to be noticed, and in this case, erosion was the prominent release mechanism. If the FITC-dextran molecule was smaller than the pore size, both erosion and diffusion contributed to the overall FITC-dextran release from HES–HEMA matrix. The release in comparison with the related technique DEX-HEMA is more accelerated and can apply a different spectrum of demands. A possible application can be the biphasic release of growth factors, which can be, for example, deposited in the maxillary cavity after bone injury.

Acknowledgments

The authors wish to thank the Deutsche Forschungsgemeinschaft for support of their projects in SFB 578 "Development of Biotechnological Processes by Integrating Genetic and Engineering Methods: From Gene to Product". We also like to thank Fresenius Kabi Deutschland GmbH, Friedberg, Germany, and BASF AG, Ludwigshafen, Germany for material support.

References

[1] A. Granberg, R. Stankiewicz, Biotechnology and the transformation of the pharmaceutical value chain and innovation system. Rough Draft/Working Material, University of Lund, Research Policy Institute, 2002.
[2] M.C. Manning, K. Patel, R.T. Borchardt, Stability of protein pharmaceuticals, Pharm. Res. 6 (1989) 903–918.
[3] W.N.E. van Dijk-Wolthuis, J.A.M. Hoogeboom, M.J. van Steenbergen, S.K.Y. Tsang, W.E. Hennink, Degradation and release behavior of dextran based hydrogels, Macromolecules 30 (1997) 4639–4645.
[4] N.A. Peppas, P. Bures, W. Leobandung, H. Ichikawa, Hydrogels in pharmaceutical formulations, Eur. J. Pharm. Biopharm. 50 (2000) 27–46.
[5] T. Meyvis, S. De Smedt, B. Strubbe, On the release of proteins from degrading dextran methacrylate hydrogels and the correlation with the rheologic properties of the hydrogels, Pharm. Res. 18 (2001) 1593–1599.
[6] P. Lawin, J. Zander, B. Weidler (Eds.), Hydroxyethylstärke – Eine aktuelle Übersicht, Thieme, Stuttgart, 1989.
[7] W.N.E. van Dijk-Wolthuis, S.K.Y. Tsang, J.J. Kettenes-van den Bosch, W.E. Hennink, A new class of polymerizable dextrans with hydrolyzable groups: hydroxyethyl methacrylated dextran with and without oligolactate spacer, Polymer 38 (1997) 6235–6242.
[8] E. Heim, S. Harling, K. Pöhling, F. Ludwig, H. Menzel, M. Schilling, Fluxgate magnetorelaxometry of supermagnetic nanoparticles for hydrogel characterisation, J. Magn. Magn. Mater. 311 (2007) 150–154.
[9] P.-A. Albertsson, G. Johannson, F. Tjerneld, Aqueous two-phase separations, in: J.A. Asenjo (Ed.), Separation Processes in Biotechnology, Marcel Dekker, New York, 1990. pp. 287–327.
[10] O. Franssen, W.E. Hennink, A novel preparation method for polymeric microparticles without the use of organic solvents, Int. J. Pharm. 16 (1998) 1–7.
[11] S. Harling, K. Scheibe, A. Schwoerer, R. Daniels, G. Gross, H. Menzel, Drug Delivery Systeme für die kontrollierte Proteinfreisetzung, in: Berichtskolloquium 2007, Integration gen- und verfahrenstechnischer Methoden zur Entwicklung biotechnologischer Prozesse, SFB 578, Braunschweig, 2007, pp. 163–173.
[12] R J.H. Stenekes, O. Fransen, E.M.G. van Bommel, D.J.A. Crommelin, W.E. Hennink, The preparation of dextran microspheres in an all-aqueous system: effect of the formulation parameters on particle characteristics, Pharm. Res. 15 (1998) 555–559.
[13] K. Scheibe., Hydrogele auf der Basis von Hydroxyethylstärke als Drug Delivery Systeme für die kontrollierte Freisetzung von Proteinen, PhD Thesis, TU-Braunschweig, 2007.
[14] J.B. Leach, C.E. Schmidt, Characterization of protein release from photocrosslinkable hyaluronic acid–polyethylene glycol hydrogel tissue engineering scaffolds, Biomaterials 26 (2005) 125–135.
[15] B. Vollmert, Polymer Chemistry, Springer, New York, 1973.
[16] O. Franssen, L. Vandervennet, P. Roders, W.E. Hennink, Degradable dextran hydrogels: controlled release of a model protein from cylinders and microspheres, J. Controll. Release 60 (1999) 211–221.