Novel in Situ Forming, Degradable Dextran Hydrogels by Michael Addition Chemistry: Synthesis, Rheology, and Degradation

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Received October 25, 2006; Revised Manuscript Received November 28, 2006

ABSTRACT: Various vinyl sulfone functionalized dextrans (dex-VS) ($M_{n,dextran}$ = 14K or 31K) with degrees of substitution (DS) ranging from 2 to 22 were conveniently prepared by a one-pot synthesis procedure at room temperature. This procedure involved reaction of a mercaptoalkanoic acid with an excess amount of divinyl sulfone yielding vinyl sulfone alkanoic acid, followed by conjugation to dextran using *N,N′*-dicyclohexylcarbodiimide (DCC)/4-(dimethylamino)pyridinium 4-toluenesulfonate (DPTS) as a catalyst system. By using two different mercaptoalkanoic acids, 3-mercaptopropionic acid (**1a**) and 4-mercaptobutyric acid (**1b**), dex-VS conjugates with either an ethyl spacer (denoted as dex-Et-VS) or a propyl spacer (denoted as dex-Pr-VS) between the thioether and ester groups were obtained. Linear and four-arm mercaptopoly(ethylene glycol) (M_n = 2.1K) with two or four thiol groups (denoted as PEG-2-SH and PEG-4-SH, respectively) were also prepared. Hydrogels were rapidly formed in situ under physiological conditions by Michael type addition upon mixing aqueous solutions of dex-VS and multifunctional PEG-SH at a concentration of 10−20% w/v. The gelation time ranged from 0.5 to 7.5 min, depending on the DS, concentration, dextran molecular weight, and PEG-SH functionality. Rheological studies showed that these dextran hydrogels are highly elastic. The storage modulus increased with increasing DS, concentration, and dextran molecular weight, and hydrogels with a broad range of storage moduli from 3 to 46 kPa were obtained. Swelling/degradation studies revealed that these dextran hydrogels have a low initial swelling and are degradable under physiological conditions. The degradation time varied from 3 to 21 days depending on the DS, concentration, dextran molecular weight, and PEG-SH functionality. Interestingly, dex-Pr-VS hydrogels showed prolonged degradation times, but otherwise similar properties compared to dex-Et-VS hydrogels. The hydrolysis of the linker ester bonds of the dex-VS conjugates under physiological conditions was confirmed by ^{1}H NMR. The results showed that the hydrolysis kinetics were independent of the DS and the dextran molecular weight. Therefore, the degradation rate of these hydrogels can be precisely controlled.

Introduction

Hydrogels are three-dimensional, hydrated networks of cross-linked hydrophilic polymers. They have been studied extensively for biomedical applications, such as drug delivery[1,2] and tissue engineering,[3] due to their excellent biocompatibility. Hydrogels that can be formed in situ under physiological conditions have received much attention recently due to their many favorable characteristics. Bioactive compounds and/or cells can be mixed homogeneously with the polymer solutions prior to gelation, and the in situ gelation allows preparation of complex shapes and applications using minimally invasive surgery. In situ formed, physically cross-linked hydrogels have been prepared by stimuli-responsive block copolymers,[4−6] stereocomplexation between poly(L-lactide) and poly(D-lactide) blocks of poly(ethylene glycol)−poly(lactide) (PEG−PLA) or dextran−PLA copolymers,[7−10] β-sheet or coiled-coil formation of peptides,[11,12] inclusion complexation between α-cyclodextrins and PEG,[13] and ionic interactions between microparticles of dextran−(2-hydroxyethyl methacrylate) (dextran−HEMA) copolymerized with methacrylic acid (MAA) or (dimethylamino)ethyl methacrylate (DMAEMA).[14] The cross-linking conditions for these types of hydrogels are generally mild, thus allowing for the entrapment of labile compounds, such as proteins. The main drawback of physically cross-linked hydrogels is however that they are generally mechanically weak. Chemically cross-linked hydrogels are generally stronger compared to physically cross-linked hydrogels. The most common in situ formed, chemically cross-linked hydrogels are based on UV irradiation of (meth)acrylate functionalized polymers.[15−20] Their in situ formation in vivo is however limited by the low penetration depth of the UV light due to the absorption by the skin.[21] Hydrogels prepared by Michael type addition reaction between thiols and either

Table 1. Synthesis of Dextran Vinyl Sulfone Derivatives, Dex-Et-VS and Dex-Pr-VS

entry	dextran derivative	$M_{n,GPC}$ dextran × 10^{-3}	molar feeding ratio of mercaptoalkanoic acid to AHG of dextran[a]	DS[e]
1	dex-Et-VS	14	0.30	2
2			0.45	4
3			0.60	8
4			0.60[b]	13
5			0.90	22
6	dex-Et-VS	31	0.30	2
7			0.45	4
8			0.53[c]	9
9			0.60[d]	13
10	dex-Pr-VS	14	0.53	10
11		31	0.90	8

[a] Dextran concentration is 3.3% w/v. [b] Dextran concentration is 4.7% w/v. [c] Dextran concentration is 3.7% w/v. [d] Dextran concentration is 4.3% w/v. [e] Degree of substitution (DS), defined as the number of vinyl sulfone groups per 100 AHG of dextran, was determined by ^{1}H NMR by comparing the peak areas corresponding to the dextran glucosidic protons (δ 3.4−4.1, 5.2, and 5.4) and the protons of the vinyl sulfone group (δ 6.5 and 6.9).

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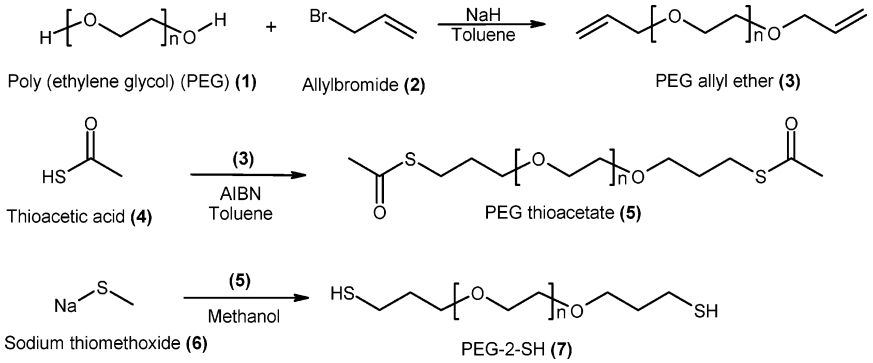
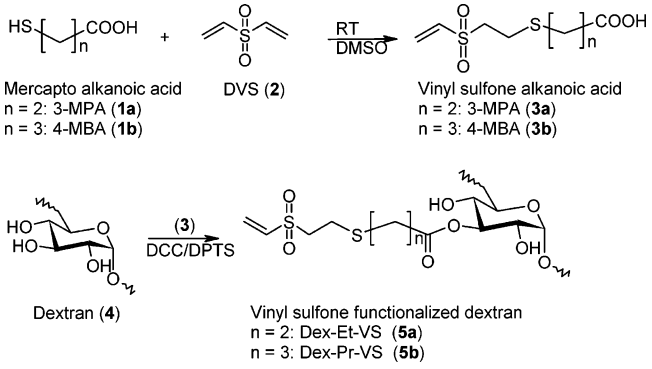
10.1021/ma062468d CCC: $37.00
Published on Web 01/11/2007

Scheme 1. Schematic Representation of the Three-Step Synthesis of Mercapto-PEG, Shown for PEG-2-SH



Scheme 2. Schematic Representation of the One-Pot Synthesis Procedure of Dextran Vinyl Sulfone Conjugates with an Ethyl Spacer (Dex-Et-VS) or a Propyl Spacer (Dex-Pr-VS) between the Thioether and Ester Groups



acrylates or vinyl sulfones may overcome this problem, since they can be rapidly formed under physiological conditions without the aid of UV irradiation. Hubbell and Metters et al. have prepared hydrogels by Michael type addition between small molecules bearing several thiol groups and multiarm star PEG acrylates or vinyl sulfones.[22−27] PEG acrylate hydrogels released human growth hormone in vitro for up to a few months with preservation of the protein integrity. PEG vinyl sulfone hydrogels containing cell-binding and protease-cleavable sites allowed the ingrowth of cells due to cellular activity in vitro. Prestwich et al. have prepared hydrogels by Michael type addition between thiol-modified hyaluronic acid (HA) or chondroitin sulfate (CS) and PEG diacrylate.[28,29] These hydrogels quantitatively released basic fibroblast growth factor in vitro for up to 28 days with 55% of its original biological activity. Furthermore, when modified with cell adhesion peptides, they supported attachment and spreading of fibroblasts in vitro.

Dextran-based materials are highly hydrophilic and biocompatible and show low protein adsorption. Water-soluble dextran with molecular weights of $<\sim$30 000 can be excreted through the kidneys.[30] Dextran has many hydroxyl groups, allowing for a broad range of substitution with functional groups, in contrast to the limited number of functional groups of PEG. Cadee et al. have prepared degradable dextran hydrogels based on dextran−lactate−HEMA derivatives cross-linked by redox-initiated polymerization of the double bonds.[31] These hydrogels were biocompatible when implanted subcutaneously into rats.[32] Maia et al. prepared injectable, degradable dextran hydrogels by cross-linking oxidized dextran with adipic acid dihydrazide (AAD).[33] The gels, formed within 2−4 min, had good mechanical properties and degraded within 3 w. In this paper, we report a novel degradable hydrogel that is rapidly formed under physiological conditions by Michael type addition between dextran vinyl sulfones and multifunctional mercapto-PEG. Our results show that the gelation time, mechanical properties, and the degradation time of the dextran vinyl sulfone hydrogels can be well-controlled by the DS, concentration, and dextran molecular weight.

Materials and Methods

Materials. Dextrans ($M_{n,GPC}$ = 14K with M_w/M_n = 1.45, denoted as dex14K, and $M_{n,GPC}$ = 31K with M_w/M_n = 1.38, denoted as dex31K), linear poly(ethylene glycol) (PEG) ($M_{n,MALDI-TOF\ MS}$ = 2.1K with M_w/M_n = 1.02), calcium hydride, divinyl sulfone, and 2,2′-azobis(isobutyronitrile) (AIBN) were purchased from Fluka. Four-arm PEG ($M_{n,MALDI-TOF\ MS}$ = 2.1K, M_w/M_n = 1.01) was obtained from Nektar. Dextran and PEG were dried by azeotropic distillation with toluene. AIBN was recrystallized from ethanol. 3-Mercaptopropionic acid (3-MPA), sodium hydride, allyl bromide, and dithioerythritol (DTE) were obtained from Aldrich. *N*,*N*′-Dicyclohexylcarbodiimide (DCC), thioacetic acid (TAA), and sodium thiomethoxide (STM) were supplied by Acros. These chemicals were used as received. 4-Mercaptobutyric acid (4-MBA) was prepared by reduction of 4,4′-dithiodibutyric acid (Acros) by tripropylphosphine (Aldrich) and water in dioxane and subsequent evaporation of the solvents. 4-(Dimethylamino)pyridinium 4-toluenesulfonate (DPTS) was synthesized from 4-(dimethylamino)-pyridine (DMAP, Merck) and hydrated *p*-toluenesulfonic acid (PTSA, Fluka) and recrystallized from toluene. Dimethyl sulfoxide (DMSO), dichloromethane (DCM), ethanol, and dioxane were dried over calcium hydride. Toluene was dried over sodium wire. All solvents were distilled prior to use.

Synthesis. Dex-Et-VS. Dextran vinyl sulfone esters with an ethyl spacer between the thioether and the ester groups (denoted as dex-Et-VS) were synthesized by a one-pot synthesis procedure at room temperature from dextran, DVS, and 3-MPA. Typically, DVS (32.85 g, 278 mmol, molar ratio of DVS to 3-MPA is 20) was dissolved in DMSO (90 mL) and 3-MPA (1.476 g, 13.9 mmol, molar ratio of 3-MPA to anhydroglucosidic rings (AHG) of dextran is 0.45) was added dropwise, and the reaction was stirred for 4 h. Dextran (5.0 g, 30.9 mmol AHG, 3.3% w/v concentration), DPTS (0.62 g, 2.1 mmol, molar ratio of DPTS to 3-MPA is 0.15), and DCC (4.346 g, 21.1 mmol, molar ratio of DCC to 3-MPA is 1.5) were dissolved in DMSO (60 mL) and added to the DVS/3-MPA mixture, and the reaction was stirred for another 24 h. Subsequently, the formed *N*,*N*-dicyclohexylurea (DCU) salt was removed by filtration, and the product was recovered by precipitation in cold ethanol. The precipitate was washed with ethanol, dissolved in water (pH 8), and purified by ultrafiltration (MWCO 5000). The final product was obtained by lyophilization. DS (^{1}H NMR): 4. Yield: 4.75 g, 95%. ^{1}H NMR (D_2O): δ 2.8−3.0 (m, $-CH_2-CH_2-S-CH_2-CH_2-$), 3.4−4.1 (m, dextran glucosidic protons), 5.0 (s, dextran anomeric proton), 5.2 and 5.4 (m, glucosidic protons linked to vinyl sulfone substituents), 6.5 (m, $-SO_2CH{=}CH_2$), 6.9 (m, $-SO_2CH{=}CH_2$).

Different degrees of substitution (DS) were obtained by using different molar ratios of 3-MPA to AHG of dextran (ratios were 0.30, 0.45, 0.53, 0.60, and 0.90, Table 1).

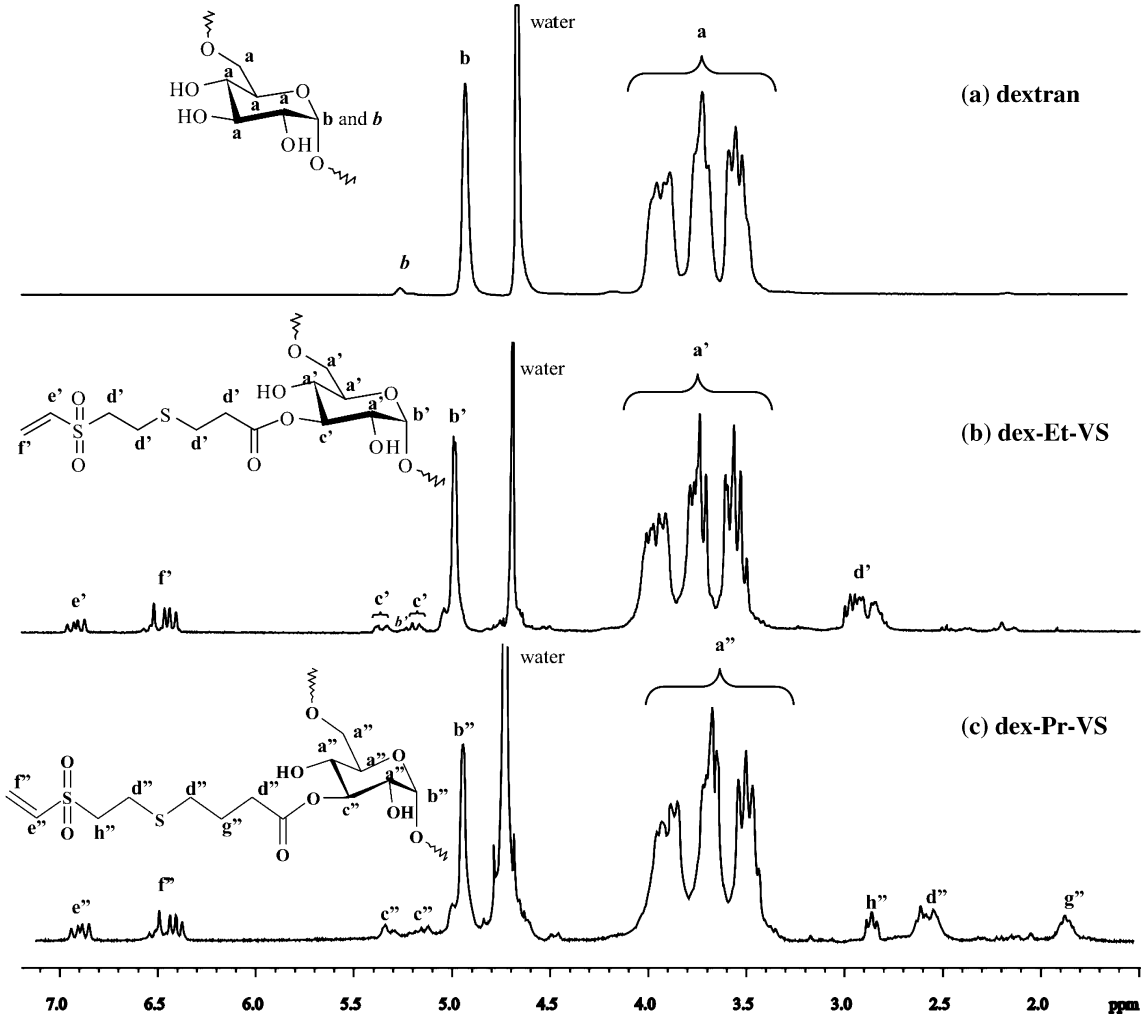


Figure 1. ^{1}H NMR spectra (D_2O) of (a) dextran, (b) dex-Et-VS (Table 1, entry 3), and (c) dex-Pr-VS (Table 1, entry 10). The substitution at position C-3 is given as an example.

Dex-Pr-VS. Dextran vinyl sulfone esters with a propyl spacer between the thioether and the ester groups (denoted as dex-Pr-VS) were synthesized similarly to dex-Et-VS with the exception that 4-MBA was used instead of 3-MPA. Typically, DVS (65.64 g, 556 mmol, molar ratio of DVS to 4-MBA is 20) was dissolved in DMSO (90 mL), a 4-MBA/tripropylphosphine mixture (10.007 g, 27.8 mmol, molar ratio of 4-MBA to AHG is 0.90) was added dropwise, and the reaction was stirred for 4 h. Dextran (5.0 g, 31 mmol AHG, 3.3% w/v concentration), DPTS (1.239 g, 4.2 mmol, molar ratio of DPTS to 4-MBA is 0.15), and DCC (8.684 g, 42.1 mmol, molar ratio of DCC to 4-MBA is 1.5) were dissolved in DMSO (60 mL) and were added to the DVS/4-MBA mixture, and the reaction was stirred for another 24 h. Subsequently, the formed DCU salt was removed by filtration, and the product was recovered by precipitation in cold ethanol. The precipitate was washed with ethanol, dissolved in water (pH 8), and purified by ultrafiltration (MWCO 5000). The final product was obtained by lyophilization. DS (^{1}H NMR): 10. Yield: 3.77 g, 75%. ^{1}H NMR (D_2O): δ 2.0 (m, $-CH_2-CH_2-CH_2-CH_2-S-$), 2.5−2.7 (m, $-CH_2-CH_2-CH_2-CH_2-S-$ and $-S-CH_2-CH_2-SO_2-$), 2.9 (t, $-S-CH_2-CH_2-SO_2-$), 3.4−4.1 (m, dextran glucosidic protons), 5.0 (s, dextran anomeric proton), 5.2 and 5.4 (m, dextran glucosidic protons linked to vinyl sulfone substituents), 6.5 (m, $-SO_2CH=CH_2$), 6.9 (m, $-SO_2CH=CH_2$).

Mercapto-PEG. Linear and four-arm mercapto poly(ethylene glycol) (denoted as PEG-2-SH and PEG-4-SH, respectively) were obtained by a three-step synthesis procedure as reported previously by Goessl et al.[34] (Scheme 1).

First, the hydroxyl groups were converted to allyl groups, which were subsequently reacted with thioacetic acid to yield thioacetate groups. The thioacetate groups were removed by reaction with a base. To convert the hydroxyl groups of PEG into allyl groups, typically linear PEG (**1**, 40 g) was dissolved in toluene (432 mL, hydroxyl group concentration is 93 mM) at 25 °C. Sodium hydride (2.88 g, 120 mmol, 3 times molar excess to hydroxyl groups) was suspended in a small volume of toluene and was added to the solution. After hydrogen evolution, allyl bromide (**2**, 17.4 mL, 200 mmol, 5 times molar excess to hydroxyl groups) was added dropwise to the solution, and the reaction was stirred overnight. Subsequently, the sodium salts were removed by filtration and toluene was evaporated. The product was dissolved in DCM, extracted four times with water, and subsequently the organic phase was dried over anhydrous sodium sulfate. The PEG allyl ether (**3**) was recovered by two times precipitation in cold hexane and dried in vacuo. Conversion (^{1}H NMR): 92%. Yield: 33.37 g, 83%. ^{1}H NMR ($CDCl_3$): δ 3.5−3.7 (m, PEG main chain protons), 4.0 (m, $-O-CH_2-CH=CH_2$), 5.2−5.3 (m, $-O-CH_2-CH=CH_2$), 5.8−6.0 (m, $-O-CH_2-CH=CH_2$).

To obtain the PEG thioacetate (PEG-TA), typically linear PEG allyl ether (**3**, 20 g) was dissolved in toluene (120 mL, allyl group concentration is 160 mM), and the mixture was degassed for 30 min by argon bubbling. Subsequently, AIBN (30.58 g, 192 mmol, 10 mol equiv to allyl groups), and TAA (**4**, 10.9 mL, 154 mmol, 10 mol equiv to allyl groups) were added to the solution. TAA was added in five equal aliquots during an hour, and the reaction proceeded for 24 h at 65 °C. The PEG-TA (**5**) was recovered by three times precipitation in cold diethyl ether and dried in vacuo. Conversion: 100%. Yield: 19.16 g, 96%. ^{1}H NMR ($CDCl_3$): δ 1.8−1.9 (q, $-O-CH_2-CH_2-CH_2-S-$), 2.3 (s, $-CH_2-S-CO-CH_3$), 2.9−3.0 (t, $-O-CH_2-CH_2-CH_2-S-$), 3.5 (t, $-O-CH_2-CH_2-CH_2-S-$), 3.6−3.8 (m, PEG main-chain protons).

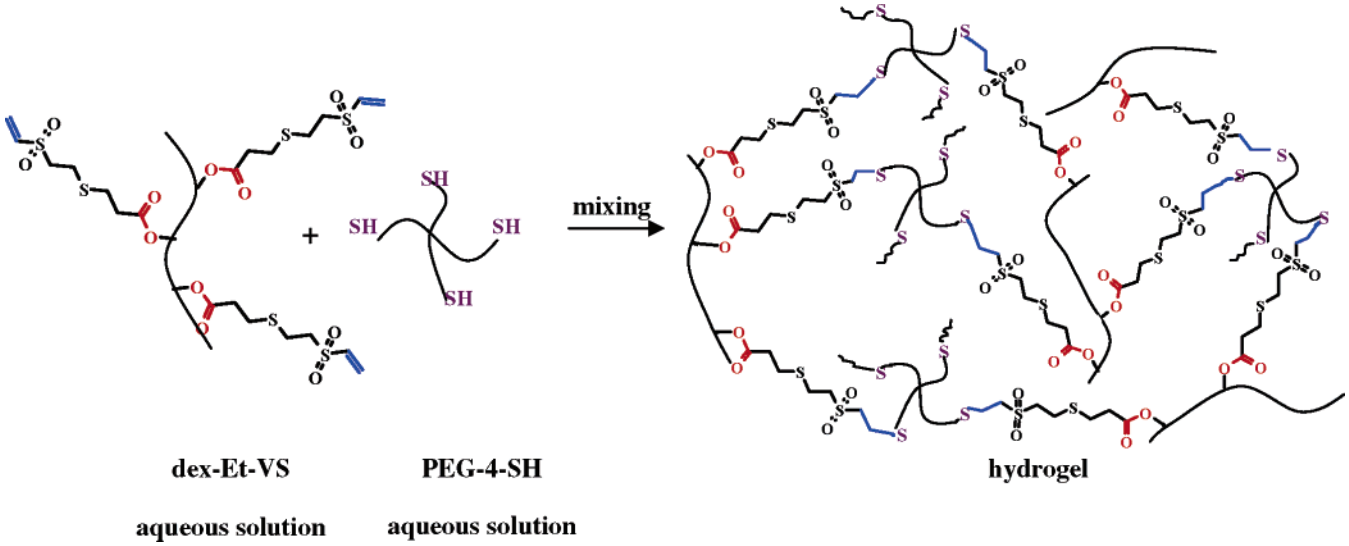


Figure 2. Schematic representation of the Michael type addition between dex-VS and PEG-SH, shown for dex-Et-VS and PEG-4-SH.

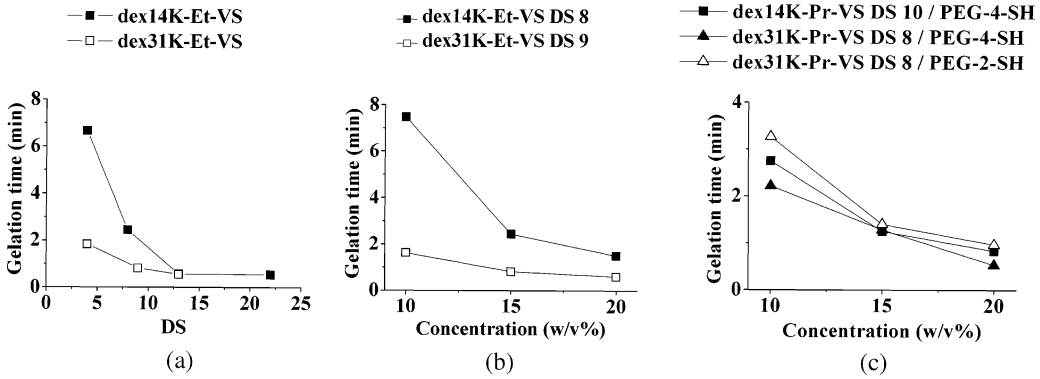


Figure 3. Gelation times (±5 s) determined by the vial tilting method after mixing solutions of dex-VS and PEG-SH (molar ratio of SH to VS is kept at 1.1) in HEPES buffered saline at pH 7 and 37 °C: (a) dex14K-Et-VS and dex31K-Et-VS with PEG-4-SH as a function of the DS at 15% w/v concentration; (b) dex14K-Et-VS DS 8 and dex31K-Et-VS DS 9 with PEG-4-SH as a function of the concentration; (c) dex14K-Pr-VS DS 10 with PEG-4-SH and dex31K-Pr-VS DS 8 with PEG-4-SH or PEG-2-SH as a function of the concentration.

To remove the thioacetate groups and obtain the PEG-2-SH, linear PEG-TA (**5**, 10.25 g) was dissolved in methanol (5 mL, TA concentration is 100 mM). STM (**6**) was dissolved in methanol (92 mL, 1 M) and added to the PEG-TA solution. After 30 min of reaction, the solution was added to 0.1 M HCl (10 mL) and extracted four times with DCM. The organic layer was subsequently washed with brine and dried over anhydrous magnesium sulfate. The solvents were removed under vacuum, and after dissolution in deionized water a small amount of DTE was added to reduce possibly formed disulfide bonds. Finally, PEG-2-SH (**7**) was purified by ultrafiltration against deionized water under a nitrogen atmosphere (MWCO 1000) and obtained by lyophilization. Yield: 5.72 g, 56%. The Ellman test showed a thiol functionality of 85%. ^{1}H NMR ($CDCl_3$): δ 1.80−1.90 (m, −O−CH_2−CH_2−CH_2−SH), 2.52 − 2.60 (q, −O−CH_2−CH_2−CH_2−SH), 3.47−3.53 (t, −O−CH_2−CH_2−CH_2−SH), 3.53−3.80 (m, PEG main-chain protons).

PEG-4-SH was synthesized similarly to PEG-2-SH. The Ellman test showed a thiol functionality of 89%.

Characterization. Molecular weights of dextran were determined by gel permeation chromatography (GPC) using a Viscotek GPCmax with Viscotek 302 triple detection array. As eluent 0.1 M $NaNO_3$ was used with a flow of 1 mL/min. Molecular weights of PEG were determined by MALDI-TOF mass spectrometry (MS) performed on a Voyager (Applied Biosystems) in the reflector mode using ditranol as matrix. ^{1}H NMR spectra were recorded on a Varian Inova spectrometer (Varian, Palo Alto, CA) operating at 300 MHz. The DS of dex-VS is defined as the amount of substituents per 100 AHG. The DS was calculated from the ^{1}H NMR spectra (D_2O) based on the glucosidic protons of dextran (δ 3.4−4.1, 5.2, and 5.4) and the protons of the vinyl sulfone group (δ 6.5 and 6.9). The conversion of the PEG derivatives was calculated from the PEG main-chain protons at δ 3.5−3.7 and protons from the characteristic functional end groups. For PEG allyl ether the protons of the allyl group at δ 5.1−5.3 and 5.8−6.0 were used, and for PEG-TA the protons of the thioacetate group at δ 2.3 were used. The number of free thiol groups of PEG-SH was determined by the Ellman test.[35] Absorption of diluted PEG-SH solutions (PBS buffer, pH 7, 100 mM) was recorded at 412 nm on a Cary 300 Bio UV−vis spectrophotometer (Varian). The concentration of free thiol groups was calculated using a calibration curve derived from mercaptoethanol standard solutions.

Gelation Time and Swelling Tests. To determine the gelation time, solutions of dex-VS with various degrees of substitution and concentrations and PEG-SH (molar ratio of thiol to vinyl sulfone groups is kept at 1.1) in 250 μL of HEPES buffered saline (pH 7, 100 mM, adjusted to 300 mOsm with NaCl) were mixed at 37 °C by vortexing. The gelation time was determined by the vial tilting method. When the sample showed no flow within 20 s, it was regarded as a gel. Subsequently, 3 mL of buffer solution was put on top of the hydrogels, and the hydrogels were allowed to swell at 37 °C. The swollen hydrogels were weighed at regular time intervals after removal of the buffer. After each weighing the buffer was refreshed. The swelling ratio of the hydrogels was calculated from the initial hydrogel weight after preparation (W_0) and the swollen hydrogel weight after exposure to buffer (W_t):

$$\text{swelling ratio} = \frac{W_t}{W_0}$$

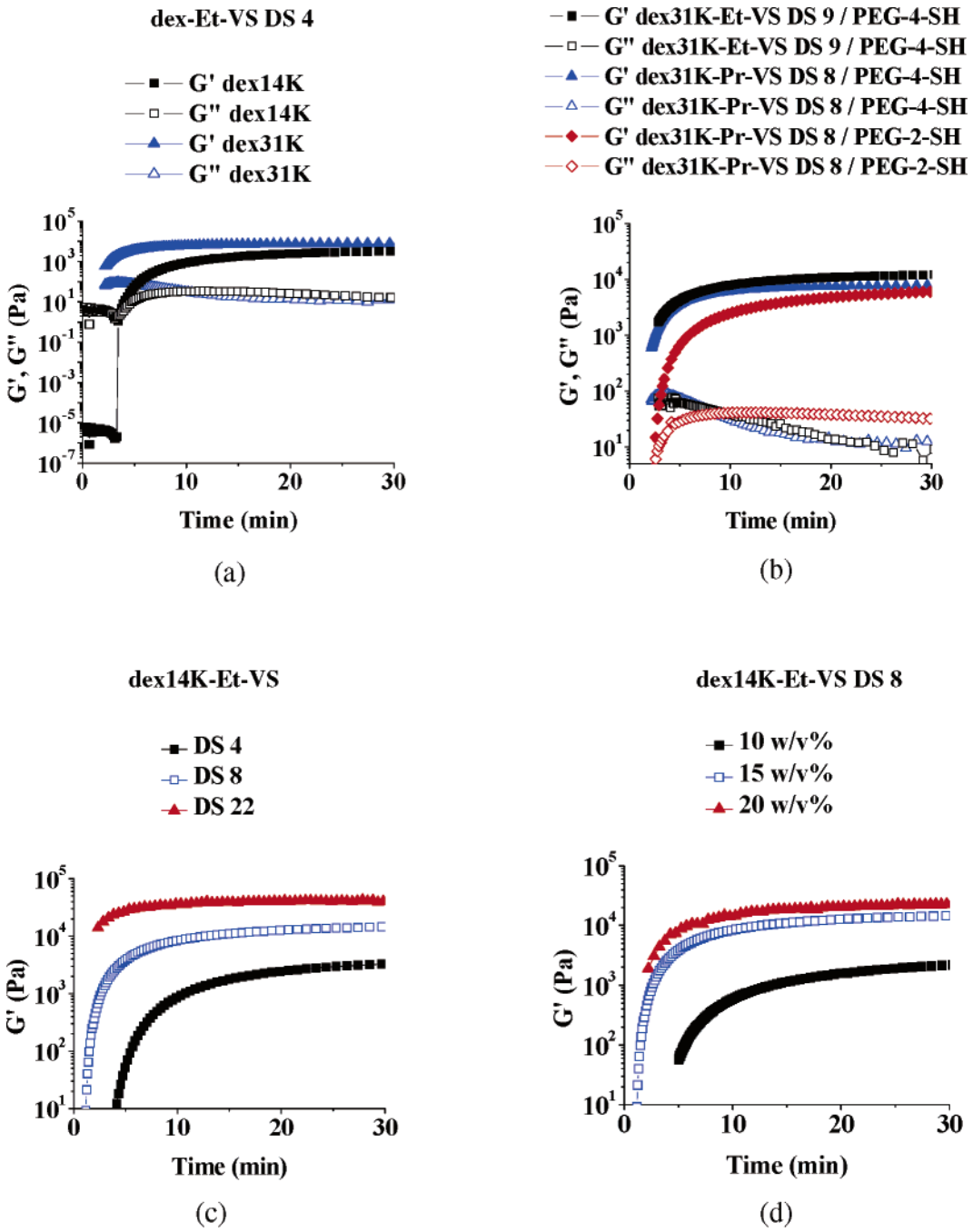


Figure 4. Storage modulus (G') and the loss modulus (G'') as a function of time of dex-VS/PEG-SH mixtures in HEPES buffered saline at pH 7 and 37 °C: (a) dex14K-Et-VS DS 4 and dex31K-Et-VS DS 4 with PEG-4-SH at 15% w/v concentration; (b) dex31K-Et-VS DS 9 with PEG-4-SH and dex31K-Pr-VS DS 8 with PEG-4-SH or PEG-2-SH at 10% w/v concentration; (c) dex14K-Et-VS at DS 4, 8, and 22 at 15% w/v concentration; (d) dex14K-Et-VS DS 8 at 10, 15, and 20% w/v concentration.

Degradation of Dex-VS Materials. The kinetics of the ester bond hydrolysis of the dex-Et-VS and dex-Pr-VS materials at 37 °C in PBS (pH 7, 100 mM, adjusted to 300 mOsm with NaCl) were followed. Dex-VS solutions were placed in dialysis bags (MWCO 3000), which allows complete removal of the vinyl sulfone alkanoic acid degradation byproduct. At regular time intervals samples were taken, and after lyophilization the DS of the dex-VS conjugates was determined by ^{1}H NMR (D_2O).

Rheology. Rheology experiments were performed at 37 °C on a US 200 rheometer (Anton Paar). Solutions of dex-VS and PEG-SH in HEPES buffered saline were mixed (molar ratio of thiol groups to vinyl sulfone groups is kept at 1.1) and quickly applied to the rheometer using a double-barreled syringe with a mixing chamber (Mixpac). To prevent evaporation, a thin layer of oil was applied. Parallel plates (25 mm in diameter) with an adjustable gap were used to have a normal force of maximal 0.1 N, and a frequency of 1 Hz was applied. The strain was adjusted to the torque limits of the machine and was 1% or 0.1%. Both strains are within the linear viscoelastic region.

Results and Discussion

Synthesis of Dextran Vinyl Sulfone Conjugates and Mercaptopoly(ethylene glycol)s. Dextran vinyl sulfone derivatives were prepared by a one-pot synthesis procedure at room temperature using dimethyl sulfoxide (DMSO) as a solvent (Scheme 2).

3-Mercaptoproponic acid (3-MPA, **1a**) was first reacted with 20 times excess of divinyl sulfone (DVS) (**2**) for 4 h. Test reactions, using ^{1}H NMR, showed 100% conversion of **1a**, yielding the corresponding vinyl sulfone propionic acid (**3a**). The formed **3a**, without isolation, was subsequently coupled to

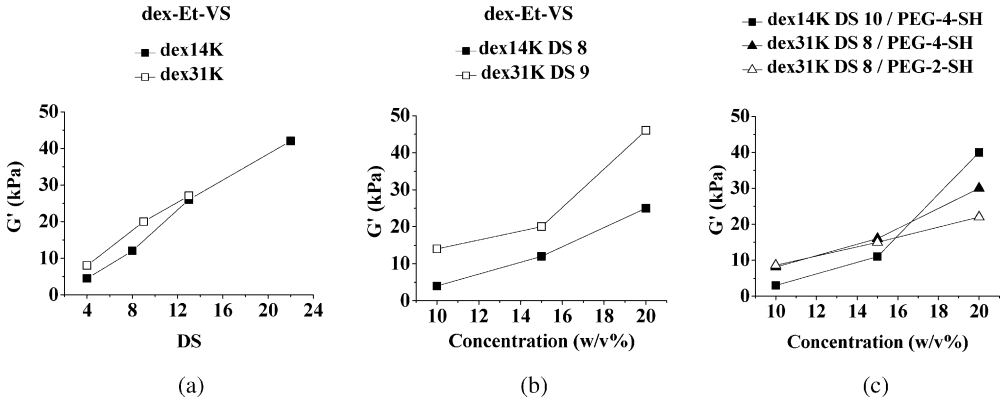


Figure 5. Storage modulus plateau values of dex-Et-VS and dex-Pr-VS hydrogels in HEPES buffered saline at pH 7 and 37 °C: (a) dex14K-Et-VS and dex31K-Et-VS with PEG-4-SH as a function of the degree of substitution (DS); (b) dex14K-Et-VS and dex31K-Et-VS with PEG-4-SH as a function of the concentration; (c) dex14K-Pr-VS DS 10 with PEG-4-SH and dex31K-Pr-VS DS 8 with PEG-4-SH or PEG-2-SH as a function of the concentration.

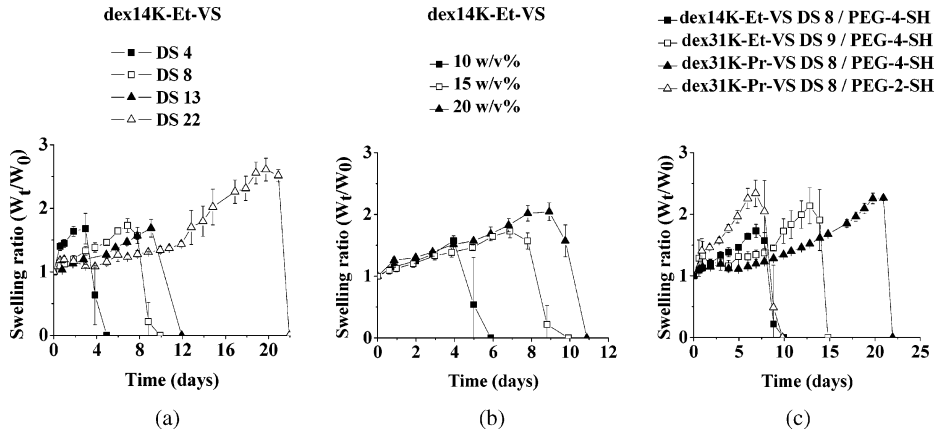


Figure 6. Swelling ratio (W_t/W_0) profiles of dex-VS hydrogels in HEPES buffered saline at pH 7 and 37 °C ($n = 3$): (a) dex14K-Et-VS with PEG-4-SH at DS 4, 8, 13, and 22 at 15% w/v concentration; (b) dex14K-Et-VS DS 8 with PEG-4-SH at concentrations of 10, 15, and 20% w/v; (c) dex14K-Et-VS DS 8 and dex31K-Et-VS DS 9 with PEG-4-SH and dex31K-Pr-VS DS 8 with PEG-4-SH or PEG-2-SH at 15% w/v concentration.

dextran (**4**) using *N*,*N*′-dicyclohexylcarbodiimide (DCC)/4-(dimethylamino)pyridinium 4-toluenesulfonate (DPTS) as a catalyst system. The reaction was allowed to proceed for 24 h, and the resulting dextran vinyl sulfone conjugates (**5a**) were isolated by filtering off the DCU salt, precipitation in cold ethanol, ultrafiltration against water, and lyophilization. Yields of 68−98% were obtained.

The vinyl sulfone derivatization of dextran was confirmed by ^{1}H NMR. Figure 1b shows, besides signals attributed to dextran, new peaks at δ 6.5 and 6.9 (peaks e′ and f′) due to the vinyl sulfone protons (Figure 1b). The vinyl sulfone derivatization was further confirmed by the presence of small peaks at δ 5.2 and 5.4 (peaks c′) due to the peak shift of glucosidic protons of the anhydroglucose unit upon reaction with the vinyl sulfone acid (Figure 1b). We did not study in detail the position at which the substitution took place. The degree of substitution (DS) was determined by comparing the peak areas corresponding to the vinyl sulfone protons at δ 6.5 and 6.9 and the dextran glucosidic protons at δ 3.4−4.1, 5.2, and 5.4. The DS is defined as the number of substituents per 100 anhydroglucosidic rings (AHG). Dextrans with two different molecular weights, 14K and 31K, were used to study the effect of the molecular weight on the hydrogel formation. The DS of dex14K-Et-VS ranged from 2 to 22 when varying the molar ratio of 3-MPA to the AHG of dextran from 0.3 to 0.9 (Table 1, entries 1−5). Likewise, dex31K-Et-VS materials with DS 2−14 were obtained by varying the molar ratio 3-MPA to the AHG of dextran from 0.3 to 0.6 (Table 1, entries 6−9). The DS is proportional to the molar feeding ratio of 3-MPA and AHG of dextran, using the same reaction conditions. At higher dextran concentrations, but otherwise the same conditions, higher DSs could be obtained (Table 1, entry 4). Dextran vinyl sulfone derivatives with a propyl spacer between the thioether and the ester groups (dex-Pr-VS) were also prepared in a similar way (Scheme 2). 4-Mercaptobutyric acid (4-MBA, Scheme 2, **1b**) was obtained by reduction of 4,4′-dithiodibutyric acid using tripropylphosphine and was used without further purification. From the literature it is known that with increased spacing between the thioether and the ester bond the hydrolytic susceptibility of the ester bond decreases.[36] Therefore, the hydrogels derived from dex-Pr-VS are expected to degrade slower compared to the dex-Et-VS hydrogels. The ^{1}H NMR spectrum (Figure 1c) of dex-Pr-VS also showed peaks at δ 6.5 and 6.9 (peaks e″ and f′) due to the presence of the vinyl sulfone substituents and at δ 5.2 and 5.4 (peaks c″) due to the glucosidic protons linked to the vinyl sulfone substituents. Dex14K-Pr-VS with DS 8 and dex31K-Pr-VS with DS 10 were synthesized using molar feeding ratios of 4-MBA to the AHG of dextran of 0.53 and 0.90, respectively (Table 1, entries 10 and 11). This one-pot synthesis procedure is a convenient method to prepare vinyl sulfone-functionalized dextrans with a broad range of substitution degrees.

Mercaptopoly(ethylene glycol)s were synthesized in three steps as reported previously by Goessl et al.[34] In order to investigate the influence of the thiol functionality on the hydrogel formation, two mercaptopoly(ethylene glycol)s with two and four thiol groups (denoted as PEG-2-SH and PEG-4-SH, respectively) were prepared. Both the linear and four-arm

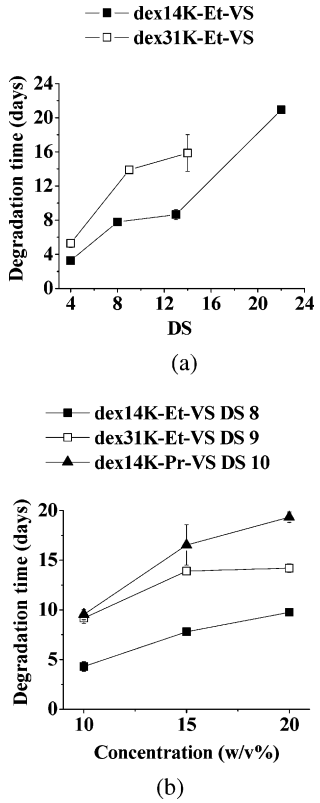


Figure 7. Plots of degradation times vs DS or concentration of dex-Et-VS hydrogels cross-linked with PEG-4-SH in HEPES buffered saline at pH 7 and 37 °C ($n = 3$): (a) dex14K-Et-VS and dex31K-Et-VS as a function of the DS at 15% w/v concentration; (b) dex14K-Et-VS DS 8, dex31K-Et-VS DS 9, and dex14K-Pr-VS DS 10 as a function of the concentration.

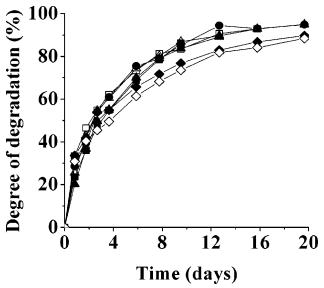


Figure 8. Degree of degradation of dex-VS conjugates in PBS at pH 7 and 37 °C as determined by ^{1}H NMR: dex14K-Et-VS DS 4 (■), DS 8 (□), DS 13 (▲), and DS 22 (△); dex31K-Et-VS DS 13 (●), dex14K-Pr-VS DS 10 (◆), and dex31K-Pr-VS DS 8 (◇).

PEG have a molecular weight of 2.1K, as determined by MALDI-TOF MS. Ellman tests[35] showed a thiol functionality of 85 and 89% for PEG-2-SH and PEG-4-SH, respectively.

In Situ Hydrogel Formation. Dextran hydrogels were formed in situ via Michael type addition between dex-VS and PEG-SH in HEPES buffered saline at pH 7 and 37 °C (Figure 2). The molar ratio of thiol to vinyl sulfone groups was kept at 1.1, since thiol groups may form some disulfide bonds due to exposure to air, thus lowering the effective concentration of free thiol groups. In the concentration range studied (10−20% w/v) these hydrogels were transparent. The gelation time was determined by the vial tilting method. The concentration is defined as the total dry weight of both PEG and dextran per volume of buffer. Figure 3a shows the gelation time as a function of the DS for dex14K-Et-VS and dex31K-Et-VS cross-linked with PEG-4-SH at a constant concentration of 15% w/v. The gelation time decreased with increasing DS and was 7 min for dex14K-Et-VS with DS 4 and 0.5 min for dex14K-Et-VS with DS 13. A further increase in DS did not alter the gelation time.

In Figure 3b the gelation time is shown as a function of the concentration for dex14K-Et-VS DS 8 and dex31K-Et-VS with a comparable DS, cross-linked with PEG-4-SH. The gelation time for dex14K-Et-VS DS 8 decreased from ca. 7.5 to 1.5 min by increasing the concentration from 10 to 20% w/v. Similarly, the gelation time for dex31K-Et-VS DS 9 decreased from 1.5 to 0.5 min when increasing the concentration from 10 to 20% w/v (Figure 3b). In Figure 3c the gelation times are shown of dex14K-Pr-VS DS 10, dex31K-Pr-VS DS 8 cross-linked with PEG-4-SH, and of dex31K-Pr-VS DS 8 cross-linked with PEG-2-SH as a function of the concentration. Similar to dex-Et-VS the gelation time of dex-Pr-VS decreased by increasing the concentration from 10 to 20% w/v and by increasing the dextran molecular weight from 14K to 31K. Increasing the PEG thiol functionality from two to four somewhat decreased the gelation time (from ca. 3 to 2 min) at 10% w/v concentration, while the gelation times were comparable at 15 and 20% w/v concentrations (Figure 3c). Dex31K-Et-VS DS 9 and dex31K-Pr-VS DS 8 showed comparable gelation times at the same concentration (Figure 3, b and c), indicating that the spacer between the thioether and the ester groups has little influence on the gelation process. In general, the gelation times of these dex-VS materials are short compared those (ca. 15 min or longer) reported by Lutolf et al. for four-arm PEG vinyl sulfones cross-linked with dithiol peptides at similar conditions (pH 7, 10% w/v solutions).[37] This is most likely due to the generally higher cross-linking functionality of the dex-VS as compared to the PEG vinyl sulfones. Dex-VS hydrogels could also be formed by using dithioerythritol (DTE) instead of PEG-4-SH or PEG-2-SH. Gelation times ranged from 0.5 to 7 min in a concentration range of 10−20% w/v. In order to be able to compare the influence of different thiol functionalities, only PEG-2-SH and PEG-4-SH were used for further studies. Hydrogels could not be formed at DS 2 at 15% w/v concentration for dex14K-Et-VS and dex31K-Et-VS cross-linked with PEG-4-SH. Apparently, at these conditions the number of reacted groups is lower than the critical cross-linking density at which the three-dimensional network can be formed. For dex-VS with DS 4 or higher, gelation occurred on a time scale of 0.5−7.5 min, which is particularly appealing for application as injectable hydrogels.

Rheology. The mechanical properties of the dextran hydrogels were studied by oscillatory rheology experiments on solutions in HEPES buffered saline at pH 7 and 37 °C. Dex-VS and PEG-SH solutions (molar ratio of thiol groups to vinyl sulfone groups is kept at 1.1) were mixed by a double-barreled syringe with a mixing chamber and quickly applied to the rheometer. Subsequently, the kinetics of the gelation were followed by monitoring the storage modulus (G') and loss modulus (G'') in time. Figure 4a shows that the storage modulus sharply increases after mixing of dex14K-Et-VS DS 4 and PEG-4-SH at 15% w/v concentration. The gelation point is reached 4 min after mixing as indicated by the crossing of the storage and loss modulus. The vial tilting method showed a somewhat longer gelation time of 7 min (Figure 3a). This is attributed to the fact that a certain yield stress is needed to have zero flow at vial tilting. Li et al. found for PEG−poly(butylene oxide) diblock copolymer hydrogels that a yield stress of at least ca. 65 Pa is needed to have zero flow at vial tilting.[38] Dex31K-Et-VS DS 4 cross-linked with PEG-4-SH at 15% w/v concentration showed faster gelation compared to the corresponding dex14K-Et-VS DS 4 mixture due to the higher number of vinyl sulfone groups per

dextran molecule (Figure 4a). The higher number of vinyl sulfone groups per molecule of dex31K-Et-VS also yields higher storage moduli compared to dex14K-Et-VS at the same DS and concentration. Dex31K-Et-VS DS 4 showed a storage modulus of 4.5 kPa, while dex14-Et-VS DS 4 showed a storage modulus of 8 kPa when cross-linked with PEG-4-SH at 15% w/v concentration. A plot of the storage modulus vs the DS (Figure 5a) showed that this effect leveled off at higher DS. Figure 4b shows that both the gelation rate and the storage moduli of dex31K-Pr-VS DS 8 and dex31K-Et-VS DS 9 cross-linked with PEG-4-SH at 10% w/v concentration are similar, indicating that the nature of the spacer between the thioether and the ester groups does not affect the mechanical properties. Dex31K-Pr-VS DS 8 cross-linked with PEG-4-SH gelated faster compared to when PEG-2-SH is used as a cross-linker (Figure 4b).

A plot of the storage modulus vs the concentration (Figure 5c) shows that the storage moduli of dex31K-Pr-VS DS 8 cross-linked with PEG-4-SH or PEG-2-SH are similar at 10 and 15% w/v concentration (ca. 8 and 15 kPa, respectively), and at 20% w/v the storage modulus is somewhat higher for the PEG-4-SH hydrogel (30 vs 22 kPa). The similar storage modulus values may be due to the high vinyl functionality of the dextran, wherein increasing the number of thiol groups from two to four per PEG molecule hardly influences the gel storage modulus. Generally, the damping factors ($\tan \delta = G''/G'$) of these dex-VS hydrogels were lower than 0.01, indicating that these hydrogels are highly elastic. The loss moduli of dex-VS hydrogels with DS higher than 4 at either 15 or 20% w/v concentrations were too low to be accurately measured, and therefore only the evolutions of the storage modulus of these hydrogels are shown. Figure 4c shows that the gelation rate increases considerably with increasing DS for dex14K-Et-VS hydrogels at 15% w/v concentration. At DS 22 the storage modulus plateau value is reached within a few minutes, while at DS 4 this takes ca. 20 min. Dex31K-Et-VS hydrogels showed a similar increase in gelation rate with increasing DS. A plot of the storage modulus vs the DS (Figure 5a) shows that the storage moduli of both dex31K-Et-VS and dex14K-Et-VS hydrogels increase almost linearly with increasing DS. At DS 4 dex14K-Et-VS hydrogels have a storage modulus of 4.5 kPa, while at DS 22 the storage modulus is 42 kPa. As shown in Figure 4d, the gelation rate of dex14K-Et-VS cross-linked with PEG-4-SH increases by increasing the concentration from 10 to 20% w/v. Plots of the storage modulus vs the polymer concentration (Figure 5b,c) show that the storage modulus of dex-Et-VS and dex-Pr-VS hydrogels increases with increasing concentration. For example, at 10% w/v dex14K-Et-VS DS 8 hydrogels have a storage modulus of 4 kPa, while at 20% w/v the storage modulus is 25 kPa (Figure 5b). In summary, dex-VS hydrogels with storage moduli ranging from 3 to 46 kPa could be obtained by varying the DS, concentration, and dextran molecular weight.

Hydrogel Swelling and Degradation. The dex-VS hydrogels were degradable under physiological conditions. To study the rate of degradation of these hydrogels, solutions of dex-VS and PEG-SH were mixed in HEPES buffered saline at pH 7 and 37 °C (molar ratio of thiol groups to vinyl sulfone groups is kept at 1.1). After the hydrogels were formed, HEPES buffer was applied on top, and the gels were allowed to swell at 37 °C. At regular time intervals, the swelling ratio was calculated by rationing the swollen hydrogel weight with the initial hydrogel weight (W_t/W_0). Figure 6 shows that in general the initial swelling ratio of the dex-VS hydrogels is low. All hydrogels displayed gradual swelling in time, until they rapidly dissolved. This is caused by the hydrolytic cleavage of the ester bonds between the dextran backbone and the thioether group. The degradation time is defined as the time after which the hydrogel is almost or completely dissolved.

A plot of the degradation time vs the DS (Figure 7a) revealed that the hydrogel degradation time increased with increasing DS. The corresponding dex31K-Et-VS hydrogels follow the same trend and showed degradation times of 5 and 14 days at DS 4 and DS 13, respectively (Figure 6a). In Figure 6b, the swelling ratio profiles are shown of dex14K-Et-VS DS 8 hydrogels cross-linked with PEG-4-SH at 10, 15, and 20% w/v concentrations. Increasing the concentration from 10 to 20% w/v increased the hydrogel degradation time from 3 to 9 days. For the corresponding dex31K-Et-VS DS 9 hydrogels the degradation time increased from 9 to 14 days when increasing the concentration from 10 to 15% w/v, while the effect leveled off at 20% w/v (Figure 7b).

Figure 7b shows that by increasing the dextran molecular weight from 14K to 31K the hydrogel degradation time almost doubles due to the higher number of vinyl sulfone groups per dextran molecule. Interestingly, as shown in Figure 6c, the use of a propyl spacer instead of an ethyl spacer between the thioether and ester groups considerably increases the hydrogel degradation time. The hydrogel degradation times are 14 and 21 days for dex31K-Et-VS DS 9 and dex31K-Pr-VS DS 8 cross-linked with PEG-4-SH at 15% w/v concentration, respectively. This is due to the lower positive charge on the carbonyl carbon when more methylene groups are spaced between the thioether and the ester linkage, rendering the ester linkage less susceptible to hydrolysis.[36] Figure 7b shows that, similar to dex-Et-VS hydrogels, the degradation time of the dex-Pr-VS hydrogels increases with increasing concentration from 10 to 20% w/v. The degradation times were 10 and 19 days at 10 and 20% w/v, respectively. As shown in Figure 6c, the degradation time decreases with decreasing the number of thiol groups per PEG molecule (8 vs 21 days).

The degradation of dex-VS conjugates in PBS at pH 7 and 37 °C was followed by ^{1}H NMR. A dialysis bag (MWCO 3000) was used, and at regular time intervals samples were removed and lyophilized and the remaining DS was determined by rationing the peak areas of the dextran glucosidic protons and protons of the vinyl sulfone group.

Figure 8 shows that degradation rates for dex14K-Et-VS conjugates with DS 4, 8, 13, and 22 and of dex31K-Et-VS DS 13 are similar, indicating that the hydrolysis kinetics are independent of the DS and the dextran molecular weight. On the other hand, dex14K-Pr-VS DS 10 and dex31K-Pr-VS DS 8 degrade somewhat slower than dex-Et-VS conjugates. This agrees well with the slower degradation of the dex-Pr-VS hydrogels compared to the corresponding dex-Et-VS hydrogels. In summary, the degradation rate of dex-VS hydrogels can be readily controlled by the DS, concentration, dextran molecular weight, PEG-SH functionality, and the length of the spacer between the thioether and ester groups.

Conclusions

Dextrans with pendent vinyl sulfone groups linked by a hydrolytically susceptible ester bond were synthesized by a one-pot synthesis procedure to a broad range of degrees of substitution. Hydrogels were rapidly formed in situ under physiological conditions by mixing aqueous solutions of vinyl sulfone-functionalized dextrans and multifunctional mercapto-PEG. Their mechanical and degradation properties are readily controlled by the degree of vinyl sulfone substitution, concentration, dextran molecular weight, and PEG thiol functionality. The

hydrogels showed storage moduli ranging from 3 to 46 kPa and degraded within 3−21 days. Furthermore, hydrogels with similar mechanical properties, but decreased degradation rates, could be prepared by increasing the spacer length between the thioether and the ester groups. These hydrogels are very promising for use in biomedical applications, since they can be rapidly formed in situ in the body by co-injection of aqueous solutions of vinyl sulfone dextran and multifunctional mercapto-PEG. Also, they offer a broad range of degradation and mechanical properties. Furthermore, in principle, bioactive molecules, such as proteins and peptides, can readily be incorporated by using thiol-containing biomolecules to give biomimetic scaffolds.

Acknowledgment. This work was funded by the Netherlands Organization for Scientific Research (NWO).

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MA062468D