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The Preparation of Dextran Microspheres in an All-Aqueous System: Effect of the Formulation Parameters on Particle Characteristics

**Robert J. H. Stenekes,[1,2,3] Okke Franssen,[1]
Elvira M. G. van Bommel,[2] Daan J. A. Crommelin,[1]
and Wim E. Hennink[1]**

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Purpose. The purpose of this study was to investigate the effect of the formulation parameters on the characteristics of dextran-based microspheres, prepared in an all-aqueous system.

Methods. Dextran microspheres were formed by polymerization of methacryloyl groups attached to dextran (dexMA), emulsified in an aqueous poly(ethylene glycol) (PEG) solution. DexMA/PEG/water phase diagrams were established.

Results. The binodals in the phase diagrams shifted to higher concentrations of dextran and PEG with decreasing molecular weight of both polymers, and with increasing degree of MA substitution. The volume-number mean diameter of the microspheres, varied between 2.5 and 20 μm. For a given formulation, the particle size was independent of the PEG/dexMA volume ratio > 40 and increased for volume ratios < 40. Furthermore, larger particles were obtained with decreasing viscosity of the continuous phase and increasing viscosity of the discontinuous phase.

Conclusions. Particle characteristics of dextran microspheres prepared in an all-aqueous system, among which the size and initial water content, can be tailored by adjusting the formulation parameters.

KEY WORDS: aqueous two phase system; phase diagram; microsphere preparation; dextran; hydrogel; poly(ethylene glycol).

INTRODUCTION

Recently, we reported on the use of dextran hydrogels for the controlled release of proteins (1–3). For therapeutic applications of these protein-loaded gels, injectable dosage forms are required. An attractive method to prepare polymeric microspheres in an all-aqueous system, avoiding the use of organic solvents, was recently described (4) and is based on the phenomenon that in aqueous two-polymer systems phase separation can occur.

The biodistribution of microspheres after intravenous administration or the uptake after oral administration by the M-cells strongly depends on the size of the particles (5–9). In addition, the release kinetics of entrapped proteins from macroscopic dextran gels is dependent on the initial water content and the crosslink density of the hydrogels. We demonstrated that the release of proteins from highly hydrated dextran gels could be effectively described by the free volume theory, whereas in strongly crosslinked gels screening occurred (1). This means that for pharmaceutical applications of protein loaded hydrogel-based microspheres, it is necessary to have full control over the size, the initial water content and the crosslink density of the (hydrated) particles. The aim of this study was therefore to gain insight into the formulation parameters that affect these characteristics of dextran microspheres. Since the microsphere preparation process is based on liquid-liquid phase separation, the PEG/dexMA/water phase diagrams were the first subject of investigation.

Theoretical Background

In a ternary system consisting of two water-soluble polymers and water, liquid-liquid phase separation can occur. This phenomenon depends on the molecular weight and concentration of the polymers. Well known is the phase separation in aqueous systems of PEG and dextran (10–12). From a thermodynamic point of view, phase separation in these systems occurs when the change in Gibbs free energy of mixing (ΔG_{mix}) is positive: $\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix} > 0$, where ΔH_{mix} is the enthalpy of mixing, T is the absolute temperature and ΔS_{mix} is the entropy of mixing. Further, the enthalpy and the entropy of mixing can be given by $\Delta H_{mix} = kT \Sigma n_i v_j \chi_{ij}$ and $\Delta S_{mix} = -k \Sigma n_i \ln v_i$ respectively, where k is Boltzmann's constant, T is the absolute temperature, n_i is the number of molecules of component i, v_j is the volume fraction of component j and χ_{ij} is the interaction parameter between component i and j (13).

For obvious reasons, the entropy of mixing is positive. However, for polymers it has a relatively small value because it depends on the number of moles present. The solvent-polymer interaction parameter for PEG/water and dextran/water is 0.54 and 0.38–0.47, respectively (14–15), causing that both polymers can be dissolved in water. This means that in aqueous systems of dextran and PEG, phase separation is caused by an unfavorable dextran/PEG interaction parameter (large χ parameter). In other words, when the gain in entropy of mixing is not large enough to compensate for the repulsive dextran/PEG interaction enthalpy, mixing of the two polymers is thermodynamically not favorable and phase separation occurs. Fig. 1 shows the phase diagram of an aqueous system containing two water-soluble polymers.

MATERIALS AND METHODS

Materials

PEG 4.000 (M_w 5.400; M_n 4.200), PEG 10.000 (M_w 12.000; M_n 8.700), PEG 20.000 (M_w 19.700; M_n 12.600) and potassium peroxodisulfate were obtained from Merck (Darmstadt, Germany). Dex 6.000 (M_w 7.800; M_n 4.700), Dex 40.000 (M_w 38.800; M_n 16.400); Dex 220.000 (M_w 23.3000; M_n 68.000) and *N,N,N',N'*-tetramethylethylenediamine were purchased from Fluka (Buchs, Switzerland). M_w and M_n refer to the weight and the number average molecular weight, respectively

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[1] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), participant in the Groningen-Utrecht Institute for Drug Exploration (GUIDE), University of Utrecht, PO Box 80082, 3508 TB Utrecht, The Netherlands.

[2] OctoPlus B.V., Niels Bohrweg 11-13, 2333 CA Leiden, The Netherlands.

[3] To whom correspondence should be addressed. (e-mail: R.J.H.Stenekes@far.ruu.nl)

ABBREVIATIONS: DexMA, Methacrylated Dextran; DS, Degree of Substitution; PEG, Polyethylene Glycol.

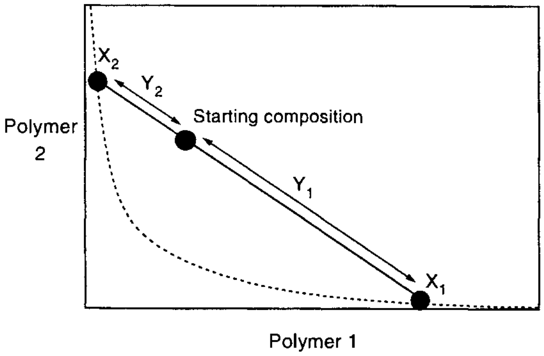

Fig. 1. Phase diagram of a water/polymer 1/polymer 2 ternary system. When the starting-composition is below the binodal (----), a one phase system is present, whereas above the binodal, two coexisting phases are formed: one enriched in polymer 1 (composition x_1) and the other enriched in polymer 2 (composition x_2). x_1 and x_2 are connected via a tie-line (——). All systems prepared using starting-compositions on the same tie-line separate into phases of constant composition. For a given starting-composition, the volume ratio of the coexisting phases x_1/x_2 equals y_2/y_1.

(determined by GPC). Endo-dextranase D-1508, specific activity 250–500 U/mg protein (1 U releases 1 μmol reducing oligosaccharides per min at 37°C and pH 6.0) was obtained form Sigma NV (Bornem, Belgium). Two batches of MA derivatized dextrans (dexMA) differing in degree of substitution were used. The degree of substitution (DS: the number of MA groups per 100 dextran glucopyranosyl monomer units) was 10 and 30 respectively. DexMA was prepared as described by Van Dijk-Wolthuis et al. (3,16).

Phase Diagrams of Dextran(MA) and PEG

A number of (typically 4 or 5) systems (total weight 10 g) were chosen, for which phase separation was expected. E.g. for PEG 10.000 and dexMA 40.000 (DS 10): 8% w/w dexMA, 8 % w/w PEG; 10-10; 12-12; 13-13 and 14-14% w/w. For a given starting composition, the % w/w of dexMA (or PEG) is defined as weight of dexMA/(weight of dexMA + PEG + water) * 100%. These systems were vigorously shaken for 1 min in a 15 ml polystyrene Falcon® 2095 conical tube and subsequently centrifuged (type: Sigma 4 K 10) for 30 min at 2000 rpm and 25°C. From the two phases a sample of about 1 ml was taken and analyzed for the PEG and dextran concentration using GPC analysis as described below.

Gel Permeation Chromatography

The concentrations of dextran and PEG in the coexisting phases were determined by gel permeation chromatography (GPC) with a LC Module 1 (consisting of a 6000A HPLC pump and a 715 autoinjector), a Model 410 differential refractometer (both from Waters Associates Inc., Milford, MA, USA), and three thermostated (35 °C) Shodex KB-800 series columns (OHpak KB-800P, 6 mm × 50 mm, guard-column; OHpak KB-802, 8 mm × 300 mm, exclusion limit 4.10^3; OHpak KB-806M, 8 mm × 300 mm, exclusion limit 2.10^7; Showa Denko, Tokyo, Japan). Degassed 5 mM ammonium acetate buffer (pH = 5.5) in reversed osmosis water was used as the mobile phase. The flow rate was 1.0 ml/min. The columns were calibrated with dextran standards of known molecular weight and narrow molecular weight distribution (Fluka Chemie, AG). The chromatograms were analyzed with Millenium 2010® V.2.15 software (Waters Associates Inc.). Calibration curves (peak area versus injected amount of polymer) were established for both dextran and PEG (linear relationship for 0–6 mg/g). Since PEG and dextran (for the molecular weights used) had about the same retention time, a method based on the selective degradation of dextran was developed to determine the concentrations of both polymers in a single solution. In detail: a sample was diluted (15–70 times) with 5 mM ammonium acetate buffer pH 5.5, followed by the addition of dextranase (final concentration 0.04 U/ml). Next, the sample was incubated at 37°C for 72 hours to fully degrade dextran into oligosaccharides. From the obtained GPC chromatograms of both a dextranase-treated and an untreated sample (Fig. 2) the concentration of both polymers was determined. This method was validated using solutions containing a known concentration of dextran and PEG.

Preparation of Microspheres

Standard procedure: aqueous solutions of PEG and dexMA in 0.22 M KCl were flushed for 10 min with nitrogen and subsequently transferred into a scintillation vial. The total weight of the PEG and dexMA solution was 5 g. The two phase system was vigorously mixed (vortex, type Scientific Industries, Vortex Genie 2, Model G-560E, maximum intensity) for 60 seconds to create a water-in-water emulsion. Next, the emulsion was allowed to stabilize for 15 minutes (ambient conditions), followed by the addition of *N,N,N′,N′*-tetramethylethylenediamine (100 μl, 20 % v/v, adjusted to pH 7 with 4 M HCl) and potassium peroxodisulfate (180 μl, 50 mg/ml). This system was incubated for 30 min at 37°C to polymerize the methacryloyl groups coupled to the dextran chains. The PEG/dex volume ratio was calculated as demonstrated in Fig. 1 (ratio of the distances between the starting composition and the compositions of the coexisting phases on the experimentally determined tie-line).

The particle size and size distribution was measured with a laser light blocking technique (Accusizer™, model 770, Particle Sizing Systems, Santa Barbara, CA, USA) calibrated with monodisperse latex microspheres (ranging from 1 to 100 μm; Duke Scientific Corp., Palo Alto, CA, USA).

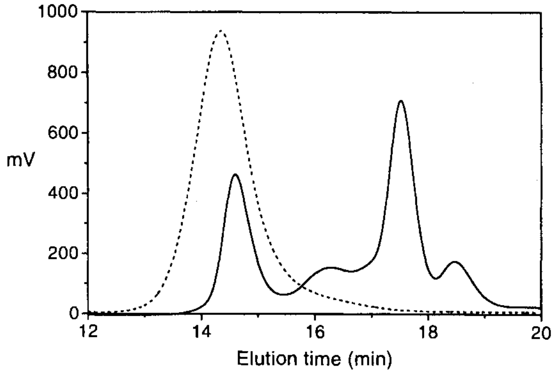

Fig. 2. GPC chromatograms of a mixture of PEG 10.000 and dex 40.000; untreated (----) and treated with dextranase (——).

Viscosity Measurements

The viscosity of PEG and dextran solutions in water was determined with a Contraves Epprecht Rheomat 15 rotation viscosimeter. Calibration curves of viscosity as function of concentration were measured: for dextran 40.000 from 15 to 50 % w/w, for dextran 220.000 from 10 to 35 % w/w, for PEG 4.000 from 20 to 50 % w/w, and for PEG 10.000 and PEG 20.000 from 10 to 50 % w/w. For all solutions, the shear rate (0–750 s^{-1}) versus shear stress plot gave a linear relationship, demonstrating that the solutions behave Newtonian. The viscosities of the coexisting phases were calculated using the concentrations predicted by the phase diagram.

RESULTS AND DISCUSSION

The average size of particles prepared using emulsion technology, depends on the volume ratio of the discontinuous/continuous phase and the viscosity of both phases. For the preparation of dextran microspheres using a water-in-water emulsion, the viscosity of the coexisting phases is dependent on the concentration and molecular weight of PEG and dexMA. It is therefore necessary to know the phase diagrams of PEG and dexMA in order to study the effect of the formulation parameters on the microparticle characteristics.

Phase Diagrams

In Figure 3A, the phase diagrams for the ternary systems of water, PEG 10.000 and dex(MA) 40.000 with different degrees of substitution (DS = 0, 10, 30) are presented. Interestingly, the binodal shifts to higher PEG/dex concentrations with an increasing degree of MA substitution. Obviously, the PEG/dexMA interaction parameter is decreasing with an increasing degree of MA substitution (smaller ΔH_{mix}) indicating a favorable interaction between the methacryloyl groups and PEG. In Figure 3B, the phase diagrams for PEG 10.000 and dexMA (DS 10) with varying molecular weights are presented. As expected, the binodal shifts to higher concentration with decreasing molecular weight of dexMA, because at a fixed concentration, a lower molecular weight corresponds with more molecules per volume and thus a larger ΔS_{mix}. The phase diagrams were also established for PEG (varying molecular weight)/dexMA 40.000 (DS 10)/water ternary systems. Again, the binodal shifts to higher concentrations with decreasing molecular weight of PEG (results not shown).

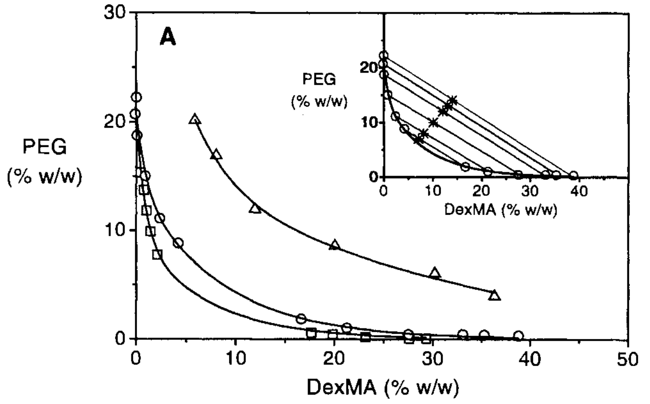

Fig. 3A. Phase diagrams for PEG 10.000 with dexMA 40.000 with varying degrees of substitution of 0 (□), 10 (○) and 30 (Δ). The insert gives the phase diagram for PEG 10.000 and dexMA 40.000 (DS 10) including tie-lines and starting compositions (*).

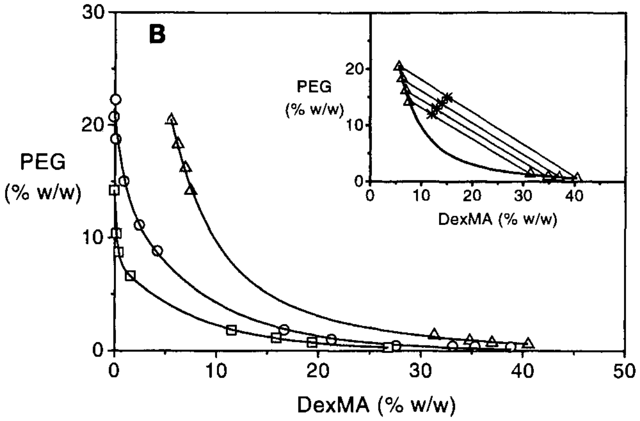

Fig. 3B. Phase diagrams for PEG 10.000 with dexMA 6.000 (Δ), dexMA 40.000 (○) and dexMA 220.000 (□) (all DS 10). The insert gives the phase diagram for PEG 10.000 and dexMA 6.000 including tie-lines and starting compositions (*).

It should be mentioned that when aqueous solutions of dextran and PEG were mixed, in the equilibrium state the PEG/dex volume ratio might differ from the starting composition, e.g. the PEG/dex volume ratio using a starting composition of 15 % w/w dexMA 6.000 (DS 10) and 15% w/w PEG 10.000 (Fig. 3B (insert)) was 1, whereas in the equilibrium state this ratio was 2.8.

Microspheres

The microspheres prepared after polymerization of dexMA in the discontinuous phase, typically have a particle size distribution as shown in Figure 4 (starting composition: 1% w/w DexMA 220.000 (DS 10) and 28.5% w/w PEG 10.000). The microsphere preparation was very reproducible: for 6 independently prepared batches using PEG 10.000 and dexMA 40.000 (DS 10) (starting composition: 22.2 and 1.0% w/w, respectively), the volume-number mean diameter was 7.4 ± 0.7 μm.

The volume weighted mean particle size decreased from 13.6 to 9.7 μm with increasing vortex intensities (formulation:

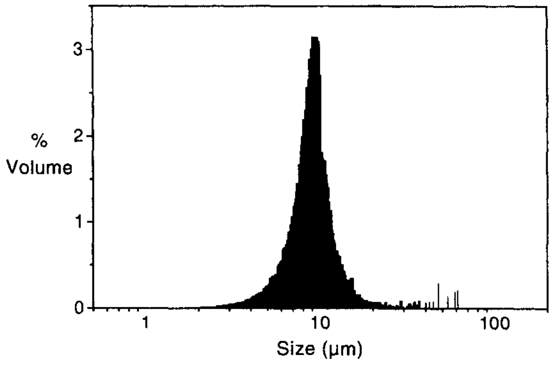

Fig. 4. Volume weight diameter distribution of dextran microspheres (starting composition: 1 % w/w DexMA 220.000 (DS 10) and 28.5% w/w PEG 10.000) prepared using the water-in-water emulsion technique.

22.2 and 1.0% w/w for PEG 10.000 and dexMA 40.000 (DS 10), respectively). On the other hand, the particle size was not affected by either the time applied to vortex the two phase system (15 s–10 min) or the 'stabilization times' (15 s–15 min). However, for stabilization times > 60 min, larger particles were formed probably caused by the coalescence of the water-in-water emulsion. In the remainder of this study, vortex times of 1 min at maximum vortex intensity were used with a stabilization time of 10 – 15 min

Knowledge of the dexMA/PEG/water phase diagrams, including the tie-lines, was used to calculate for each starting composition of PEG/dexMA/water, the concentration of PEG and dexMA in the coexisting phases. Microspheres were prepared at a fixed dexMA concentration of the discontinuous phase (36% w/w dexMA) and a fixed PEG concentration of the continuous phase (21% w/w PEG), but with varying volume ratios of the two phases and varying PEG molecular weights. This was established by selecting starting compositions on tie-lines of the different phase diagrams (Fig. 5, insert). Fig. 5 shows for three PEG molecular weights the effect of the PEG/dexMA volume ratio on the particle size of the formed dextran microspheres. At PEG/dexMA volume ratios > 40, the volume-number mean particle diameter (9, 5 and 3 μm for PEG 4.000, 10.000 and 20.000, respectively) was not dependent on the volume ratio, suggesting that the size of the emulsified dexMA droplets was independent of the PEG/dexMA volume ratio. However, at volume ratios < 40, the particle size increased with decreasing volume ratio. Most likely, at a volume ratio > 40, the dextran phase is well emulsified in the continuous phase. However, at smaller PEG/dexMA volume ratios, the dextran phase was less well emulsified and/or merging of droplets occurred, which resulted in larger particles. Figure 5 also shows that, at any PEG/dexMA volume ratio and fixed concentrations in the coexisting phases (21 and 36% w/w for PEG and dexMA, respectively), larger dextran microspheres were formed when lower PEG molecular weights were used. The viscosity of the discontinuous phase was constant for all formulations (220 mPa.s), whereas the viscosity of the continuous phase decreased with decreasing PEG molecular weight (60 – 10 mPa.s for PEG 20.000 - PEG 4.000). Therefore, during vortexing of the two phase system the shear forces on the dextran phase are smaller with lower PEG molecular weight and viscosity of its phase, ultimately resulting in larger particles (17–18).

We also studied the effect of a varying viscosity of the discontinuous dexMA phase (varying molecular weight) at a fixed viscosity of the continuous phase on the average particle size (Fig. 6). In agreement with the results shown in Fig. 5, an almost constant volume-number mean particle diameter (3 and 5 μm for dex 40.000 and 220.000, respectively) was observed for PEG/dexMA volume ratios > 40, whereas for a volume ratio < 40, larger particles were obtained with decreasing volume ratio. Furthermore, at a volume ratio > 15, the average particle size increased with enhanced viscosity of the dexMA phase (220 and 1640 mPa.s for dexMA 40.000 and 220.000, respectively). This might be explained by an increased resistance of the discontinuous dexMA phase against the shear forces of the continuous PEG phase, during vortexing. Interestingly, the particle size was independent of the viscosity of the discontinuous phase at PEG/dexMA volume ratios < 15. This indicates that at small volume ratios (relatively large volume of dextran phase), the dextran droplets collide frequently, which results for the less viscous phase (dexMA 40.000) in merging and thus in growth of the particles compared to the more viscous phase (dexMA 220.000), where merging is less probable. These effects were also observed when PEG 10.000 was used (results not shown).

We also prepared dextran microspheres at varying PEG/dexMA volume ratios for three different dexMA/PEG concentrations. This was established by selecting several different starting compositions on three different tie-lines (Fig. 7, insert). Table I gives the viscosity of the different phases. Fig. 7 shows that at a volume ratio > 40, the particle size (volume-number mean diameter = 5 μm) is almost constant for the three dexMA/PEG concentrations used. Apparently, the increase in viscosity of the discontinuous dexMA phase is compensated by an increase in the viscosity of the continuous PEG phase. In agreement with the results presented in Figs. 5 and 6, at a volume ratio < 40, the average particle size increases with decreasing PEG/dexMA volume ratio. It should be noted that although at volume ratios > 40, the size of the different particles was the same, they differ substantially in initial water content. In a previous study it was demonstrated that gels derived from dexMA with a DS > 10 were dimensionally stable. This means

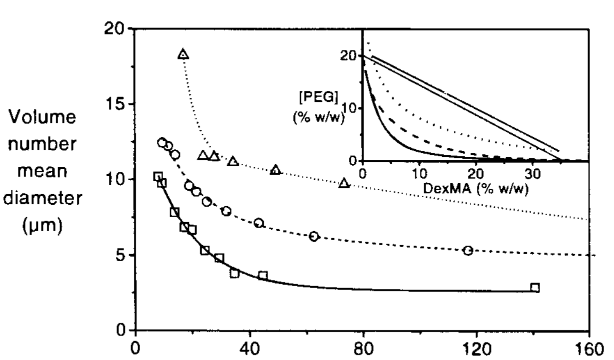

Fig. 5. Volume number mean diameter as function of PEG/dexMA volume ratio for dexMA 40.000 (DS 10) and PEG 4.000 (···Δ···), PEG 10.000 (--○--) and PEG 20.000 (—□—). The insert gives the corresponding phase diagrams and tie-lines.

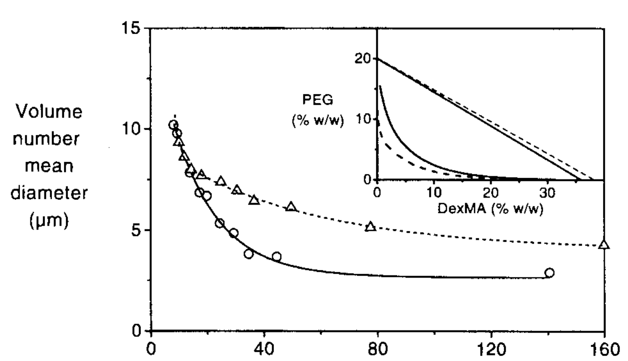

Fig. 6. Volume number mean diameter as function of PEG/dexMA volume ratio for PEG 20.000 with dexMA 40.000 (—○—) and 220.000 (--Δ--) (both DS 10). The insert gives the corresponding phase diagrams and tie-lines.

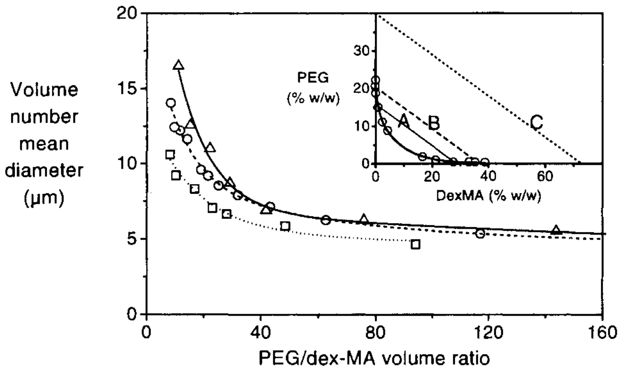

Fig. 7. Volume number mean diameter as function of PEG/dexMA volume ratio for PEG 10.000 and DexMA 40.000 (DS 10) for several compositions on three different tie-lines: A (—Δ—), B (--○--) and C (····□····). The insert gives the corresponding phase diagram and tie-lines.

Table I. Calculated Viscosities of PEG and dexMA Phase for Different Concentrations

Tie-line (Fig. 7, insert)	PEG (% w/w) in PEG phase	η_{PEG} (mPa.s)	DexMA (% w/w) in dex phase	η_{Dex} (mPa.s)
A	15.0	16	27.5	66
B	20.7	30	35.3	202
C	40.6	264	73.0	45600

that the water content of the microsphere is equal to the water content of the dexMA phase used to prepare the microsphere. Therefore, an initial water content of 73, 65 and 27% w/w can be expected for particles prepared using starting compositions on tie-line A, B, and C, respectively (Fig. 7). The initial water content and the crosslink density of the particles are expected to be important parameters affecting the release of entrapped proteins.

CONCLUSIONS

Microspheres based on crosslinked dextrans can be prepared by polymerization of an aqueous solution of methacrylated dextran emulsified in an aqueous solution of PEG. Knowledge of the PEG/dex phase diagrams enables the evaluation of the effects of different formulation parameters (molecular weight and concentration of PEG and dextran in both phases and the PEG/dex volume ratio) on the average particle diameter. The results presented in this study demonstrate that it is possible to tailor the average particle diameter (from 2.5 until 20 μm) and the initial water content (from 73 until 27%). Presently, we are studying the release characteristics of protein-loaded dextran microspheres which vary in water content, crosslink density and size.

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REFERENCES

1. W. E. Hennink, H. Talsma, J. C. H. Borchert, S. C. de Smedt, and J. Demeester. Controlled release of proteins from dextran hydrogels. *J. Contr. Rel.* **39**:47–55 (1996).
2. O. Franssen, O. P. Vos, and W. E. Hennink. Delayed release of a model protein from enzymatically-degrading dextran hydrogels. *J. Contr. Rel.* **44**:237–245 (1997).
3. W. N. E. van Dijk-Wolthuis, J. J. Kettenes-van den Bosch, A. van der Kerk-van Hoof, and W. E. Hennink. Reaction of dextran with glycidyl methacrylate: An unexpected transesterification. *Macromolecules* **30**: 411–3413 (1997).
4. O. Franssen and W. E. Hennink. A novel preparation method for polymeric microparticles without the use of organic solvents. *Int. J. Pharm.* in press (1998).
5. J. H. Eldridge, C. J. Hammond, J. A. Meulbroek, J. K. Staas, R. M. Gilley, and T. R. Rice. Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administered biodegradable microspheres target the Peyer's patches. *J. Contr. Rel.* **11**:205–214 (1990).
6. B. C. Thanoo, W. J. Doll, R. C. Mehta, G. A. Digenis, and P. P. DeLuca. Biodegradable Indium-111 labeled microspheres for *in vivo* evaluation of distribution and elimination. *Pharm. Res.* **12**:2060–2064 (1995).
7. M. Mora and J. Pato. Preparation of egg albumin microparticles for oral application. *J. Contr. Rel.* **25**:107–113 (1993).
8. I. Sjoholm and P. Edman. Acrylic microspheres *in vivo*. I. Distribution and elimination of polyacrylamide microparticles after intravenous and intraperitoneal injection in mouse and rat. *J. Pharmacol. Exp. Ther.* **211**:656–662 (1979).
9. C. Li, D. J. Yang, L. R. Kuang, and S. Wallace. Polyamino acid microspheres: preparation, characterization and distribution after intravenous injection in rats. *Int. J. Pharm.* **94**:143–152 (1993).
10. P. A. Albertsson. *Partition of cell-particles and macromolecules,* Wiley-Interscience, New York, 1986.
11. C. A. Haynes, R. A. Beynon, R. S. King, H. W. Blanch, and J. M. Prausnitz. Thermodynamic properties of aqueous polymer solutions: poly(ethylene glycol)/dextran. *J. Phys. Chem.* **93**:5612–5617 (1989).
12. H. Walter and G. Johansson. Partitioning in aqueous two-phase systems: an overview. *Anal. Biochem.* **155**:215–242 (1986).
13. P. J. Flory. *Principles of Polymer Chemistry,* Cornell University Press, New York, 1953.
14. A. Gustafsson, H. Wennerstrom, and F. Tjerneld. The nature of phase separation in aqueous two-polymer systems. *Polymer* **27**:1768–1770 (1986).
15. K. Gekko. *Solution properties of polysaccharides,* in: D. A. Brant (Ed.), *ACS Symp. Ser.* **150**:415–438, American Chemical Society, Washington DC, 1981.
16. W. N. E. van Dijk-Wolthuis, O. Franssen, H. Talsma, M. J. van Steenbergen, J. J. Kettenes-van den Bosch, and W. E. Hennink. Synthesis, characterization and polymerization of glycidyl methacrylate derivatized dextran. *Macromolecules* **28**:6317–6322 (1995).
17. S. P. Sanghvi and J. G. Nairn. Effect of viscosity and interfacial tension on particle size of cellulose acetate trimellitate microspheres. *J. Microencapsulation* **9**:215–227 (1992).
18. Y. F. Maa and C. C. Hsu. Effect of primary emulsions on microsphere size and protein-loading in the double emulsion process. *J. Microencapsulation* **14**:225–241 (1997).