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Polymerization kinetics of dextran-bound methacrylate in an aqueous two phase system

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Abstract

Cross-linking of dextran can be established by derivatization of the polysaccharide with methacryloyl groups followed by polymerization of an aqueous solution of this methacrylated dextran with an initiator system consisting of potassium peroxydisulfate (KPS) and *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED). Microspheres with a hydrogel character can be obtained by performing the polymerization in an aqueous two phase system of PEG and methacrylated dextran. In order to reach a maximal conversion of methacrylate groups, using a minimal amount of KPS and TEMED, the aim of this work was to study the polymerization kinetics as a function of the reaction parameters, e.g. the KPS or TEMED concentrations, the degree of methacrylate substitution (DS), temperature, the polymer concentration in both phases and the volume ratio of the phases. As expected, the polymerization rate was greater when higher concentrations of KPS and TEMED were used. A higher methacrylate concentration yielded a greater polymerization rate as well. A quantitative analysis of the kinetics of the reaction revealed that the order was 0.41 ± 0.02, 0.53 ± 0.03 and 0.99 ± 0.29 for KPS, TEMED and methacrylate, respectively. These orders were in agreement with a kinetic model derived for the polymerization reaction. The activation energy was 16.1 ± 1.4 kJ/mol. When the equilibrium water content of the dextran phase was 70%, the final conversion of methacrylate groups was around 90% and was reached within one hour, even at relatively low concentrations of KPS and TEMED. At water contents of 50%, a lower final conversion (75%) was observed. Further, a higher viscosity of the dextran enriched phase resulted in a lower polymerization rate. The results presented in this paper give insights into the kinetics of the polymerization of dextran-bound methacryloyl groups, which can be exploited to prepare protein loaded dextran microspheres using minute amounts of initiating species.

Keywords: Methacrylated dextran; Polymerization kinetics; Microspheres

1. Introduction

Polymeric carriers are widely studied as controlled release systems for pharmaceutically active proteins. Until now, the most popular polymers for the preparation of drug delivery systems are poly(lactic acid) (PLA) and its co-polymers with glycolic acid, poly(lactic-co-glycolic acid) (PLGA) [1–3]. However, a number of drawbacks are associated with the use of these polymers. Firstly, organic solvents have to be used for the preparation of the drug loaded system, and secondly, owing to acidic degradation products, a low pH inside the device may occur. Both effects are known to adversely affect protein stability [4–7]. Hydrogels are three-dimensional hydrophilic polymer networks that absorb large amounts of water, which have attracted attention for the delivery of pharmaceutically active proteins [8–10]. The high water content causes good compatibility with proteins and body tissue [11–13]. Further, the release of the protein can be well controlled by the swelling and/or degradation of the hydrogel matrix. We recently demonstrated that hydrogels based on cross-linked dextrans are very suitable systems for the controlled release of proteins. Cross-linking of dextran was accomplished by coupling methacrylate groups to dextran, followed by a radical polymerization initiated by potassium peroxydisulfate (KPS) and *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED) [14,15]. Degradation of these gels could be accomplished by entrapment of dextranase in the hydrogel matrix as well as by introduction of hydrolytically sensitive groups in the cross-links [16–18]. Depending on the average mesh size of the matrix and the hydrodynamic diameter of the protein, the release of the protein from the gels could be governed by either Fickian diffusion [19] or by the degradation rate of the matrix [16,20].

To obtain injectable dosage forms, we developed a

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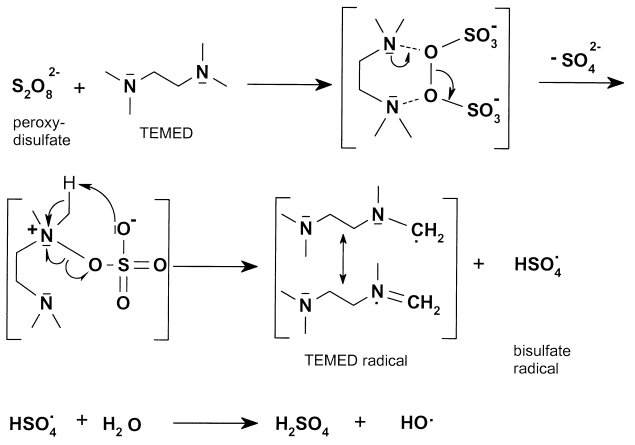

Fig. 1. Formation of the polymerization initiating radicals from peroxydisulfate and *N,N,N′,N′*-tetramethylethylenediamine (from Ref. [26]).

method to prepare hydrogel-based dextran microparticles with a size ranging from 2 to 25 μm, avoiding the use of organic solvents [21,22]. This method is based on phase separation between aqueous solutions of PEG and methacrylated dextran (dexMA). This phase separated system was used to prepare a water-in-water emulsion with a continuous phase enriched in PEG and a discontinuous phase enriched in dexMA. Upon addition of an initiator system (KPS and TEMED) to this water-in-water emulsion, the dextran-bound methacrylate groups were polymerized, which ultimately resulted in the formation of dextran microspheres. Proteins were encapsulated in these microspheres with a very high efficiency and the release could be fully controlled by the degradation rate of the particles [20,23]. The initiator system, however, may cause unwanted oxidation of, e.g. methionine residues in the protein to be entrapped in the dextran matrix [24,25]. To reduce the possible risk of protein oxidation, the amount of KPS and TEMED should therefore be minimized. On the contrary, to obtain a well-defined network with reproducible controlled release characteristics, the conversion of methacrylate groups should be as high as possible. Therefore, the aim of this study was to investigate the kinetics of the polymerization of dextran-bound methacryloyl groups in the PEG/dextran two phase system.

1.1. Kinetic model

The polymerization of the dextran-bound methacryloyl groups can be initiated with potassium peroxydisulfate (KPS) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED). Feng et al. [26] proposed the following initiation mechanism (Fig. 1) in which TEMED accelerates the homolytic scission of peroxydisulfate, yielding the bisulfate free radical ($HSO_4^-\cdot$). In addition, the TEMED free radical [$(CH_3)_2NCH_2CH_2N(CH_3)CH_2\cdot$] and the hydroxyl free radical OH· are generated. These free radicals are responsible for the initiation of the polymerization of the methacrylate groups.

Based on the steady state kinetics for a free radical polymerization [27], a model can be derived for a radical polymerization initiated by KPS and TEMED:

Dissociation :

$$\mathrm{KPS} + \mathrm{TEMED} \xrightarrow{k_d} 2\mathrm{R}\cdot \qquad v_d = 2k_d[\mathrm{KPS}][\mathrm{TEMED}]$$

$$\text{Initiation} : \mathrm{R}\cdot + \mathrm{M} \xrightarrow{k_i} \mathrm{M}_1\cdot \qquad v_i = k_i[\mathrm{R}\cdot][\mathrm{M}]$$

$$\text{Propagation} : \mathrm{M}_i\cdot + \mathrm{M} \xrightarrow{k_p} M_{i+1}\cdot \qquad v_p = k_p[\mathrm{M}_i\cdot][\mathrm{M}]$$

$$\text{Termination} : \mathrm{M}_i\cdot + \mathrm{M}_j\cdot \xrightarrow{k_t} \mathrm{M}_i + \mathrm{M}_j \qquad v_t = k_t[\mathrm{M}_i\cdot][\mathrm{M}_j\cdot]$$

where v_d, v_i, v_p and v_t are the rate of dissociation, initiation, propagation and termination, respectively and k_d, k_i, k_p and k_t are the corresponding constants.

Using steady state assumptions: $d[R\cdot]/dt = v_d - v_i = 2k_d[KPS][TEMED] - k_i[R\cdot][M] = 0$ and $d[M\cdot]/dt = v_i - v_t = 2k_d[KPS][TEMED] - 2k_t[M\cdot]^2 = 0$, the following expression for the rate of polymerization can be derived: $v_p = k_p[M][M\cdot] = k_p(k_d/k_t)^{0.5}[TEMED]^{0.5}[KPS]^{0.5}[M]$.

2. Materials and methods

2.1. Materials

Sodium hydroxide pellets, acetic acid, 37% hydrochloric acid, 70% perchloric acid, PEG 10,000 (M_w 12,000; M_n 8700) and potassium peroxydisulfate were obtained from Merck (Darmstadt, Germany). Dex 40,000 (M_w 38,800; M_n 16,400); Dex 220,000 (M_w 233,000; M_n 68,000), DMSO and *N,N,N′,N′*-tetramethylethylenediamine were purchased from Fluka (Buchs, Switzerland). Methacrylic acid was from Acros (Geel, Belgium) and acetonitrile (HPLC grade) was obtained from Biosolve Ltd (Valkenswaard, The Netherlands). M_w and M_n refer to the weight and the number average molecular weight, respectively and were determined by GPC. Dextran derivatized with methacryloyl groups (abbreviated as DexMA) was synthesized by reaction of dextran with glycidyl methacrylate as described previously [14,15]. The degrees of substitution (DS: the number of MA groups per 100 dextran glucopyranosyl monomer units) used were 7, 12, 14, 21 and 27.

2.2. Preparation of microspheres

The microspheres were essentially prepared as reported in previous papers [21,22]. In short, deoxygenated aqueous solutions of PEG and dexMA in 0.22 M KCl were transferred into a crimp-top vial with a rubber septum. The starting conditions were selected in such a way that a phase separated system was formed. The PEG/dex volume ratio

ranged from 20/1 to 80/1 and the water content of the dextran-enriched phase ranged from 70 to 50% [28]. The total mass of the PEG/dex system amounted to 5 g. The vial was deoxygenated by applying vacuum and subsequently nitrogen was introduced via a needle. This step was repeated two times. The two phase system was vigorously stirred (vortex, type Scientific Industries, Vortex Genie 2, Model G-560E, maximum intensity) for 60 s to create a water-in-water emulsion. Next, the emulsion was allowed to stabilize for 10–15 min, followed by the addition of 100 μl of a *N*,*N*,*N*′,*N*′-tetramethylethylenediamine solution in 0.22 M KCl (adjusted to pH 7 with 4 M HCl) of desired concentration (2, 4, 10, 15 or 20% (v/v)) and 180 μl of an aqueous potassium peroxydisulfate solution of desired concentration (1, 2, 10, 25 or 50 mg/ml) via the septum using a syringe. The 'standard polymerization conditions' refer to a water content [28] of the dextran phase of 70% (w/w), a PEG/dextran volume ratio of 40, a DS of 12 and a polymerization temperature of 37°C.

2.3. Polymerization kinetics

At different time points (0, 1, 2, 3, 5, 10, 15, 30 and 60 min), a sample of the reaction mixture was taken via the septum using a syringe. Of this sample, 100 μl was immediately transferred to a vial with 4900 μl of 0.02 M NaOH solution to stop the polymerization reaction. Next, this sample was incubated at 37°C for 30 min to hydrolyze unreacted (dextran-bound) methacrylates. It was demonstrated by Van Dijk-Wolthuis et al. [18,29] that under these conditions only hydrolysis of the unreacted methacryloyl groups occurs, resulting in methacrylic acid (MAA). The conversion (defined as $(1 - (\text{moles of unreacted methacrylate groups at time } t/\text{moles of methacrylate groups originally coupled to dextran})) \times 100\%$) was determined by measuring the amount of MAA by reversed phase HPLC [29].

2.4. Macroscopic hydrogels

Hydrogels (initial weight 1.5 g) were obtained by free radical polymerization of aqueous solutions of methacrylated dextran according to the following general procedure [14].

To 1.29 g of a 35% (w/w) of methacrylated dextran in 0.2 M KCl in a 2 ml eppendorf cup, 135 μl of 7.2 mg/ml KPS in H_2O was added and mixed well. Subsequently, 75 μl of 1.6% (v/v) TEMED in an aqueous solution of 0.22 M KCl (adjusted to pH 7 with 4 N HCl) was added and vigorously mixed. At different time points (0, 1, 2, 3, 5, 10, 15, 30 and 60 min), a sample was taken and immediately frozen in liquid nitrogen to stop the polymerization. Next, the samples were freeze-dried and rehydrated in 10 ml of 0.02 M NaOH and incubated at 37°C for 30 min to hydrolyze unreacted (dextran-bound) methacrylates.

2.5. HPLC analysis

Methacrylic acid was determined by HPLC essentially as described previously [29]. In brief, prior to the HPLC measurement, 1000 μl (for microsphere samples) or 2000 μl (for macroscopic hydrogel samples) of a 2 M acetic acid solution was added to the samples to convert the methacrylate anion into methacrylic acid. Of this mixture, 100 μl (for microsphere samples) or 10 μl (for macroscopic hydrogel samples) was injected onto a RP-18 column (Lichrosphere, Merck, Darmstadt, Germany) using a LC Module 1 (consisting of a 600A HPLC pump and a 715 autoinjector) and a Model 486 UV detector (all Waters Associates Inc.). A degassed 90/10 reversed osmosis water/acetonitrile mixture, adjusted to pH 2 with perchloric acid was used as the mobile phase. The flow rate was 1.0 ml/min. The chromatograms were analyzed with Millennium V3.0 software (Waters Associates Inc.). A calibration curve was obtained by injecting various volumes of a 100 μM MAA solution in eluent and plotting the peak area versus the amount of MAA. The conversion was calculated from the peak area using the calibration curve, relative to the amount of MAA in the sample taken before addition of KPS and TEMED. The degree of methacrylate substitution of the unpolymerized dexMA calculated in this way corresponded well with the degree of substitution according to ^{1}H-NMR [14,15].

2.6. Partition coefficients

The partition coefficient of KPS (or TEMED), defined as the concentration of KPS (or TEMED) in the PEG enriched phase over the concentration of KPS (or TEMED) in the dextran enriched phase ($K = c_{PEG}/c_{Dex}$), was determined by measuring the concentration of KPS (or TEMED) in both phases. A KPS solution (180 μl, 50 mg/ml) or TEMED solution (90, 180 and 270 μl, 20% (v/v), adjusted to pH 7 with HCl) was added to a two phase system consisting of (non-methacrylated) dextran and PEG and vortexed one minute. Subsequently, the two phase system was centrifuged (type: Sigma 4 K 10) for 20 min at 4500 rpm and 25°C, to separate the phases. From both phases, a sample was taken and analyzed for their concentrations of KPS or TEMED. KPS was determined by measuring the peroxide value, essentially according to the European Pharmacopoeia [30]. TEMED was determined by titration using a Metrohm 636 titroprocessor and a Metrohm Dosimat E 635. Prior to the analysis, the sample was adjusted to pH 2 by 4 N HCl, and subsequently titrated with 0.1 M NaOH.

3. Results and discussion

3.1. Partition

The polymerization of the dextran-bound methacryloyl groups was initiated by addition of the initiator system

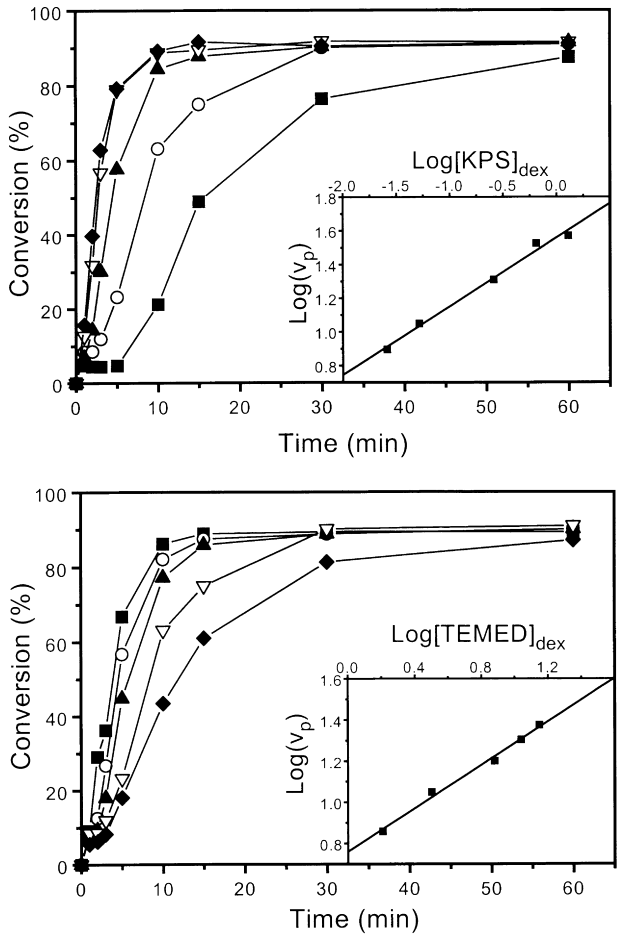

Fig. 2. Conversion versus time under standard polymerization conditions with: (a) constant concentration of TEMED (5.0 mmol/l) and different concentrations of KPS in the dextran enriched phase (◆ 4.8, ▽ 2.4, ▲ 1.0, ○ 0.19 and ■ 0.10 mmol/l) and with (b) constant concentration of KPS (0.19 mmol/l) and different concentrations of TEMED (◆ 2.5, ▽ 5.0, ▲ 12.6, ○ 18.9 and ■ 25.2 mmol/l) in the dextran enriched phase. The inserts give the double logarithmic plots of the rate of polymerization versus: (a) the KPS concentration and (b) TEMED concentration in the dextran enriched phase.

(KPS and TEMED) to a preformed water-in-water emulsion of dexMA and PEG. To know the actual concentrations of the initiating species in the dextran enriched phase, it was necessary to know the partition of KPS and TEMED over both phases. The partition coefficient for KPS ($K = c_{PEG}/c_{Dex}$) was 1.37 ± 0.15 and 1.92 ± 0.02 for a system with coexisting PEG and dextran phases composed of 16% PEG/30% dextran (corresponding to a water content of 70% in the dextran enriched phase) and 28% PEG/50% dextran (corresponding to a water content of 50% in the dextran enriched phase), respectively. The partition coefficient was independent of the volume ratio, and equilibrium was reached within 1 min. The partition coefficient of TEMED was close to unity (1.06 ± 0.03) and independent of the volume ratio or the water content of the coexisting phases. In the remainder of this paper the KPS and TEMED concentrations mentioned, refer to their concentrations in the dextran enriched phase.

3.2. Polymerization kinetics

In a previous paper, we studied the polymerization kinetics of dexMA using FTIR spectroscopy [14]. We now used HPLC analysis to determine the methacrylate conversion. As compared with FTIR analysis, HPLC analysis is more accurate and sensitive, and therefore also allowed studying the polymerization kinetics of dexMA with a relatively low DS. Figs. 2(a) and (b) show the effects of the concentrations of KPS (at a fixed concentration of TEMED (5.0 mmol/l)) and TEMED (at a fixed concentration of KPS (0.19 mmol/l)) in the dextran enriched phase on the methacrylate conversion versus time under standard polymerization conditions (see Section 2.2). Independent of the concentrations of KPS and TEMED used, the final conversions were about 90%. At the highest concentrations of KPS and TEMED investigated, this conversion was obtained after polymerization times of about 10 min. So, despite the relative low concentration of monomer, the polymerization was very rapid. This has also been observed for the aqueous polymerization of methacryloyl groups in poly(ethylene oxide) macromers and was attributed to association of the hydrophobic methacryloyl groups in the aqueous environment. This association may also account for the rapid polymerization of dexMA in water [31]. As expected and in agreement with previously published data [14], decreasing KPS and TEMED concentrations resulted in a slower polymerization and can be attributed to lower concentrations of initiating species (Fig. 1).

From Figs. 2(a) and (b), the rate of polymerization v_p was calculated (slope of conversion versus time). The reproducibility of the polymerization rate and the final conversion was investigated for two formulations under standard polymerization conditions: at a concentration of 0.19 mmol/l KPS and 5.0 mmol/l TEMED in the dextran enriched phase, v_p was 12.7 ± 1.7 mmol/l/min and the final conversion was $89.2 \pm 1.4\%$, respectively ($n = 4$). When 4.8 mmol/l KPS and 25.2 mmol/l TEMED in the dextran enriched phase was used (other reaction parameters unchanged), these values were 50.7 ± 2.5 mmol/l/min and $93.0 \pm 1.7\%$, respectively ($n = 3$). The inserts in both figures give the double logarithmic plot of the v_p versus the concentration of KPS and TEMED, respectively. The order of the reaction was calculated from these figures and was 0.41 ± 0.02 and 0.53 ± 0.03 for KPS and TEMED, respectively. This is in good agreement with the kinetic model derived, where the order of the reaction with respect to both KPS and TEMED was 0.5 (see Section 1.1).

We also studied the effect of the monomer concentration on the rate of polymerization under standard polymerization conditions and using dexMA's with different degrees of substitution (Fig. 3). It was observed that the final conversion decreased with higher degrees of substitutions. This can be

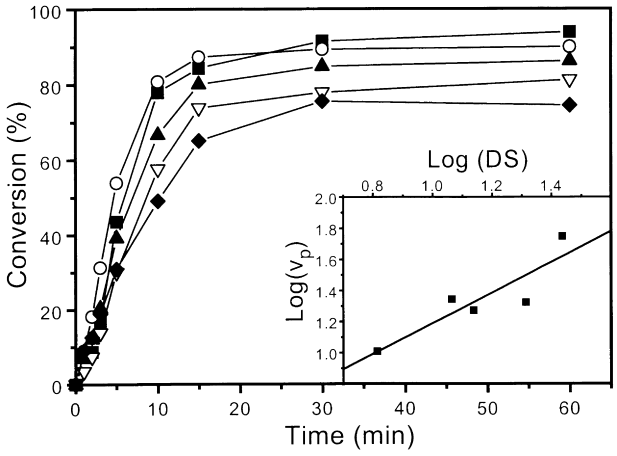

Fig. 3. Conversion versus time under standard polymerization conditions and dexMA with different degrees of substitution (■ 7, ○ 12, ▲ 14, ▽ 21 and ◆ 27) and 0.19 mmol/l KPS and 12.6 mmol/l TEMED in the dextran enriched phase. The insert gives the double logarithmic plot of the (initial) rate of polymerization versus the DS.

explained by the fact that the gel-point is reached at lower conversion using dexMA with higher DS, resulting in severe mobility restrictions of the unreacted methacryloyl groups [14]. The insert of Fig. 3 shows the rate of polymerization as function of the degree of methacrylate substitution (which is under standard polymerization conditions proportional to the concentration of methacrylate monomer). The order of the reaction with respect to the DS was 0.99 ± 0.29, which is again in good agreement with the derived kinetic model. The rate of polymerization was therefore proportional to: $v_p \sim [\text{KPS}]^{0.41} \cdot [\text{TEMED}]^{0.53} \cdot [\text{methacrylate}]^{0.99}$.

In Fig. 4, the effect of temperature on the polymerization rate under standard polymerization conditions is shown. A higher reaction temperature resulted in faster polymerization,

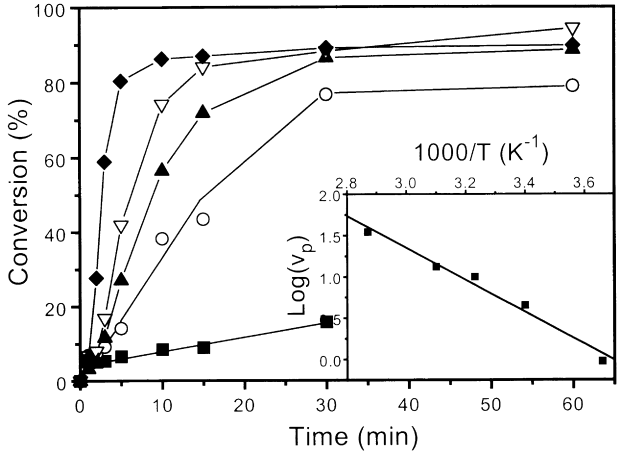

Fig. 4. Conversion versus time under standard polymerization conditions at various temperatures (■ 0, ○ 21, ▲ 37, ▽ 50 and ◆ 75°C) using 0.19 mmol/l KPS and 5.0 mmol/l TEMED in the dextran enriched phase. The Arrhenius plot is presented in the insert.

which can be ascribed to faster formation of radicals at elevated temperatures. The final conversion was constant at temperatures of >37°C (90%), but tended to decrease at lower temperatures. For the polymerization at 0°C, no maximum in conversion was found during the reaction time investigated; the observed conversion was 32% after 60 min (not shown in Fig. 4). The apparent activation energy (E_a) was calculated using an Arrhenius plot (Fig. 4, insert) and amounted to 16.1 ± 1.4 kJ/mol. This agrees well with the values reported in similar polymerization systems [32] (15–25 kJ/mol).

The effect of the PEG/dex volume ratio (ranging between 20 and 80) on the rate of polymerization was also studied. Both v_p and the final conversion were independent of the volume ratio (results not shown). This can be explained by the fact that the partition of KPS and TEMED over both phases is independent of the volume ratio, resulting in equal KPS and TEMED concentrations in the dextran enriched phases, regardless of its volume.

Further, the effect of the water content of the dextran enriched phase on the polymerization kinetics was investigated. At a lower water content of the dextran phase both a lower final conversion and a lower polymerization rate were observed. A representative example is shown is Fig. 5. The lower final conversion can be explained by the fact that for a dexMA solution with a lower water content, the gel-point is reached at a lower conversion, resulting in screening of the radicals in an earlier stage of the polymerization reaction [14]. When the polymerization rate at a low and high water content of the dexMA phase is compared, three factors that potentially affect this kinetic process can be identified. Firstly, at a lower water content the concentration of dextran-bound methacryloyl groups increases, which will result in a faster polymerization rate. On the contrary, a higher PEG/dextran partition coefficient of KPS at lower water contents of the dextran phase (see Section 3.1) will result in a lower concentration of initiating species in the dextran enriched phase, which will give a lower polymerization rate. Finally, the higher viscosity of the dextran phase at lower water contents might cause a lower mobility of the radicals and thus a decreased polymerization rate. To further evaluate the effect of viscosity of the dexMA enriched phase on the rate of polymerization, we studied the polymerization of a methacrylated dextran with a higher molecular weight (Mr 220,000). The remaining formulation parameters were according to the standard polymerization conditions, which means that the only variable is the viscosity of the dextran enriched phase. Fig. 6 indeed shows that the rate of polymerization decreased (from 53.4 to 29.6 mmol/l/min) when the high molecular weight dextran was used. This means that the viscosity of the dextran enriched phase does indeed affect the rate of polymerization and does therefore contribute to the decreased polymerization rate at lower water contents (Fig. 5).

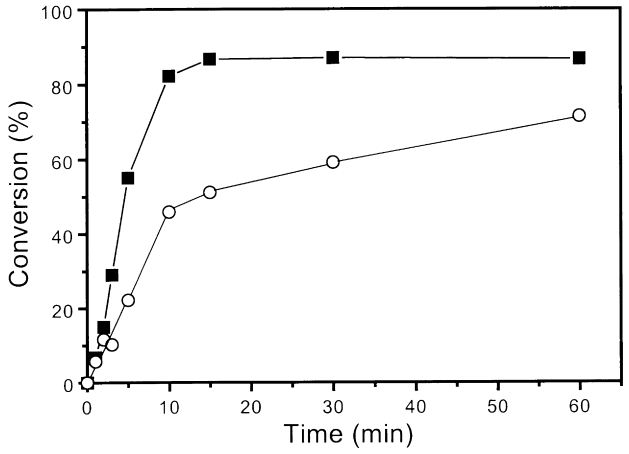

Fig. 5. Conversion versus time under standard polymerization conditions with water contents of 70 (■) and 50% (○) using a 2 mg/ml KPS solution and a 20% (v/v) TEMED solution.

3.3. Comparison between microspheres and macroscopic hydrogels

The kinetics of the dexMA polymerization of microspheres were compared with that of a macroscopic hydrogel [14]. A macroscopic hydrogel was prepared using concentrations of KPS and TEMED that were equal to the concentration of these compounds in the dextran enriched phase used in microsphere preparation. From Fig. 7, it is obvious that the polymerization rates were initially similar, but after 3 min the rate of polymerization was lower in the gel than in the microspheres. In the macroscopic gel, the KPS concentration decreased gradually in time, whereas for the microspheres it can be envisaged that the KPS concentration remained constant due to the partition equilibrium, which exists between KPS in the PEG and dextran phase. This can explain the observed greater polymerization rate and a higher final conversion in microspheres as compared with a macroscopic gel.

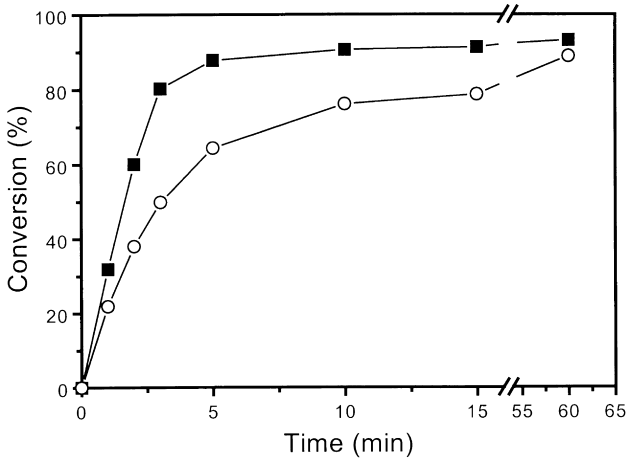

Fig. 6. Conversion versus time under standard polymerization conditions with dextran molecular weights of 40,000 (■) and 220,000 (○) using a 50 mg/ml KPS solution and a 20% (v/v) TEMED solution.

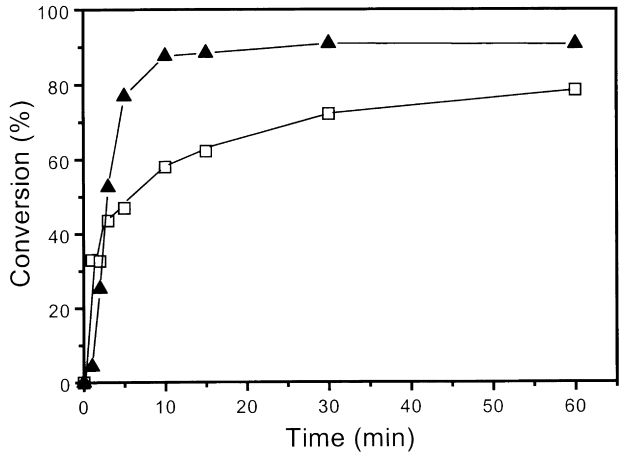

Fig. 7. Conversion versus time for a hydrogel (□) and microspheres (▲) using similar KPS (2.4 mmol/l) and TEMED (5.0 mmol/l) concentrations.

4. Conclusions

This study shows that the polymerization kinetics of dextran-bound methacrylates were in good agreement with a derived model. High conversions can be obtained even at reduced KPS and TEMED concentrations. Presently, we are investigating the extent of oxidation of especially methionine residues of proteins under conditions with low concentrations of KPS and TEMED identified in this paper.

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