Enzymatic Degradation of Cross-Linked Dextrans

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ABSTRACT: The enzymatic degradation of intramolecularly polymerized methacrylated dextran [dex-poly(MA)] and hydrogels based on methacrylated dextran was studied. Dex-poly(MA) was synthesized as a soluble model compound to investigate the enzymatic degradation of dextran hydrogels. After degradation of hydrogels and dex-poly(MA) by dextranase, the formed products were analyzed by gel permeation chromatography (GPC) and electrospray mass spectrometry (ES-MS). GPC analysis revealed that besides low-molecular-weight fractions (180−1100 Da) a soluble high-molecular-weight fraction (9−94 kDa) also was formed for both dextran hydrogels and dex-poly(MA). The high-molecular-weight products in degraded dextran hydrogels and dex-poly(MA) increased both in relative amount and in molecular weight with increasing degree of methacrylate substitution. This fraction most likely consisted of oligosaccharides bound to polymerized methacrylate groups. ES-MS showed that the low-molecular-weight fractions consisted of glucose, isomaltose and some larger unsubstituted oligosaccharides. The same products were formed after enzymatic degradation of native dextran. The enzymatic degradation of dex-poly(MA) as well as dextran hydrogels was described with kinetic models. Combination of enzyme kinetics, GPC and ES-MS lead to the conclusion that the enzymatic degradation can be divided in two processes. First, long unsubstituted chains with a length of probably about 18 or more glucopyranose residues are hydrolyzed at a similar rate and extent as native dextran. Second, the enzyme binds to unsubstituted chain segments of 6 to about 18 glucopyranose residues, which are hydrolyzed slower. Shorter unsubstituted chain segments are not enzymatically degraded.

Introduction

Enzymatic degradation of polymers is widely studied for several biomedical applications. To introduce functional properties in polymeric systems, chemical modifications of the enzymatically degradable chains are often required. Besides derivatization of the monomeric units in linear polymers (e.g., polysaccharides[1−4]), these modifications include the introduction of cross-links for the formation of hydrogels.[5−10] However, the enzymatic degradability of these derivatives is often reduced.[2,3,6,7]

Recently, we reported on the enzymatic degradation of methacrylated dextrans (dex-MA).[4] Dex-MA could be degraded to a similar extent as native dextran by dextranase, even at a high degree of substitution (DS, the number of methacrylates per 100 glucopyranose residues). Mass spectrometry revealed that the degradation of dextran by dextranase resulted mainly in the formation of glucose and isomaltose, with small amounts of larger oligosaccharides. Next to these products, methacrylated oligosaccharides were detected after the enzymatic degradation of dex-MA. Furthermore, combination with data obtained by enzyme kinetics suggested a favorable interaction between a methacrylate group and a binding subsite of the enzyme. We also demonstrated that dextranase was capable of hydrolyzing a glycosidic bond between a substituted and an unsubstituted glucose residue. However, the maximum degradation rate was reduced for methacrylated substrates.

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Dex-MA can be used to prepare enzymatically degrading dextran hydrogels, which are suitable for the controlled release of proteins.[6] The degradation rate of these gels was dependent on the amount of dextranase in the gel as well as on the DS. Dextran hydrogels with DS 4 could be completely degraded by dextranase, whereas hydrogels with DS 37 did not degrade. The latter observation is in contrast with the results obtained with dex-MA.

The aim of this study is to gain insight into the factors that cause the observed differences between the enzymatic degradation of dextran hydrogels and dex-MA. For this purpose, a soluble model compound for dextran hydrogels was synthesized. This dextran-poly(methacylate) [dex-poly(MA)] can be used for enzyme kinetics and was synthesized by polymerization of the methacrylate groups at a low dex-MA concentration (10 mg/mL). Because of this low concentration, predominantly intramolecular cross-links are formed, (Figure 1A) and gelation by intermolecular cross-links is avoided (Figure 1B).

Experimental Section

Materials. Dextran [T40, M_n = 15 kDa, M_w = 39 kDa as determined by gel permeation chromatography (GPC)] from *Leuconostoc ssp.*, dextran standards from *Leuconostoc mesenteroides*, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and maltose, maltotriose, -tetraose, -pentaose and -hexaose (further referred to as malto-oligomers) were obtained from Fluka, Buchs, Switzerland. Potassium peroxodisulfate (KPS) was purchased from Merck, Darmstadt, Germany. Glucose was purchased from Genfarma, Maarssen, The Netherlands. Dextranase D-1508 from *Penicillium funiculosum* [EC 3.2.1.11, specific activity 250−500 U/mg protein, one unit is defined as the amount of enzyme that produces 1 μmol/min reducing oligosaccharides, with dextran (10 mg/mL) as substrate at pH 5.5 and 37 °C] was from Sigma, Bornem, Belgium. Dialysis

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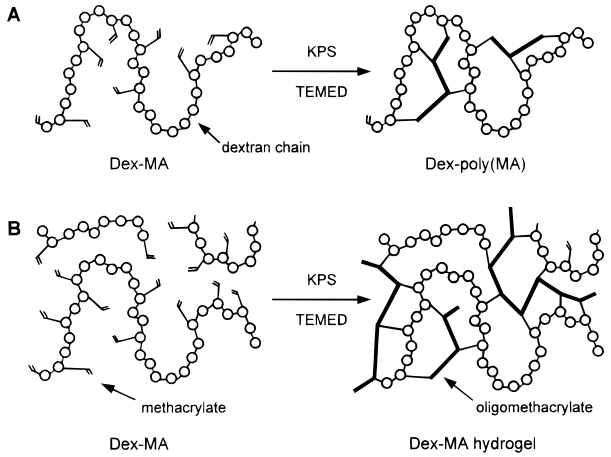

Figure 1. Schematic representation of the formation of (A) dex-poly(MA) with only intramolecular cross-links and (B) a hydrogel with intramolecular and intermolecular cross-links.

tubes (cellulose; molecular-weight cutoff 12−14 kDa) were obtained from Medicell International, London, U.K. Several dex-MA batches differing in DS were synthesized and characterized according to Van Dijk-Wolthuis et al.[11,12]

Synthesis and Characterization of Soluble Dextran-poly(methacrylate). Dex-MA (1 g; DS 4, 8, or 12) was dissolved in deoxygenated phosphate buffer (10 mM; pH 7.0; 100 mL). Polymerization of the methacrylate groups was started by addition of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (1 mL; 20% (v/v); pH adjusted to 7 with 4 M HCl) and potassium peroxodisulfate (1.8 mL; 50 mg/mL). The mixture was stirred overnight at room temperature under a nitrogen atmosphere. Subsequently, the reaction mixture was extensively dialyzed against water at 4 °C and lyophilized. Yields were 80−90%. The conversion of the methacrylate groups was determined after incubation of dex-poly(MA) with 20 mM NaOH for 30 min at room temperature. Residual, unreacted methacrylates are hydrolyzed quantitatively under these conditions.[13] Subsequently, the solution was brought to pH 2 with acetic acid, and the amount of methacrylic acid was quantitatively determined by HPLC as described by Van Dijk-Wolthuis et al.[13] The same method was used to determine the DS of the starting material, dex-MA. The DS of dex-poly(MA), DS_p, is now defined as the DS for dex-MA, minus the unreacted methacrylates. Dex-poly(MA) was further characterized by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and GPC. ^{1}H NMR samples were prepared in DMSO-*d*6 and D_2O, as described by Van Dijk-Wolthuis et al.[11,12]

Preparation of the Hydrogels. Dex-MA hydrogels were obtained by radical polymerization of aqueous solutions of dex-MA. As an example, the preparation of a dex-MA hydrogel [initial water content 70% (w/w)] containing dextranase (1 U/g gel) is given. All solutions were flushed with nitrogen for 10 min before use. Dex-MA (600 mg) was dissolved in 920 μL of phosphate buffer (10 mM; pH 8.0), followed by the addition of dextranase (200 μL; 10 U/ml) in citrate buffer (5 mM; pH 6.0). The polymerization was started after adding 100 μL TEMED solution [20% (v/v) in water; pH adjusted to 8.0 with 4 M HCl] and KPS (180 μL, 50 mg/mL) in phosphate buffer (10 mM; pH 8.0). Part of the polymerizing solution was immediately transferred into a 1 mL polypropylene syringe (radius 0.23 cm). The hydrogels were allowed to polymerize for about 1 h at room temperature and were subsequently removed from the syringe. This procedure yielded cylindrical hydrogels with a weight of about 1 g.

Gel Permeation Chromatography and Electrospray Mass Spectrometry of Enzymatically Degraded Dextran Derivatives. To characterize the products after the enzymatic degradation was completed, dextran, dex-MA, or dex-poly(MA) solutions (10 mg/mL) in ammonium acetate (5 mM; pH 5.5) were degraded by dextranase (0.1 U/ml) at 37 °C for 3 days. Dextran hydrogels were degraded under the same conditions

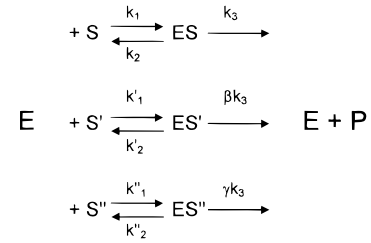

Figure 2. Reaction scheme of an enzyme (E) acting on three different substrates (S, S′, and S″) resulting in a product (P).[4,14] *S*″ is assumed to be zero in the corresponding two-substrate model.

(37 °C; pH 5.5) for 5 days and with a higher amount of dextranase (2 U/g gel).

For GPC, the samples were filtered [polypropylene filters (diameter 4 mm, pore size 0.45 μm); Alltech, Deerfield, IL] and diluted to a concentration of 1 mg/mL in ammonium acetate buffer (5 mM; pH 5.5). The system for GPC consisted of a Waters 510 HPLC pump and a Waters 410 differential refractometer (Waters Associates, Inc., Milford, MA) with three thermostated (35 °C) columns (Shodex KB series, OH pak KB 800P (6 × 50 mm, precolumn); OH pak KB 802 (8 × 300 mm, exclusion limit 4 × 10^3 Da); OH pak 80M (8 × 300 mm, exclusion limit 2 × 10^7 Da); Showa Denko, Tokyo, Japan). Degassed ammonium acetate buffer (5 mM; pH 5.5) in reversed osmosis water was used as mobile phase. The flow rate was 1 mL/min. Dextran standards with narrow molecular weight distributions and malto-oligomers were used for calibration. The chromatograms were analyzed with Waters Millennium 2010 version 2.15 software.

For electrospray mass spectrometry (ES-MS), samples were diluted with acetonitrile to obtain water/acetonitrile 50:50 (v/v) and were directly injected (10−20 μL) on a VG Platform II mass spectrometer (cone voltage 25−50 V; capillary voltage 3.95 kV; scan range *m*/*z* 200−1100; scan duration 9.6 s; flow 10 μL/min; source temperature 100 °C; Fisons Instruments, Cheshire, U.K.). Positively charged ions were produced using a Fisons electrospray probe. Calibration was done with malto-oligomers (ranging from glucose to maltohexaose). The mass spectra were analyzed using Fisons Masslynx version 2.1 software.

Enzyme Kinetics on Soluble Dextran Derivatives. Dextran or dex-poly(MA) was degraded at different concentrations (0.25−10 mg/mL) in ammonium acetate buffer (5 mM; pH 5.5) at 37 °C with dextranase (0.1 U/ml). Samples were taken every 10 min and heated for 15 min at 95 °C to inactivate the enzyme. The concentration of reducing oligosaccharides in the samples was determined spectrophotometrically with Sumner reagent as described by Franssen et al.[6] The degradation rate (in μmol/U min) was calculated as the initial slope (at $t = 0$) of the amount of reducing oligosaccharides plotted against time.

The obtained degradation rates (v) were interpreted with a multiple substrate model, which was successfully used for the enzymatic degradation of dex-MA.[4] In this model, the substrates S, S′ and S″ have different maximum degradation rates, V_{max}, βV_{max} and γV_{max} as stated in Figure 2, and all substrates have their specific Michaelis−Menten constants, K, K', and K'', respectively. For the enzymatic degradation of dex-MA, S, S′, and S″ were defined as unsubstituted, monosubstituted, and multiply substituted chains, respectively. The following equation can be derived from the scheme in Figure 2:[4,14]

$$v = \frac{V_{max}[S]/K + \beta V_{max}[S']/K' + \gamma V_{max}[S'']/K''}{1 + [S]/K + [S']/K' + [S'']/K''} \qquad (1)$$

For dex-poly(MA), only products with a low DS_p ($DS_p < 12$) could be synthesized. Therefore, binding of the enzyme is assumed to be restricted to unsubstituted and monosubstituted substrates, as the concentration of multiply substituted substrates is low ($S'' \approx 0$). For dex-poly(MA), the three-substrate

Table 1. Data on the Synthesis of Dex-poly(MA)[a]

initial DS	conversion (%)	unreacted DS	DS_p	M_n (kDa)	M_w (kDa)
4.3	67	1.4	2.9	22	62
7.7	87	1.0	6.7	25	73
12.3	89	1.3	11.0	31	107

[a] The DS for the polymerized methacrylates, DS_p, is calculated as the difference between the initial and unreacted DS of dex-MA.

model (equation 1) is therefore simplified to a two-substrate model:

$$v = \frac{V_{max}[S]/K + \beta V_{max}[S']/K'}{1 + [S]/K + [S']/K'} \quad (2)$$

The substrate concentrations can be calculated by the following approach. Assuming a number of oligosaccharides (n) that is involved in binding to the enzyme, the fractional amount of S and S' can be calculated from the total substrate concentration (S_{total}):

$$S = S_{total}(1 - DS/100)^n \quad (3)$$

The fraction of monosubstituted substrate, S′, is then:

$$S' = S_{total} n(DS/100)(1 - DS/100)^{n-1} \quad (4)$$

Equations 3 and 4 can now be substituted into eq 2. Subsequently, global nonlinear regression analysis (SPSS 7.5 for Microsoft Windows 95) with the obtained equation was applied on the acquired data, in which β, V_{max}, K, K', and n were calculated as unconstrained constants with S_{total} and DS as independent and v as a dependent variable. Note that the model has an extreme case, $\beta = 0$, which equals simple competitive inhibition. In that case, S′ acts as an inhibitor, or in other words, the monosubstituted substrate can bind to the enzyme, but the enzyme is unable to hydrolyze the chain (therefore, $\beta V_{max} = 0$).

Enzyme Kinetics on Dextran Hydrogels. Dextran hydrogels [water content 70% (w/w), 1 U/g dextranase] were cut into 10 pieces of about 0.1 g and incubated at 37 °C in 10 mL ammonium acetate buffer (5 mM; pH 5.5). Periodically, samples of the supernatant were taken and replaced by fresh buffer. In addition, pieces of the hydrogels were removed from the incubation buffer, heated for 15 min at 95 °C to inactivate the enzyme, washed twice, and lyophilized. The dried gel was powdered before further analysis. The concentration of reducing oligosaccharides in the samples and the gels was determined spectrophotometrically with Sumner reagent as described by Franssen et al.[6] This procedure allows the separate determination of both soluble reducing oligosaccharides and reducing end-groups that are linked to the hydrogel network.

Results

Synthesis and Characterization of Dextran-poly(methacrylate). The aim of the synthesis of dex-poly(MA) was to obtain a soluble model compound that closely resembles the structure of dextran hydrogels. Therefore, the polymerization of dex-MA was performed at low concentration [1% (w/v)] to favor the formation of intramolecular cross-links. This method indeed yielded soluble products for dex-MA DS 4, 8, and 12. GPC analysis showed that M_w ranged from 62 to 107 kDa (see Table 1; compare $M_w = 40$ kDa for native dextran), indicating that, besides intramolecular cross-links, some limited intermolecular cross-linking had occurred. At DS 27, however, small gel particles were formed during the polymerization, making it impossible to obtain soluble dex-poly(MA) with high DS_p.

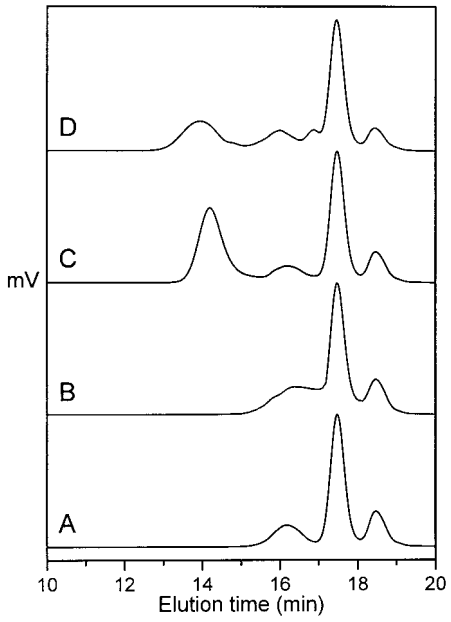

Figure 3. GPC chromatograms of enzymatically degraded dextran derivatives. See Table 2 for the amounts and molecular weights of the high-molecular-weight fractions: (A) dextran; (B) dex-MA DS 13; (C) dex-poly(MA) DS_p 7; and (D) dextran hydrogel DS 4 with water content 70% (w/w).

^{1}H NMR spectra of dex-poly(MA) consisted of very broad signals in the dextran region (3.4−4.2 ppm). The protons of the polymerized methacryloyl groups were only visible as a faint, broad band at approximately 2 ppm (results not shown). Both effects can be ascribed to a strained conformation of dex-poly(MA).[15]

The amount of unreacted methacrylate groups could not be determined quantitatively by ^{1}H NMR, as no or very weak signals were present at 5.8 and 6.3 ppm.[11,12] Therefore, unreacted methacrylate groups were determined by HPLC after hydrolysis of these groups at alkaline pH. The conversion of the methacrylate groups to poly(MA) chains is shown in Table 1 and increased with the DS_p of dex-poly(MA) from 67% for DS_p 3 to 89% for DS_p 11. This corresponds to an amount of unreacted methacrylates that can be expressed as unreacted DS, as it is about 1 for all products. This implies that approximately one unreacted methacrylate is present in each dextran chain ($M_n = 15$ kDa).

Products of Enzymatically Degraded Dextran Derivatives. The degradation products of dex-poly(MA) and dextran hydrogels were characterized with GPC and ES-MS. In Figure 3, the GPC chromatograms of degraded dextran, dex-MA DS 13, dex-poly(MA) DS_p 7 and a hydrogel [DS 4, water content 70% (w/w)] are shown. The degradation products of dextran were separated in three distinctive low-molecular-weight peaks (Figure 3A), corresponding to glucose (at 18.5 min), isomaltose (at 17.5 min) and isomaltotetraose, -pentaose, -hexaose (at 16.2 min).[4,16−18] Low-molecular-weight degradation products were also formed after enzymatic degradation of dex-MA (Figure 3B). However, the peaks were less well-separated, because of the presence of methacrylated oligosaccharides.[4] In degraded dex-poly(MA), not only low-molecular-weight degradation products were found, but also an additional fraction with a higher molecular weight was observed (Figure 3C at 13−15 min, corresponding to 9 to 43 kDa). This fraction increased with the DS in molecular weight and relative amount (see Table 2). Likewise, a high-

Table 2. High-Molecular-Weight Fractions in Enzymatically Degraded Dex-poly(MA) and Dextran Hydrogels[a]

substrate	M_n (kDa)	M_w (kDa)	relative amount (%)	no. of glucopyranose residues per MA
dex-poly(MA)				
DS_p 2.9	8.9	12	18	5.9
DS_p 6.7	12	17	39	5.6
DS_p 11.0	20	42	66	5.9
Hydrogels				
DS 4, water content 90%	11	17	19	4.4
DS 4, water content 80%	14	21	23	5.4
DS 4, water content 70%	18	36	24	5.7
DS 7, water content 90%	21	33	39	5.3
DS 7, water content 80%	34	94	38	5.2

[a] Data obtained by GPC (see also Figure 3). The number of glucopyranose residues per methacrylate in the high-molecular-weight fraction was calculated from the DS and the relative amount of this fraction (and assuming that all methacrylate groups were present in the high-molecular-weight fraction).

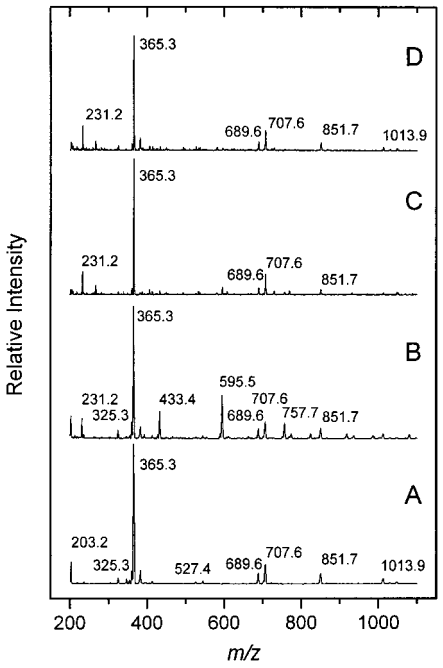

Figure 4. ES-MS spectra of enzymatically degraded dextran derivatives. For assignment of the peaks refer to Table 3 and Franssen et al:[4] (A) dextran; (B) dex-MA DS 4; (C) dex-poly-(MA) DS_p 11; and (D) dextran hydrogel DS 4 with water content 70% (w/w).

molecular-weight fraction was found in enzymatically degraded dextran hydrogels (Figure 3D and Table 2). The molecular weight of this fraction increased with the DS and with decreasing water content [up to $M_w = 94$ kDa for DS 7; water content 80% (w/w)]. Consequently, only dextran hydrogels with a relatively low DS and a high water content dissolved completely after enzymatic degradation. The existence of the high-molecular-weight fraction indicates that dex-poly(MA) and dextran hydrogels were only partially degraded.

In Figure 4, mass spectra of degraded dextran, dex-MA DS 4, dex-poly(MA) DS_p 11, and a dextran hydrogel [DS 4, water content 80% (w/w)] are shown. Ionization occurred mainly by sodium adduct formation according to the same mechanism as described earlier.[4] Assignment of the peaks in the mass spectra of dextran derivatives is given in Table 3. In agreement with GPC analysis, the same low-molecular-weight products with

Table 3. Peak Assignment of the Mass Spectra of Enzymatically Degraded Dextran Derivatives[a]

m/z	degree of polymerization	no. of methacrylates	ion description
203.2	1	0	$[M+Na]^+$
231.2	1	1	fragmentation[4]
325.3	2	0	$[M+H-H_2O]^+$
360.3	2	0	$[M+NH_4]^+$
365.3	2	0	$[M+Na]^+$
433.4	2	1	$[M+Na]^+$
527.4	3	0	$[M+Na]^+$
595.5	3	1	$[M+Na]^+$
689.6	4	0	$[M+Na]^+$
707.6	2+2	0+0	$[2M+Na]^+$
757.7	4	1	$[M+Na]^+$
851.7	5	0	$[M+Na]^+$
1013.9	6	0	$[M+Na]^+$

[a] See Figure 4.

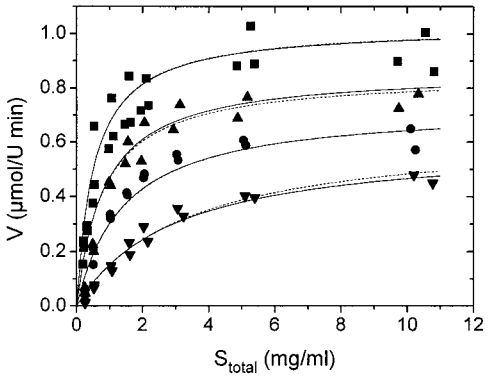

Figure 5. Initial degradation rates of dextran and several batches dex-poly(MA), differing in DS and catalyzed by dextranase. Dextran (■), dex-poly(MA) DS_p 3 (▲), dex-poly(MA) DS_p 7 (●), and dex-poly(MA) DS_p 11 (▼). The curves represent for each substrate the results of the global analysis with the model based on eqs 2−4 (see also Table 4). Monosubstituted chain segments can act as substrate (dashed line) or as inhibitor (solid line), assuming that multiply substituted chains can be neglected ($S'' \approx 0$).

similar relative intensities were found in degraded dextran, dex-poly(MA), and dextran hydrogels (Figure 4A,C,D). Isomaltose was the main degradation product (*m/z* 365.3). Sodium adducts of glucose and traces of isomaltotetraose, -pentaose and -hexaose were also found (*m/z* 203.2, 689.6, 851.7, and 1013.9, respectively). No methacrylated degradation products were observed in degraded dex-poly(MA) or dextran hydrogels (e.g., isomaltotriose-MA, *m/z* 595.5), except for an ion formed by fragmentation during the analysis (*m/z* 231).[4] Unfortunately, the high-molecular-weight fractions observed by GPC could not be detected by ES-MS.

Enzyme Kinetics on Soluble Dextran Derivatives. In Figure 5, the initial degradation rate is plotted against the total substrate concentration for dextran and several batches of dex-poly(MA) which differed in DS_p. Analysis of the data using the two-substrate model (eqs 2−4) yielded a value for β that was indistinguishable from zero (Table 4). Therefore, the analysis was repeated in an inhibitor variant ($\beta \equiv 0$). This resulted in a similar quality of the fit (R^2 global 0.984) but with more precise estimations for K' and n. K' was considerably higher than K (1.8 and 0.5 mg/mL, respectively). Interestingly, the calculation of the number of glucopyranose residues involved in binding to the enzyme (n) was 18−22 for dex-poly(MA), whereas the number of binding subsites for dextranase from *P. funiculosum* is estimated at only 9−14.[4,16−20]

Table 4. Constants Obtained by Nonlinear Regression with the Two-Substrate Model Based on Eqs 2−4[a]

parameter	*S′* substrate	*S′* inhibitor
V_{max} (μmol/U.min)	1.03 ± 0.03	1.03 ± 0.03
β	0.34 ± 0.5	0
K (mg/mL)	0.53 ± 0.05	0.53 ± 0.05
K′ (mg/mL)	1.1 ± 1	1.8 ± 0.5
n	22 ± 8	18 ± 2
R^2		
global	0.984	0.984
dextran	0.982	0.982
dex-poly(MA) DS_p 3	0.983	0.983
dex-poly(MA) DS_p 7	0.990	0.991
dex-poly(MA) DS_p 12	0.987	0.986

[a] See Figure 5. R^2 is the coefficient of determination, uncorrected for the degree of freedom in the statistical analysis.

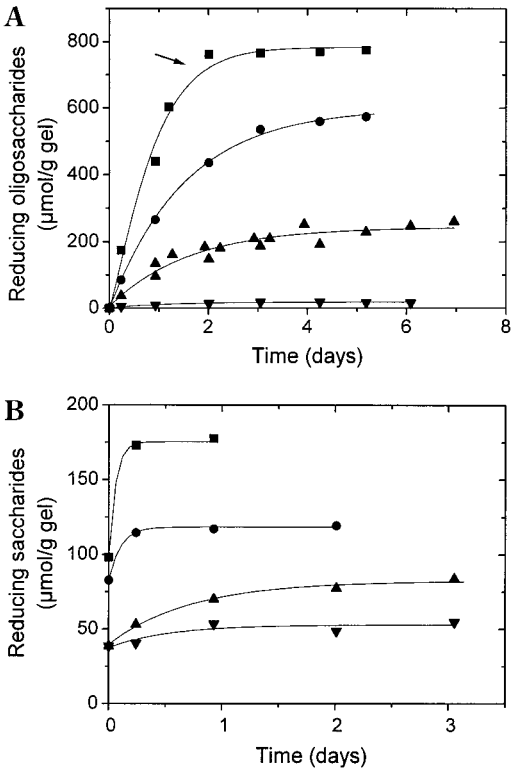

Figure 6. Formation of reducing saccharides in time for enzymatically degrading dextran hydrogels [water content 70% (w/w) and 1 U/g gel] of different DS. DS 4 (■), DS 7 (●), DS 13 (▲), and DS 37 (▼). The arrow indicates the approximate stage at which the hydrogels of DS 4 were dissolved completely. (A) the amount of soluble reducing oligosaccharides. (B) the amount of reducing saccharides inside the hydrogel.

Enzyme Kinetics on Dextran Hydrogels. Dextranase-catalyzed hydrolysis of glycosidic bonds in a hydrogel can result in the formation of both soluble reducing oligosaccharides as well as reducing saccharides that are still connected to the network. Therefore, we determined the formation of both soluble reducing oligosaccharides (Figure 6A) and of reducing end-groups inside the gel (Figure 6B). In agreement with results published earlier[4] and as shown in this paper (Figures 3 and 4), the presence of dextranase in the gel resulted in the formation of soluble oligosaccharides. The initial degradation rate as well as the final extent of degradation (the plateau value of reducing oligosaccharides) increased with decreasing DS. Most reducing saccharides were released from the gel as soluble products. Further, the amount of bound reducing saccharides reached a plateau value earlier than the amount of released oligosaccharides (compare parts A and B of Figure 6).

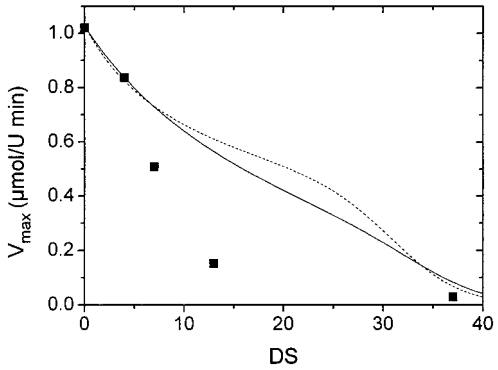

Figure 7. Degradation rate of dextran hydrogels (derived from Figure 6A,B), as a function of the DS, compared with the V_{max} predicted for both variants of the two-substrate model. The dashed line represents the predicted curve for S′ as substrate and the solid line for S′ as inhibitor (see also Figure 5 and Table 4).

Figure 7 gives the overall initial degradation rate as a function of the DS. The curves represent the inhibitor and the substrate variants of the kinetic model as described in Figure 5 and Table 4, extrapolated to hydrogel concentrations of 300 mg of solid/g of gel (corresponding to V_{max} conditions). At low and high DS (DS 4 and 37), a fair estimation for the overall initial degradation rate of the corresponding dextran hydrogel is found. However, significant deviations are found for gels with DS 7 and 13.

Discussion

In a previous study, we showed that dex-MA can be degraded by dextranase to low-molecular-weight oligosaccharides, even if the substrate has a high DS.[4] We also demonstrated that hydrogels with a low DS (DS 4) dissolved completely during enzymatic degradation. On the other hand, hydrogels based on dex-MA with DS 7 or higher were only partially degraded or not susceptible to any degradation (DS 37).[6] To elucidate the mechanistic differences between the enzymatic degradation of dex-MA and dextran hydrogels, we synthesized dex-poly(MA) as a soluble model compound and studied its enzymatic degradation.

Combination of GPC and ES-MS analysis demonstrated that dex-poly(MA) could be degraded by dextranase, resulting in the formation of two types of degradation products, namely unsubstituted low-molecular-weight oligosaccharides and a high-molecular-weight fraction (Figures 3 and 4). Most likely, the latter fraction consists of soluble polymethacrylates to which a number of glucose moieties are still bound. However, it can be expected that this product is rather heterogeneous in terms of molecular weight and composition, making the identification not yet possible with ES-MS.

Enzyme kinetics on dex-poly(MA) was analyzed with a two-substrate model with unsubstituted and monosubstituted chain segments as substrates and by omitting multiply substituted substrates; assumptions that are in line with the results from GPC and ES-MS. This model resulted in values for V_{max} and *K* for the unsubstituted substrate (S) that correspond well with data found for dextran and dex-MA.[4] Interestingly, *K′* for the monosubstituted substrate (S′) in dex-poly(MA) is greater than *K*, reflecting a lower affinity of S′ in dex-poly(MA) for the enzyme (Table 4). However, *K′* for dex-MA was

smaller than *K*, suggesting a higher affinity of S′ in dex-MA for dextranase. In addition, glycosidic bonds of S′ in dex-MA could still be hydrolyzed.[4] In contrast, it is shown in this paper that a model with S′ acting as competitive inhibitor gave a fit similar to that of S′ acting as substrate (Table 4). Probably, this means that monosubstituted chain segments in dex-poly(MA) bind to the active site of the enzyme with a relatively low affinity and that cleavage by the enzyme is difficult in these chain segments. This is in agreement with the observation from ES-MS and GPC, by which only unsubstituted low-molecular-weight compounds are found. Note however, that S′ (and S″; see eq 1−4 and Figure 2) represents a class of an almost infinite number of different substrates and inhibitors in the real situation. Therefore, the obtained values for the kinetic constants should be interpreted as indicative numbers for these classes of substrates.

The products found after enzymatic degradation of dex-MA hydrogels closely resemble the degradation products of dex-poly(MA), indicating that dex-poly(MA) is a good model compound for dextran hydrogels (Figures 3 and 4). Furthermore, the kinetic model that describes the degradation of dex-poly(MA) also gives a good estimation of the enzymatic degradation rate of hydrogels with DS 4 and 37 (Figure 7). The fair estimation at high DS demonstrates that multiply substituted substrates [not taken into account by the kinetic model (equations 1−4)] are indeed not important during enzymatic degradation, even if their concentration is relatively high. However, significant deviations between the kinetic model and the actual degradation rates were found at moderate DS. A possible explanation is that the dextran chains are not only severely strained in hydrogels but that their accessibility for dextranase is reduced as well, because of the formation of interpenetrating networks. Alternatively, diffusion of the enzyme through the dextran matrix can be reduced because some screening (i.e., the mesh size of the hydrogel is smaller than the hydrodynamic diameter of the protein) of proteins with this size (44 kDa) occurs in these gels [DS > 7; water content of 70% (w/w)].[21] Finally, the enzyme might have less conformational or rotational freedom with increasing DS.

Figure 6 shows that reducing sugars linked to the network as well as soluble products are formed for gels with DS 13 or lower. The amount of hydrogel-linked reducing oligosaccharides reached a plateau value within a few hours, whereas soluble products are released for periods up to 2−5 days. Combination of these data with enzyme kinetics suggests that the enzyme hydrolyzes initially glycosidic bonds in an unsubstituted binding site of about 18 or more glucopyranose residues. This renders reducing saccharides bound to the hydrogel matrix. Thereafter, the enzyme can act again on the opened unsubstituted chain segments, resulting in the formation of soluble, unsubstituted reducing oligosaccharides. This, however, does not lead to an increase in reducing end-groups bound to the gel matrix.

The total number of reducing saccharides (soluble and insoluble) measured for the different gels and dex-poly(MA) can be used to calculate the final extent of degradation, further expressed as degradation index (DI). The DI is defined as the amount of reducing saccharides per gram degraded dex-poly(MA) divided by the amount of reducing saccharides per gram degraded native (unsubstituted) dextran and is shown as a function of the DS in Figure 8. As expected, the DI decreases with increasing DS. Furthermore, this figure demonstrates again that dex-poly(MA) is a good model substrate for dextran hydrogels. It can be expected that the DI will be proportional to the fraction of unsubstituted chain segments that are long enough to be degraded to the same extent as dextran. The length of this chain can be calculated from the DS:

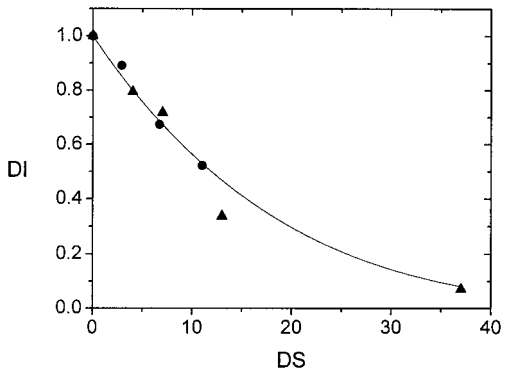

Figure 8. DI as a function of the DS. Data are given for dex-poly(MA) (●) and hydrogels [water content 70% (w/w) and 1 U/g gel dextranase, ▲). The curve represents the best fit on the combined data (derived from Figures 5 and 6) by eq 5.

$$\mathrm{DI} = (1 - \mathrm{DS}/100)^m \qquad (5)$$

Analysis of the data in Figure 8 by nonlinear regression demonstrates that the shortest degradable unsubstituted chain is only 5.9 ± 0.4 (mean ± SD) glucopyranose residues (R^2 = 0.996). The number of glucopyranose residues per methacrylate in the high-molecular-weight fraction was also calculated from the relative amount of this fraction and the DS (see Table 2). The results demonstrate that this number of glucopyranose residues after enzymatic degradation of both dex-poly(MA) and dex-MA hydrogels ranged from 4.4 to 5.9 (mean ± SD = 5.4 ± 0.5). This is in agreement with the result calculated by eq 5. It is therefore concluded that the shortest degradable chain segment is about 5−6 unsubstituted glucopyranose residues long.

Remarkably, the number of glucopyranose residues that are initially involved in binding to the enzyme (n = 18−22, as calculated by enzyme kinetics) is much higher than the number of binding subsites of the enzyme (9−14).[4,16−20] This observation, combined with the results on the final extent of degradation (m = 5−6, Table 2, Figure 8, and eq 5), can be used to propose a model for the degradation of dextran hydrogels by dextranase. Chain segments of 18 or more (n in Table 4) unsubstituted glucopyranose residues (S in eq 1) are likely to have enough conformational freedom to fold correctly in the (shorter) binding site of dextranase (Figure 9A). Therefore, they can be hydrolyzed with a rate similar to that of native (unsubstituted) dextran. These long, unsubstituted chain segments allow multiple scissions by the enzyme, which results in the formation of soluble, unsubstituted oligosaccharides. Chain segments of 6 (m in eq 5) to about 18 unsubstituted glucopyranose residues (S' in eq 1) are probably restricted in their conformational freedom by neighboring substituted glucopyranose residues (Figure 9B). However, these chain segments can still bind to dextranase but with a lower affinity than long unsubstituted chain segments ($K' > K$; see Table 4). Furthermore, these chain segments seem to act initially as

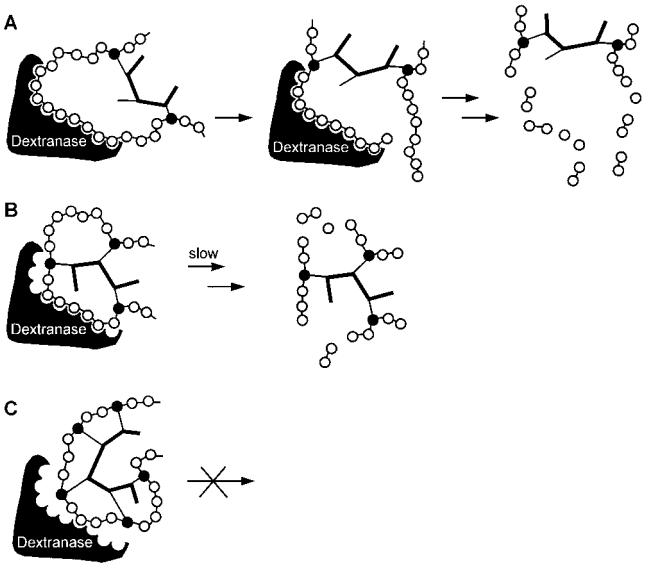

Figure 9. Model describing the enzymatic degradation of a dextran hydrogel. The open circles represent unsubstituted glucopyranose residues, and the filled circles represent substituted ones. (A) Long, unsubstituted chain segment hydrolyzed by dextranase with a similar binding affinity and hydrolysis rate as native dextran. The enzyme can act on the opened loop again. (B) Unsubstituted chain segment of moderate length, as part of a monosubstituted chain, hydrolyzed slower and with a lower affinity. (C) Short, unsubstituted chain segment, as part of a multiply substituted chain, cannot bind to the enzyme.

inhibitor ($\beta V_{max} \approx 0$; see Table 4) but are slowly hydrolyzed once the concentration of easily cleavable substrates has been decreased. On the other hand, chain segments of 6 or less unsubstituted glucopyranose residues (S'' in eq 1) are not hydrolyzed (Figure 9C), most likely because these segments do not have enough conformational freedom to bind to dextranase. This whole process finally results in the formation of soluble oligosaccharides as well as polymethacrylates with 5 to 6 glucopyranose residues per methacrylate. Although the model shown in Figure 9 gives a satisfactory explanation of the results, it should be noted that it is a simplified description of the real situation, in which an almost infinite number of different substrates and inhibiting species is present.

References and Notes

(1) Heller, J.; Pangburn, S. H.; Roskos, K. V. *Biomaterials* **1990**, *11*, 345−350.
(2) Crepon, B.; Jozefonvicz, J.; Chytry, V.; Rihová, B.; Kopecek, J. *Biomaterials* **1991**, *12*, 550−554.
(3) Vercauteren, R.; Bruneel, D.; Schacht, E.; Duncan, R. *J. Bioact. Compat. Polym.* **1990**, *5*, 4−14.
(4) Franssen, O.; Van Ooijen, R. D.; De Boer, D.; Maes, R. A. A.; Herron, J. N.; Hennink, W. E. *Macromolecules* **1997**, *30*, 7408−7413.
(5) Park, K.; Shalaby, W. S. W.; Park, H. In *Biodegradable Hydrogels for Drug Delivery*; Technomic: Basel, Switzerland, 1993.
(6) Franssen, O.; Vos, O. P.; Hennink, W. E. *J. Controlled Release* **1997**, *44*, 237−245.
(7) Brønsted, H.; Hovgaard, L.; Simonsen, L. *S.T.P. Pharma Sci.* **1995**, *5*, 60−64.
(8) Shalaby, W. S. W.; Park, K. *Pharm. Res.* **1990**, *7*, 816−823.
(9) Kurisawa, M.; Terano, M.; Yui, N. *Macromol. Rapid Commun.* **1995**, *16*, 663−666.
(10) Yeh, P.-Y.; Kopecková, P.; Kopecek, J. *Macromol. Chem. Phys.* **1995**, *196*, 2183−2202.
(11) Van Dijk-Wolthuis, W. N. E.; Franssen, O.; Talsma, H.; Van Steenbergen, M. J.; Kettenes-van den Bosch, J. J.; Hennink, W. E. *Macromolecules* **1995**, *28*, 6317−6322.
(12) Van Dijk-Wolthuis, W. N. E.; Kettenes-van den Bosch, J. J.; Van der Kerk-van Hoof, A.; Hennink, W. E. *Macromolecules* **1997**, *30*, 3411−3413.
(13) Van Dijk-Wolthuis, W. N. E.; Van Steenbergen, M. J.; Underberg, W. J. M.; Hennink, W. E. *J. Pharm. Sci.* **1997**, *86*, 413−417.
(14) Segel, I. H. In *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*; Wiley & Sons: New York, 1975.
(15) Bovey, F. A.; Kwei, T. K. In *Macromolecules: An Introduction to Polymer Science*; Bovey, F. A., Winslow, F. H., Eds.; Academic: London, 1979; pp 208−271.
(16) Walker, G. J.; Pulkownik, A. *Carbohydr. Res.* **1974**, *36*, 53−66.
(17) Walker, G. J.; Dewar, M. D. *Carbohydr. Res.* **1975**, *39*, 303−315.
(18) Sugiura, M.; Ito, A. *Chem. Pharm. Bull.* **1974**, *22*, 1593−1599.
(19) Allen, J. D.; Thoma, J. A. *Biochem. J.* **1976**, *159*, 105−120.
(20) Allen, J. D.; Thoma, J. A. *Biochem. J.* **1976**, *159*, 121−132.
(21) Hennink, W. E.; Talsma, H.; Borchert, J. C. H.; De Smedt, S. C.; Demeester, J. *J. Controlled Release* **1996**, *39*, 47−55.

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