Rapidly in Situ-Forming Degradable Hydrogels from Dextran Thiols through Michael Addition

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Thiol-functionalized dextrans (dex-SH) ($M_{n,dextran}$ = 14K or 31K) with degrees of substitution (DS) ranging from 12 to 25 were synthesized and investigated for in situ hydrogel formation via Michael type addition using poly(ethylene glycol) tetra-acrylate (PEG-4-Acr) or a dextran vinyl sulfone conjugate with DS 10 (dex-VS DS 10). Dex-SH was prepared by activation of the hydroxyl groups of dextran with 4-nitrophenyl chloroformate and subsequent reaction with cysteamine. Hydrogels were rapidly formed in situ under physiological conditions upon mixing aqueous solutions of dex-SH and either PEG-4-Acr or dex-VS DS 10 at polymer concentrations of 10 to 20 w/v%. Rheological studies showed that these hydrogels are highly elastic. By varying the DS, concentration, dextran molecular weight, and type of cross-linker, hydrogels with a broad range of storage moduli of 9 to 100 kPa could be obtained. Varying the ratio of thiol to vinyl sulfone groups from 0.9 to 1.1 did not alter the storage modulus of the hydrogels, whereas larger deviations from equimolarity (thiol to vinyl sulfone ratios of 0.75 and 1.5) considerably decreased the storage modulus. The plateau value of hydrogel storage modulus was reached much faster at pH 7.4 compared to pH 7, due to a higher concentration of the thiolate anion at higher pH. These hydrogels were degradable under physiological conditions. Degradation times were 3 to 7 weeks for dex-SH/dex-VS DS 10 hydrogels and 7 to over 21 weeks for dex-SH/PEG-4-Acr hydrogels, depending on the DS, concentration, and dextran molecular weight.

Introduction

Dextran is highly hydrophilic and biocompatible, hardly shows interactions with proteins, and can be excreted through the kidneys up to molecular weights of ca. 30 000.[1] Dextran hydrogels have been studied extensively for biomedical applications, in particular for drug delivery and tissue engineering.[2−13] The group of Chu has prepared degradable dextran hydrogels by copolymerization of dextran modified with allyl groups and poly(D,L-lactide) diacrylate. These hydrogels released indomethacin, a low molecular weight drug, with biphasic release kinetics (an initial burst effect followed by a slower sustained release), wherein 100% was released in ca. 1 month.[2,3] Logeart-Avramoglou et al. have formed hydrogels by cross-linking dextran carboxylate, sulfate, and benzylamide derivatives with sodium trimetaphosphate.[4,5] Hydrogels loaded with bone morphogenetic protein (BMP) were shown to be osteoinductive when implanted in rats. Shoichet et al. prepared cell-adhesive dextran hydrogels, by copolymerization of dextran-methacrylate (dex-MA) with aminoethyl methacrylate (AEMA) followed by coupling of cell-adhesive peptide sequences.[6] Sawicka et al. prepared glucose-responsive hydrogels by copolymerization of dextran acrylate and concanavalin A acrylate for self-regulated insulin delivery.[7]

Recently, hydrogels formed in situ under physiological conditions have received much attention, due to their many favorable characteristics. For instance, bioactive compounds and/or cells can be mixed homogeneously with the polymer solutions prior to gelation. Furthermore, in situ gelation allows preparation of complex shapes and applications using minimally invasive surgery. Hennink et al. prepared in situ forming, degradable dextran hydrogels by stereocomplexation of dextran-poly(L-lactide) and dextran-poly(D-lactide) graft copolymers.[8,9] When loaded with the protein interleukin-2 (IL-2) these hydrogels were shown to be effective tools for immunotherapy of SL2 lymphoma in mice.[10] Hydrogels were also formed in situ by ionic interactions between oppositely charged microspheres of dextran-HEMA copolymerized with methacrylic acid (MA) or dimethylaminoethyl methacrylate (DMAEMA).[11] These hydrogels showed a primarily diffusion-controlled released of lysozyme up to 25 days, wherein the enzymatic activity of lysozyme was preserved.[12] Gil et al. have synthesized in situ formed, degradable dextran hydrogels by cross-linking oxidized dextran with adipic acid dihydrazide (AAD).[13] The hydrogels formed rapidly at pH 8, 37 °C with good mechanical properties and degraded within 3 weeks.

Michael addition between thiols and either acrylates or vinyl sulfones has recently been used for in situ formation of hydrogels.[14−19] The reaction is highly selective versus biological amines[14] and can be carried out under physiological conditions. Moreover, biomimetic scaffolds can be easily obtained by incorporation of thiol-bearing molecules. Hubbell et al. prepared in situ forming hydrogels by Michael addition of multifunctional poly(ethylene glycol) (PEG) vinyl sulfones or acrylates and multifunctional thiol compounds.[14−16] These hydrogels released bovine serum albumin (BSA) with zero-order kinetics for ca. 4 days.[14] Cell-responsive hydrogels were prepared by Michael addition between four-arm PEG vinyl sulfone and a bis-cysteine, protease-cleavable peptide sequence, in the presence of a monocysteine cell-adhesion peptide sequence.[16] Fibroblasts adhered

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to these hydrogels and were able to migrate into the hydrogel. Prestwich et al. prepared hydrogels by Michael type addition between thiol-modified hyaluronic acid (HA) and PEG diacrylate.[17] These hydrogels were shown to induce angiogenesis in vivo by the dual release of vascular endothelial growth factor (VEGF) and keratinocyte growth factor (KGF).[17] Hydrogels that recruited fibroblasts in vivo were also prepared by Michael addition of HA and PEG diacrylate in the presence of a monocysteine fibronectin functional domain.[18] We have previously reported on degradable hydrogels that rapidly formed in situ by Michael addition of dextran vinyl sulfone conjugates and multifunctional mercapto PEG.[19] The gelation time, hydrogel degradation time, and storage moduli were well-controlled by the degree of substitution (DS), concentration, and dextran molecular weight. However, the hydrogels degraded within 3 to 21 days, which is too fast for certain applications, such as tissue engineering of cartilage. In this paper we report on dextran hydrogels prepared by a different approach, in which dextran thiols (dex-SH) are cross-linked with PEG tetra-acrylate (PEG-4-Acr) or a dextran vinyl sulfone conjugate (dex-VS DS 10). These hydrogels degrade much slower compared to the previously reported dex-VS/PEG-SH hydrogels, with degradation times ranging from 3 to over 21 weeks. Furthermore, the dex-SH/PEG-4-Acr hydrogels can be obtained with high storage moduli up to 100 kPa.

Materials and Methods

Materials. Calcium hydride and cysteamine were purchased from Fluka. Dithioerythritol (DTE) was obtained from Aldrich. 4-Nitrophenyl chloroformate (4-NC) and pyridine were supplied by Acros. These chemicals were used as received. Dextrans ($M_{n,GPC}$ = 14K, M_w/M_n = 1.45, denoted as dex14K, and $M_{n,GPC}$ = 31K, M_w/M_n = 1.38, denoted as dex31K) (Fluka) and 4-arm poly(ethylene glycol) (PEG) (Nektar) were dried by azeotropic distillation of toluene. Toluene was previously dried over sodium wire followed by distillation. Dimethylformamide (DMF) and dichloromethane (DCM) were dried over calcium hydride and molecular sieves 4 Å, respectively, and distilled before use. Triethylamine (TEA) (Aldrich) was dried over calcium hydride and distilled prior to use. Lithium chloride was obtained from J.T. Baker and dried in vacuo at 80 °C. Vinyl sulfone functionalized dextran ($M_{n,dextran}$ = 14K) with DS 10 (denoted as dex-VS DS 10) was prepared as reported previously.[19]

Synthesis. Dex-SH. To obtain thiol functionalized dextran (dex-SH), the hydroxyl groups were first activated with 4-NC, as reported previously by Ramirez et al.,[20] and subsequently reacted with cysteamine. Typically, dextran (7.5 g, 46 mmol anhydroglucosidic rings, AHG) was dissolved in DMF (225 mL) containing 2 w/v% of lithium chloride at 90 °C. The reaction mixture was cooled to 0 °C, and pyridine (2.1 mL, 26 mmol, molar ratio of pyridine to AHG is 0.56) was added to the dextran solution followed by 4-NC (5.25 g, 26 mmol, molar ratio of 4-NC to AHG is 0.56). The reaction mixture was stirred for 2 h at 0 °C, and finally the 4-NC activated dextran was obtained by twice precipitation in cold ethanol, washed with diethyl ether, and dried in vacuo over phosphorus pentoxide. DS (^{1}H NMR): 15. Yield: 7.38 g, 98%. ^{1}H NMR (DMSO-d_6): δ 3.1−4.0 (m, dextran glucosidic protons), 4.7 (s, dextran anomeric proton), 4.5, 4.9, and 5.1 (s, dextran hydroxyl protons), 5.3 and 5.5 (s, dextran glucosidic protons at positions which have nitrophenyl substituents), 7.5 and 8.3 (dd, aromatic protons).

In the second step, typically 4-NC activated dextran (7.0 g, 37 mmol AHG) was dissolved in DMF (56 mL, concentration of nitrophenyl groups is 0.10 M) containing 1 w/v% of lithium chloride. Cysteamine (3.49 g, 45 mmol, molar ratio of cysteamine to nitrophenyl groups is 8) was dissolved in DMF (56 mL) and added to the dextran solution. The reaction was stirred for 24 h at room temperature. Subsequently, the product was recovered by precipitation in cold ethanol and washed several times with ethanol to remove *p*-nitrophenol. A small amount of DTE was added to a solution of dex-SH in water to reduce any disulfide bonds. This solution was ultrafiltrated (MWCO 5000) against deionized water under a nitrogen atmosphere and the product was finally obtained by lyophilization. DS: 12. Yield: 4.50 g, 64%. ^{1}H NMR (D_2O): δ 2.7 (t, NHCH_2), 3.3 (d, CH_2SH), 3.4−4.1 (m, dextran glucosidic protons), 5.2 and 5.3 (s, dextran glucosidic protons at positions which have thiol group containing substituents).

PEG-4-Acr. PEG tetra-acrylate (PEG-4-Acr) was synthesized from four-arm PEG and acryloyl chloride according to the procedure reported by Hubbell et al.[14] Typically, PEG (7.0 g, 3.3 mmol) was dissolved in DCM (260 mL, hydroxyl group concentration is 54 mM). After dissolution of the PEG, TEA (2.83 g, 28 mmol, molar ratio of TEA to hydroxyl groups is 2) was added and the solution was cooled to 0 °C. Subsequently, acryloyl chloride (1.9 g, 21 mmol, 1.5 equiv to hydroxyl groups) was added dropwise and the reaction was stirred overnight at room temperature. The solution was filtered over Celite, and the filtrate was stirred with sodium carbonate for 1.5 h. After filtration and concentration, the modified PEG was precipitated by adding cold diethyl ether in an ice bath. The precipitate was further purified by ultrafiltration (MWCO 1000) against deionized water and finally recovered by lyophilization. Conversion (^{1}H NMR): 91%. Yield: 1.85 g, 26%. ^{1}H NMR ($CDCl_3$): δ 3.5−3.7 (m, PEG main chain protons), 4.3 (t, CH_2-OCO), 5.9 (d, *CH*═CH_2), 6.2 and 6.4 (dd, CH═CH_2).

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. The molecular weight of PEG was determined by MALDI-TOF mass spectrometry performed on a Voyager mass specrometer (Applied Biosystems) in the reflector mode using ditranol as a matrix. ^{1}H NMR spectra were recorded on a Varian Inova Spectrometer (Varian, Palo Alto, CA) operating at 300 MHz. The degree of substitution (DS) of dextran is defined as the number of substituents per 100 AHG. The DS of the nitrophenyl activated dextrans was calculated from the ^{1}H NMR spectra (DMSO-d_6) based on the glucosidic protons of dextran (δ 3.1−4.0, 5.3 and 5.5) and the protons of the nitrophenyl groups (δ 7.5 and 8.3). The number of free thiol groups of the dex-SH was determined by the Ellman test.[21] The absorption at 412 nm of diluted dex-SH solutions (PBS buffer, pH 7.4, 100 mM) was recorded on a Cary 300 Bio UV-Visible Spectrophotometer (Varian) and the free thiol concentration was calculated using a calibration curve derived from mercaptoethanol standard solutions. The conversion of PEG acrylate was calculated from ^{1}H NMR spectra ($CDCl_3$) based on the PEG main chain protons (δ 3.5−3.7) and the protons of the acrylate groups (δ 5.9, 6.2, and 6.4).

Gelation Time and Swelling Tests. To determine the gelation time, 250 μL solutions of dex-SH and PEG-4-Acr or dex-VS DS 10 (molar ratio of thiol to unsaturated groups was kept at 1.1) in 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 5 s, it was regarded as a gel. For the swelling test, hydrogels were allowed to swell at 37 °C after applying 3 mL of HEPES-buffered saline. The swelling experiment was performed in triplicate. The swollen hydrogels were weighed at regular time intervals after removal of the buffer and 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):

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

Rheology. Rheology experiments were performed at 37 °C on a US 200 rheometer (Anton Paar). The dex-SH and the PEG-4-Acr or dex-VS DS 10 solutions in HEPES-buffered saline were mixed (molar ratio of thiol groups to unsaturated groups was kept at 1.1, unless mentioned

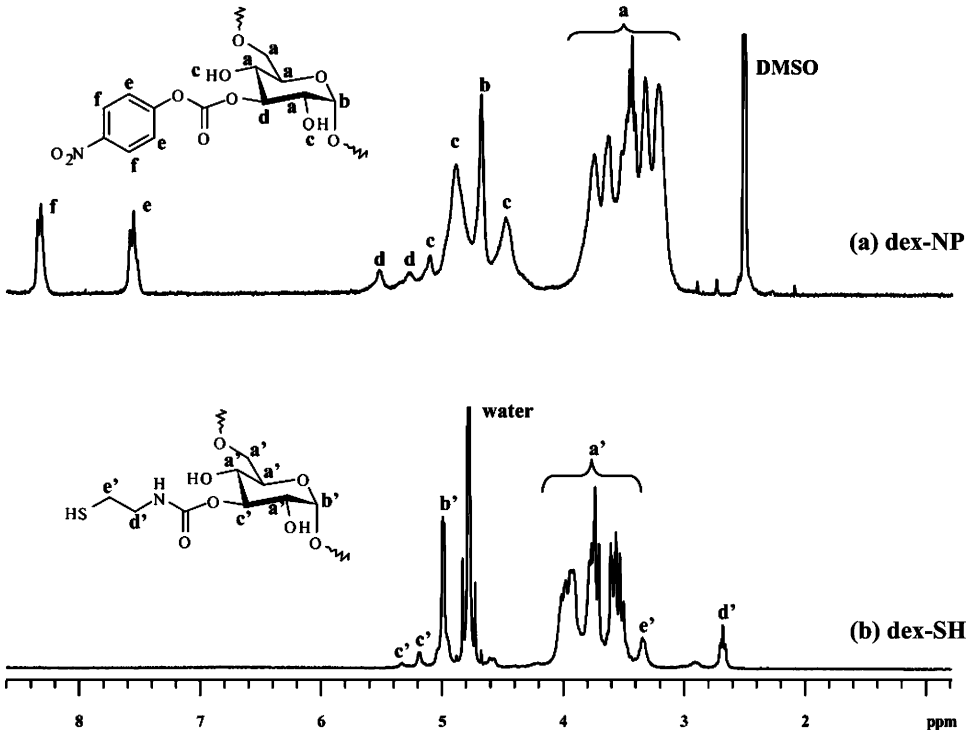
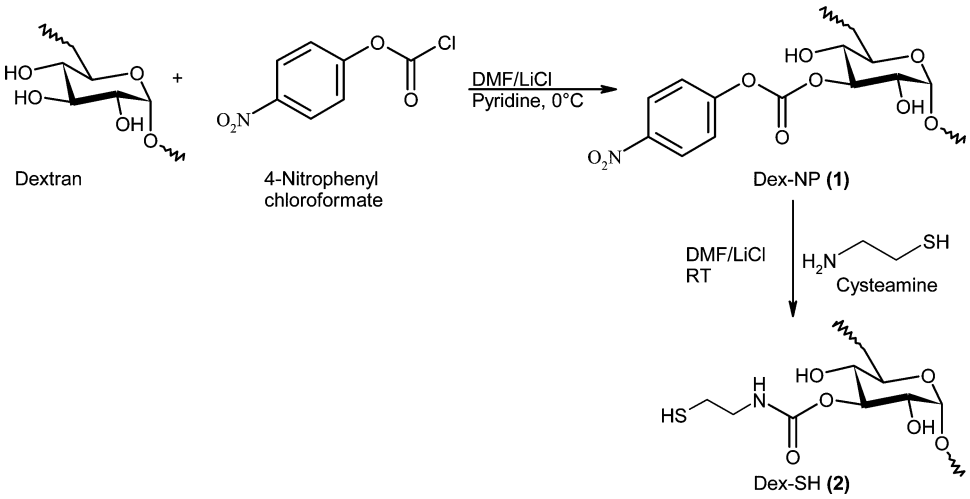


Figure 1. ^{1}H NMR spectra of (a) dex-NP (DMSO-d_6, Table 1, entry 4) and (b) dex-SH (D_2O, Table 1, entry 4). The substitution at position C-3 is given as an example.

Scheme 1. Schematic Representation of the Two-Step Synthesis of Thiol-Functionalized Dextran (dex-SH). The Substitution at Position C-3 Is Given as an Example

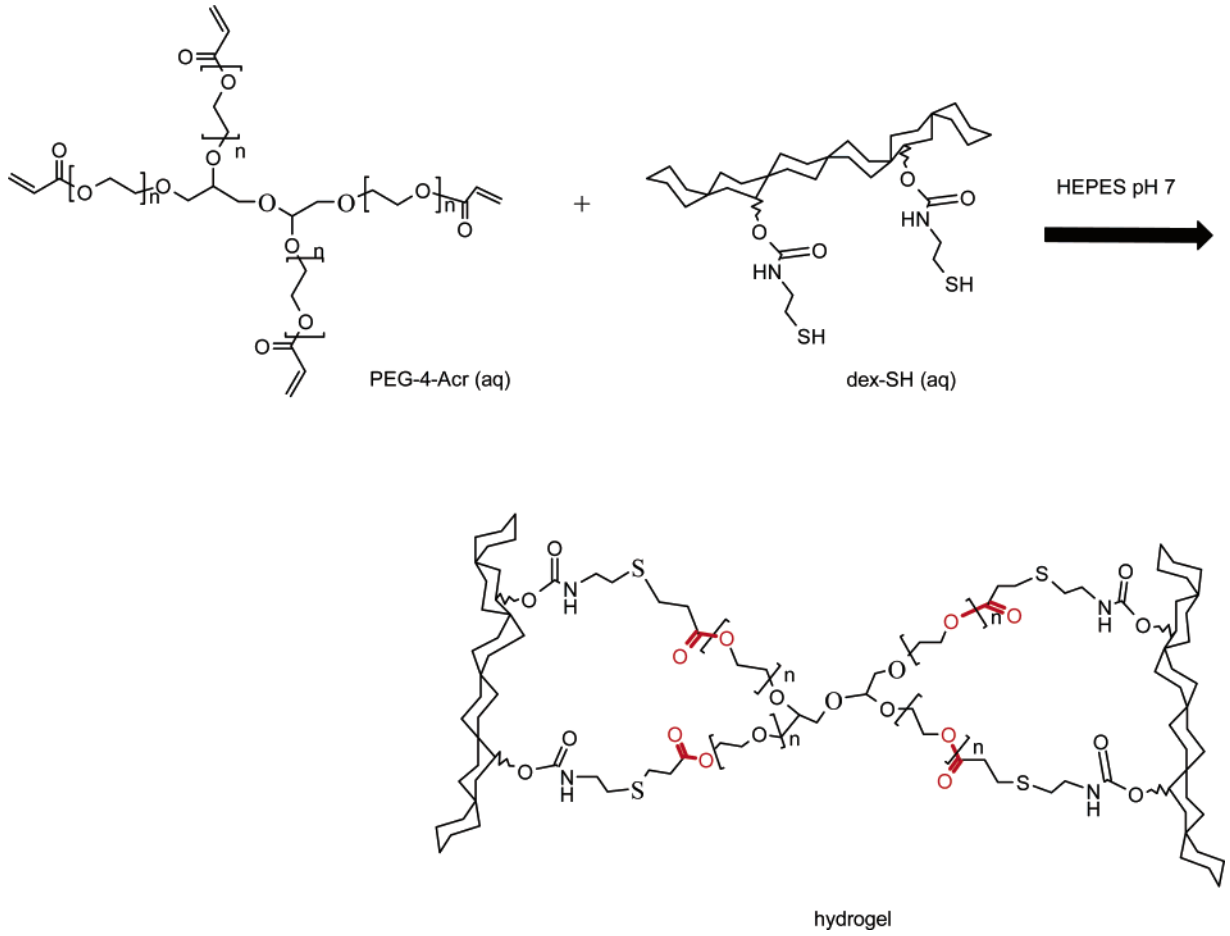


otherwise) and quickly applied to the rheometer using a double-barreled syringe with a mixing chamber (Mixpac). To prevent evaporation, a thin layer of oil was applied. Parallel plates (25 mm in diameter) were used with an adjustable gap width to keep the normal force close to 0 N (maximal normal force is 0.1 N). The storage and loss modulus were measured at a strain of 0.1% and a frequency of 1 Hz.

Results and Discussion

Synthesis of Dextran−Thiol Conjugates and Poly(ethylene glycol) Acrylate. Thiol functionalized dextrans (dex-SH) were synthesized by a two-step reaction using dimethylformamide (DMF)/LiCl as a solvent (Scheme 1). Dextrans with molecular weights of 14K (denoted as dex14K) and 31K (denoted as dex31K) were used. First, the hydroxyl groups of dextran were activated with 4-nitrophenyl chloroformate (4-NC) using pyridine as a catalyst at 0 °C for 2 h to yield nitrophenyl-substituted dextran (dex-NP) (**1**).[20] Dex-NP was purified by precipitation in cold ethanol. Then dex-NP was reacted with an excess amount of cysteamine at room temperature for 24 h. Interestingly, under the used conditions (room temperature and DMF/LiCl as a solvent) the amine group of cysteamine was much more reactive than the thiol group, leading to the exclusive formation of dex-SH. The resulting dex-SH (**2**) was recovered by precipitation in cold ethanol. After reduction of possibly formed disulfide bonds with dithioerythritol (DTE), the dex-SH was purified by ultrafiltration (MWCO 5000) against deionized water and finally obtained by lyophilization. Overall yields of 50−80% were obtained. The nitrophenyl derivatization of dextran was confirmed by ^{1}H NMR (DMSO-d_6). Figure 1a shows, besides signals of dextran, peaks due to the protons of the nitrophenyl aromatic ring (δ 7.5 and 8.3, peaks e and f). The derivatization was further confirmed by the presence of small peaks due to the shift of the glucosidic protons upon reaction with 4-NC (δ

Scheme 2. Hydrogel Formation upon Mixing Aqueous Solutions of Dextran Thiol (dex-SH) and PEG Tetra-acrylate (PEG-4-Acr)



5.3 and 5.5, peaks d). We did not study in detail the position at which the substitution took place. The degree of substitution (DS, defined as the number of substituents per 100 anhydroglucosic rings, AHG, of dextran) was determined by comparing the peak areas corresponding to the aromatic protons of the nitrophenyl group (δ 7.5 and 8.3) and the dextran glucosidic protons (δ 3.1−4.0, 5.3, and 5.5). Molar feeding ratios of 4-NC to AHG of dextran of 0.56, 0.75, and 1.13 resulted in dex31K-NP with DS 15, 21, and 25, respectively (Table 1, entries 1−3). Dex14K-NP with DS 15 was prepared by using a molar feeding ratio of 4-NC to AHG of dextran of 0.56 (Table 1, entry 4). Therefore, dex-NP with different DS could be obtained by varying the molar feeding ratio of 4-NC to the AHG of dextran.

The complete reaction of dex-NP with cysteamine was confirmed by ^{1}H NMR (D_2O). Figure 1b shows that signals attributable to the nitrophenyl aromatic ring protons have completely disappeared, whereas signals due to the methylene protons of the cysteamine residue (δ 2.7 and 3.3, peaks d′ and e′) and signals due to the peak shift of the glucosidic protons after conjugation with cysteamine (δ 5.2 and 5.3, peaks c′) are clearly detected. Ellman tests[21] showed that dex31K-SH DS 16, 22, and 25 were obtained by using dex31K-NP DS 15, 21, and 25, respectively (Table 1, entries 1−3). Dex14K-SH DS 12 was obtained by using dex14K-NP DS 15 (Table 1, entry 4). The DS could also be determined using ^{1}H NMR by comparing the peak areas corresponding to the methylene protons of the cysteamine residue (δ 2.7) and the dextran glucosidic protons (δ 3.4−4.1, 5.2, and 5.3) (results not shown). The values obtained from ^{1}H NMR were in good agreement with those from the Ellman tests. These results indicate that quantitative aminolysis of dex-NP took place upon reaction with cysteamine. Therefore, this two-step synthesis procedure provides a convenient method to prepare thiol functionalized dextrans with different DS.

Table 1. Synthesis of Dextran Nitrophenyl (dex-NP) and Dextran Thiol (dex-SH) Derivatives

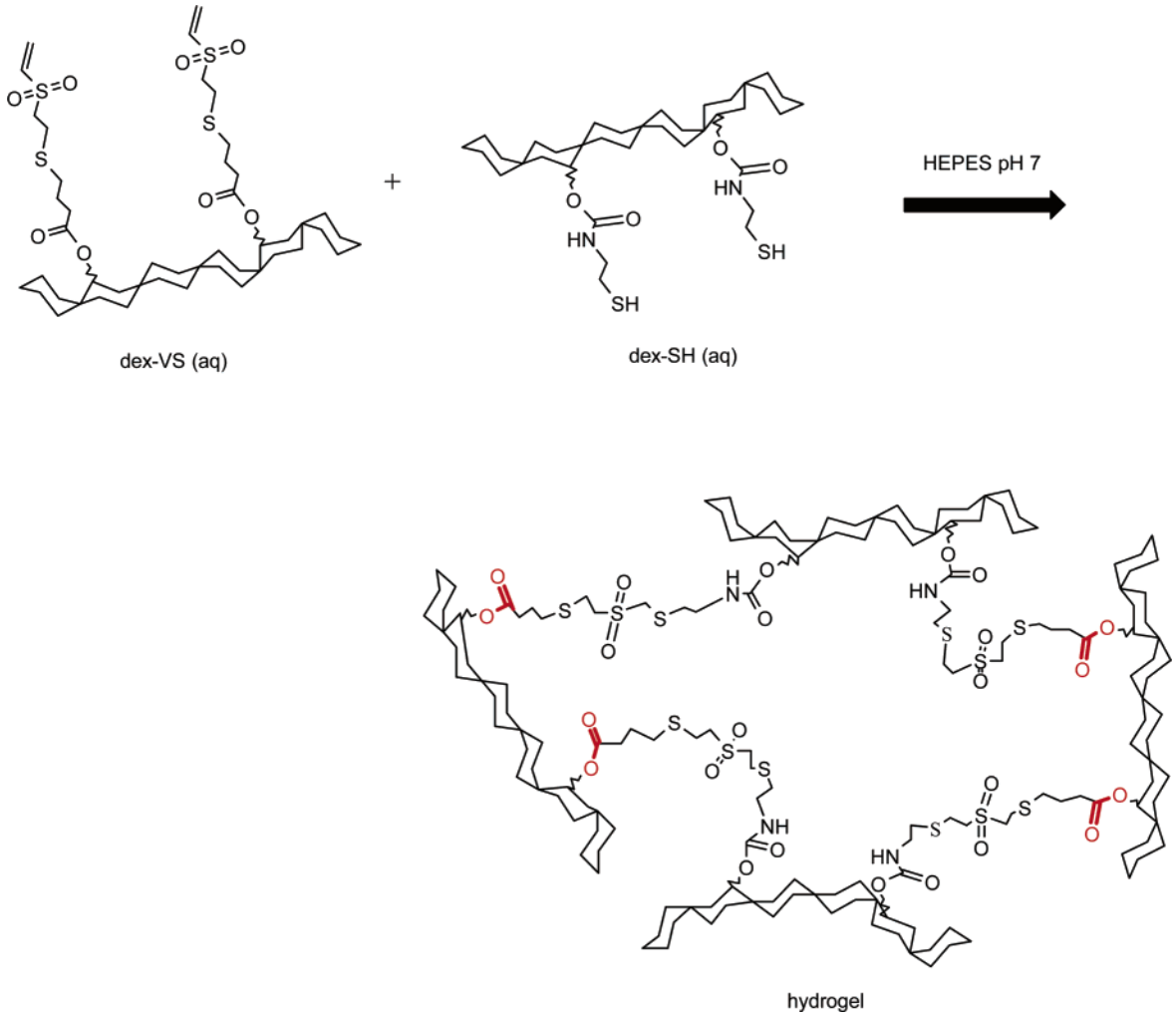
		Dex-NP		
entry	dextran	Molar feeding ratio of 4-NC to AHG of dextran	DS[a]	Dex-SH DS[b]
1	31K	0.56	15	16
2		0.75	21	22
3		1.13	25	25
4	14K	0.56	15	12

[a] The degree of substitution (DS), defined as the number of substituents per 100 AHG of dextran, was determined by ^{1}H NMR (DMSO-d_6) by comparing the peak areas corresponding to the dextran glucosidic protons (δ 3.1−4.0, 5.3, and 5.5) and the protons of the nitrophenyl group (δ 7.5 and 8.3). [b] The DS was determined by Ellman tests.[21]

To study the influence of the cross-linker on the dex-SH hydrogel formation, poly(ethylene glycol) tetra-acrylate (denoted as PEG-4-Acr) and a dextran vinyl sulfone conjugate with DS 10 (denoted as dex-VS DS 10, $M_{n,dextran}$ = 14K) were used. PEG-4-Acr ($M_{n,MALDI-TOF\ MS}$ = 2.1 K) was prepared as reported.[14] The conversion of the PEG hydroxyl groups to acrylate groups was 91%, as determined by ^{1}H NMR. Dex-VS DS 10 was synthesized as reported previously.[19]

In Situ Hydrogel Formation. Dextran hydrogels were formed in situ via Michael type addition between dex-SH and PEG-4-Acr (Scheme 2) or dex-VS DS 10 (Scheme 3) in

Scheme 3. Hydrogel Formation upon Mixing Aqueous Solutions of Dextran Thiol (dex-SH) and a Dextran Vinyl Sulfone Conjugate (dex-VS)



HEPES-buffered saline at pH 7 and 37 °C. The molar ratio of thiol to unsaturated groups was kept at 1.1, since thiol groups may form some disulfide bonds upon exposure to air, thus lowering the effective concentration of free thiol groups. The gelation time was determined by the vial tilting method. The concentration is defined as the total dry weight of both dextran and PEG per volume of buffer.

Figure 2a shows the gelation time of dex31K-SH cross-linked with PEG-4-Acr at 15 w/v% concentration as a function of the DS. The gelation time decreased from ca. 55 to 20 s when increasing the DS from 16 to 22, while a further increase to DS 25 had little influence on the gelation time. As shown in Figure 2b, the gelation times decreased from ca. 5 to 1 min for dex31K-SH DS 16/PEG-4-Acr and from ca. 90 to 40 s for dex14K-SH DS 12/PEG-4-Acr when increasing the polymer concentration from 10 to 20 w/v%. Under the same conditions, dex-VS DS 10 gave much faster gelation compared to PEG-4-Acr, which may be due to the higher cross-link functionality of the dex-VS DS 10 as compared to PEG-4-Acr as well as a higher reactivity of the vinyl sulfone group toward Michael type addition compared to the acrylate group (Figure 2b). The faster gelation of the previously reported vinyl sulfone-functionalized dextran cross-linked with tetrafunctional mercapto PEG (PEG-4-SH) compared to dex-SH cross-linked with PEG-4-Acr also indicates a higher reactivity of the vinyl sulfone group compared to the acrylate group.[19] For example, dex14K-SH DS 12 cross-linked with PEG-4-Acr gelated in ca. 90 s at 15 w/v% concentration, while dex14K-VS DS 13 cross-linked with PEG-4-SH gelated in ca. 30 s. The reactivity of the thiol group toward Michael addition depends on the pK_a of the thiol group, since the thiolate anion is the actual reactive species in the Michael addition reaction. It has been reported previously that the pK_a value of the thiol group is influenced by the electron-withdrawing ability of the neighboring group.[22] Both dex-SH and PEG-4-SH are noncharged, and in both cases, thiol groups are linked by ethylene units. Therefore, the pK_a values of dex-SH and PEG-4-SH should be similar.

Rheology. The mechanical properties of the dextran hydrogels were studied by oscillatory rheology experiments at 37 °C. Dex-SH and PEG-4-Acr or dex-VS DS 10 solutions in HEPES-buffered saline (pH 7, molar ratio of thiol groups to unsaturated groups was kept at 1.1, unless mentioned otherwise) were mixed by a double-barreled syringe with a mixing chamber and quickly applied to the rheometer. The storage modulus (G') of the hydrogels was followed in time to establish when the cross-linking reaction was completed (Figure 3). Generally, after mixing the reactants the storage modulus increased in time due to the Michael addition reaction, until reaching its plateau value, marking the end of the cross-linking process. The loss modulus (G'') of the dex-SH/PEG-4-Acr and dex-SH/dex-VS DS 10 hydrogels was too low to be accurately measured and is therefore not shown. The low loss modulus indicates that the hydrogels are highly elastic. Because of a fast gelation the gelation time could not be determined by rheology.

Figure 3 shows that the increase in the storage modulus is faster at higher concentrations. For instance, after 10 min,

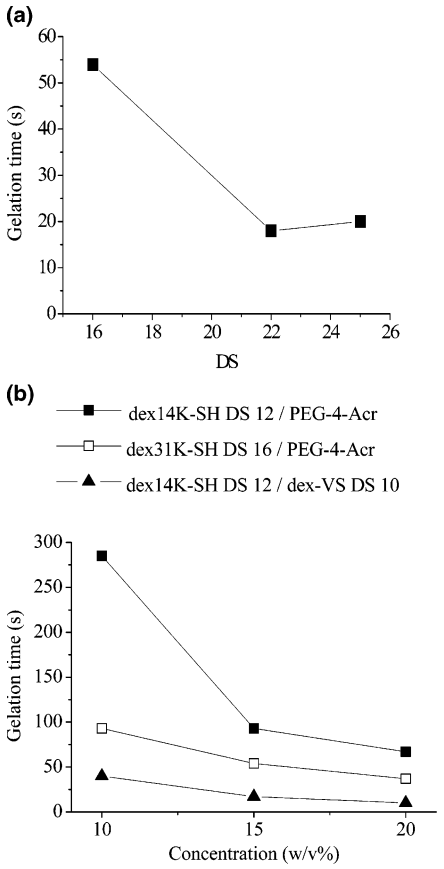


Figure 2. Gelation time (±5 s) after mixing dex-SH and PEG-4-Acr or dex-VS DS 10 solutions in HEPES-buffered saline at pH 7 and 37 °C. (a) Dex31K-SH cross-linked with PEG-4-Acr at 15 w/v% concentration as a function of the degree of substitution (DS); (b) dex14K-SH DS 12 cross-linked with and with PEG-4-Acr or dex-VS DS 10 and dex31K-SH DS 16 cross-linked with PEG-4-Acr as a function of the concentration.

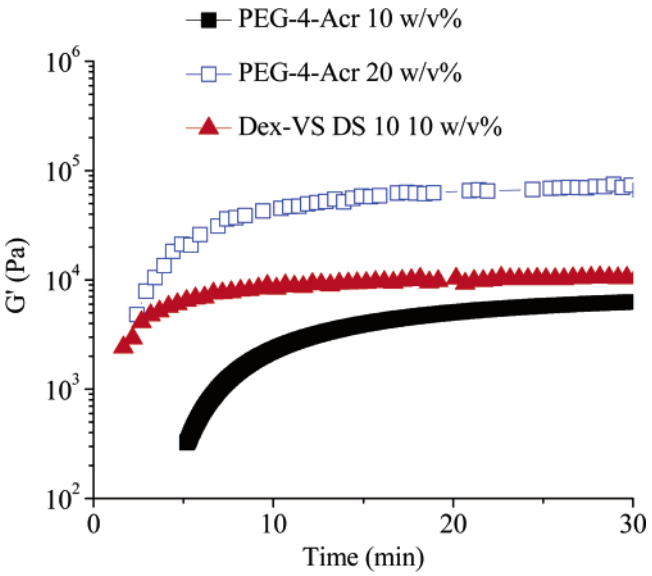


Figure 3. The evolution of the storage modulus (*G*′) at 37 °C of dex14K-SH DS 12 cross-linked with PEG-4-Acr at 10 and 20 w/v% concentration or cross-linked with dex-VS DS 10 at 10 w/v% concentration prepared in HEPES-buffered saline at pH 7. After application of the sample on the rheometer ($t = 0$ min), 1−5 min were needed to set the instrument before starting the measurement.

dex14K-SH DS 12/PEG-4-Acr hydrogels reached 25 and 59% of their storage modulus plateau value at 10 and 20 w/v% concentration, respectively. The storage modulus of dex14K-SH DS 12/dex-VS DS 10 hydrogels increased faster compared to dex14K-SH DS 12/PEG-4-Acr hydrogels (Figure 3). After 10 min dex14K-SH DS 12/dex-VS DS 10 hydrogels and dex14K-SH DS 12/PEG-4-Acr hydrogels reached 76% and 59% of their storage modulus plateau value, respectively, at 10 w/v% concentration. This may be due to the higher cross-linking functionality per molecule for dex-VS DS 10 compared to PEG-4-Acr as well as a higher reactivity of the vinyl sulfone group toward Michael type addition compared to the acrylate group. Figure 4a shows that the storage modulus plateau value increases from 9 to 83 kPa for dex14K-SH DS 12/PEG-4-Acr hydrogels and from 15 to 100 kPa for dex31K-SH DS 16/PEG-4-Acr hydrogels, when increasing the concentration from 10 to 20 w/v%. Similarly, the storage modulus plateau value of dex14K-SH DS 12/dex-VS DS 10 hydrogels increased from 11 to 51 kPa, when increasing the concentration from 10 to 20 w/v% (Figure 4a). Figure 4b shows that the storage modulus increases upon increasing the DS of dex31K-SH from 16 to 22 at 15 w/v% concentration (39 vs 70 kPa). A further increase to DS 25 did not increase the storage modulus. The storage moduli of the dex14K-SH DS 12/PEG-4-Acr hydrogels are comparable to those of the dex14K-VS DS 13/PEG-4-SH hydrogels.[19]

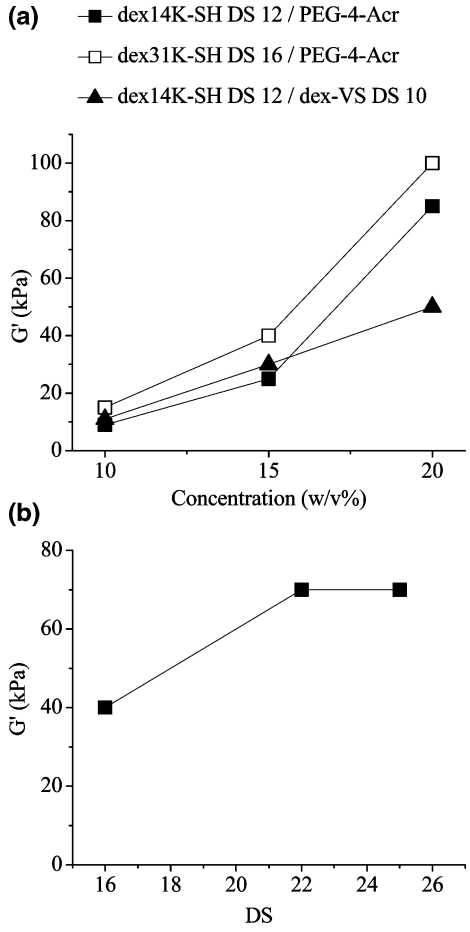


Figure 4. The storage modulus (*G*′) plateau value of hydrogels prepared in HEPES-buffered saline at pH 7 and 37 °C. (a) Dex14K-SH DS 12 cross-linked PEG-4-Acr or dex-VS DS 10 and dex31K-SH DS 16 cross-linked with PEG-4-Acr as a function of the concentration; (b) dex31K-SH cross-linked with PEG-4-Acr at 15 w/v% concentration as a function of the degree of substitution (DS).

To study the influence of the molar ratio of thiol to vinyl sulfone groups (SH:VS), dex14K-SH DS 12 was cross-linked with dex-VS DS 10 at 10 w/v% concentration and varying SH:VS ratios of 0.75, 0.9, 1, 1.1, and 1.5 (Figure 5a). All samples had already formed a gel at the start of the measurement. The

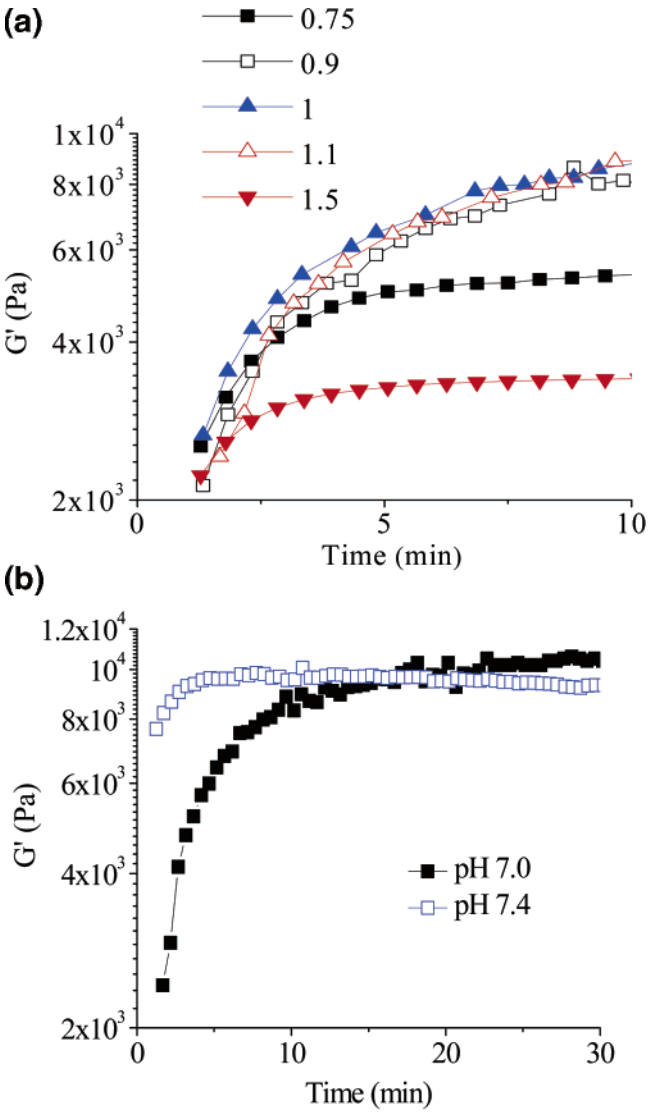


Figure 5. The storage modulus (G') as a function of time of dex14K-SH DS 12 cross-linked with dex-VS DS 10 in HEPES-buffered saline at 37 °C. (a) pH 7, 10 w/v% concentration and SH:VS molar ratio's of 0.75, 0.9, 1, 1.1, and 1.5; (b) 10 w/v% concentration at pH 7 and 7.4.

loss moduli of hydrogels formed at SH:VS ratio's of 0.9−1.1 were too low to be accurately measured. At SH:VS ratio's of 0.75 and 1.5 hydrogels showed tan δ (G''/G') values of 0.01 and 0.03, respectively, indicating that these gels are still highly elastic.

The storage moduli were similar at SH:VS ratio's of 0.9−1.1, while at SH:VS ratios of 0.75 and 1.5, the storage modulus was considerably lower (4−5 kPa vs 12 kPa). These results show that small deviations (≤10%) from equimolarity do not alter the gel formation. This indicates that a small amount of unreacted thiol or vinyl sulfone groups has little influence on the gel formation. The influence of the pH of the HEPES-buffered saline was studied for dex14K-SH DS 12 cross-linked with dex-VS DS 10 at 10 w/v% concentration. Figure 5b shows that at pH 7.4 a few minutes are needed to reach the storage modulus plateau value, while at pH 7 ca. 30 min were required, indicating that the cross-linking reaction is much faster at pH 7.4 compared to pH 7. The faster reaction at higher pH is due to the fact that increasing the pH increases the concentration of the thiolate anion, which is the actual reactive species for the Michael addition.[22]

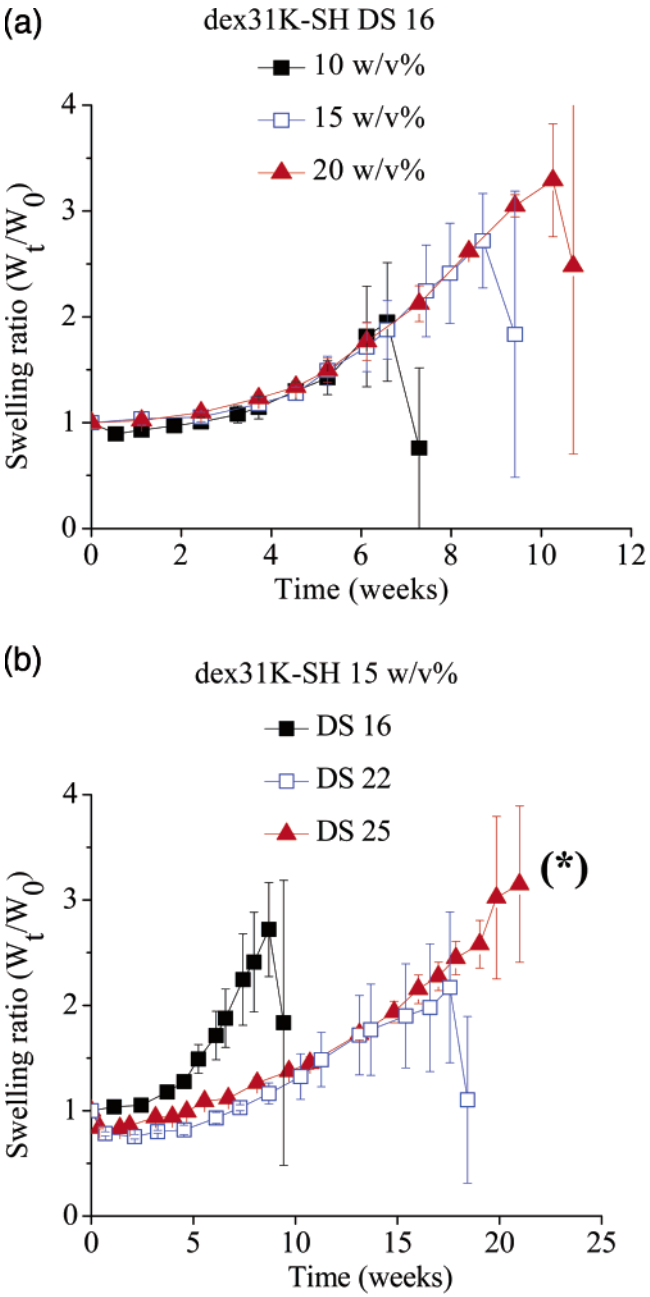


Figure 6. Swelling ratio (W_t/W_0) profiles of dex31K-SH/PEG-4-Acr hydrogels prepared in HEPES-buffered saline, at pH 7 and 37 °C (average ± S.D., $n = 3$). (a) Dex31K-SH DS 16 at 10, 15, and 20 w/v% concentration; (b) dex31K-SH DS 16, DS 22 and DS 25 at 15 w/v% concentration. (*) Dex31K-SH DS 25/PEG-4-Acr hydrogels retained their integrity after 21 weeks.

Swelling and Degradation. The dex-SH/PEG-4-Acr and dex-SH/dex-VS DS 10 hydrogels were degradable under physiological conditions. Dex-SH/PEG-4-Acr hydrogels degrade through hydrolysis of the ester bond between the thioether and PEG. Dex-SH/dex-VS DS 10 hydrogels degrade through hydrolysis of the ester bond between the sulfone group and dextran. To study the rate of degradation of these hydrogels, solutions of dex-SH and PEG-4-Acr or dex-VS DS 10 were mixed in HEPES-buffered saline at pH 7 and 37 °C (molar ratio of thiol groups to unsaturated groups was kept at 1.1). After the hydrogels were formed, HEPES-buffered saline 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 after exposure to buffer with the

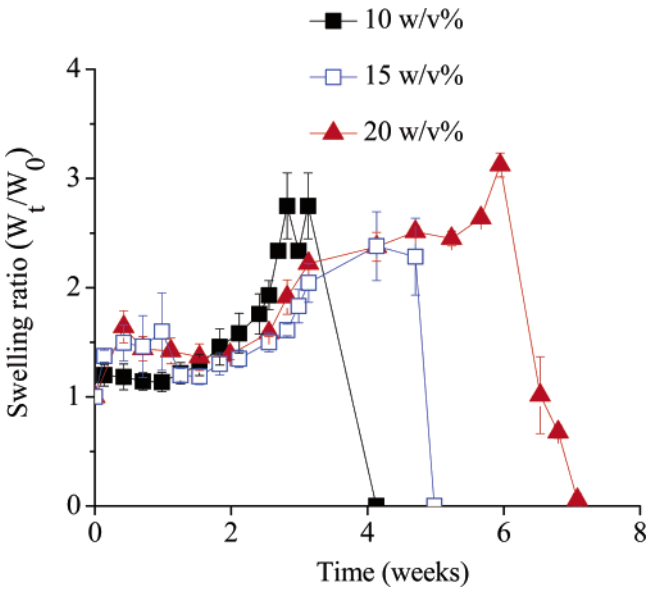


Figure 7. Swelling ratio (W_t/W_0) profiles of a dex14K-SH DS 12/dex-VS DS 10 hydrogels prepared in HEPES-buffered saline, at pH 7 and 37 °C at 10, 15, and 20 w/v% concentration (average ± SD, $n = 3$).

initial hydrogel weight after preparation (W_t/W_0). Figure 6 shows the swelling profiles of dex31K-SH hydrogels cross-linked with PEG-4-Acr. In general, the hydrogels hardly swelled for the first 4 weeks while in time the swelling gradually increased, caused by the hydrolytic cleavage of the ester bonds. At a certain point, the swelling ratios decreased due to disintegration of the hydrogel network, resulting in partial dissolution of the hydrogels. The hydrogel degradation time is defined as the time required to completely dissolve at least one of the three hydrogels. Figure 6 shows that both the concentration and DS hardly affected the initial swelling ratio. For dex31K-SH DS 16/PEG-4-Acr hydrogels, the degradation time increased from ca. 7 to 10 weeks by increasing the concentration from 10 to 20 w/v% (Figure 6a). Similarly, the degradation time of dex14K-SH DS 12/PEG-4-Acr hydrogels increased from ca. 16 to 24 weeks when increasing the concentration from 10 to 20 w/v% (results not shown). Dex31K-SH DS 16/PEG-4-Acr hydrogels degraded much faster than the corresponding dex14K-SH DS 12/PEG-4-Acr hydrogels. This may be due to a lower Michael addition conversion for dex31K-SH DS 16 compared to dex14K-SH DS 12. Figure 6b shows that the degradation time increased with increasing DS. At DS 16 and DS 22 the dex31K-SH/PEG-4-Acr hydrogels had degradation times of ca. 9 and 17 weeks at 15 w/v% concentration, respectively, while at DS 25 the hydrogel retained its integrity even after 21 weeks.

Figure 7 shows that the swelling profiles of dex14K-SH DS 12/dex-VS DS 10 hydrogels are similar to those of dex-SH/PEG-4-Acr hydrogels, with low initial swelling followed by gradual swelling and final dissolution of the hydrogel. The degradation times of dex14K-SH DS 12/dex-VS DS 10 hydrogels were ca. 3 and 7 weeks at 10 and 20 w/v% concentration, respectively. Dex14K-SH DS 12/dex-VS DS 10 hydrogels degraded much faster than the dex14K-SH/PEG-4-Acr hydrogels. This could be due to a higher susceptibility to hydrolysis of the ester bond between the sulfone group and dextran in dex-VS DS 10 conjugates compared to the ester bond between the thioether and PEG in PEG-4-Acr.

In summary, slowly degrading hydrogels could be obtained by Michael addition between dextran thiols and PEG-4-Acr or dex-VS DS 10. Hydrogel degradation times ranged from 3 to more than 21 weeks, which can be varied by the DS, polymer concentration, dextran molecular weight, and type of cross-linker (PEG-4-Acr or dex-VS DS 10). The degradation is much slower compared to previously reported hydrogels prepared by reaction of dextran vinyl sulfone conjugates with tetrafunctional mercapto PEG, which degraded within 3 weeks.[19] The slow degradation is advantageous for biomedical applications, such as tissue engineering of cartilage or release of proteins over an extended period of time.

Conclusions

Dextrans with pendent thiol groups (dex-SH) were conveniently synthesized by a two-step synthesis procedure with degrees of substitution (DS) ranging from 12 to 25. Hydrogels were rapidly formed in situ under physiological conditions by mixing aqueous solutions of dex-SH and PEG tetra-acrylate (PEG-4-Acr) or a dextran vinyl sulfone conjugate (dex-VS DS 10). Their mechanical and degradation properties could be adjusted by the DS, concentration, dextran molecular weight, and type of cross-linker (PEG-4-Acr or dex-VS DS 10). Storage moduli in a range of 9 to 100 kPa could be obtained. Degradation times of dex-SH/PEG-4-Acr ranged from 7 to more than 21 weeks, and degradation times of dex14K-SH DS 12/dex-VS DS 10 hydrogels ranged from 3 to 7 weeks. These hydrogels are very promising for use in biomedical applications, since they can be rapidly formed in situ and are biodegradable with adjustable degradation times to match a particular application. Furthermore, in principle biomimetic scaffolds can easily be obtained by incorporation of thiol-containing bioactive molecules, such as proteins and peptides.

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