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In Situ Formation of Biodegradable Dextran-Based Hydrogel Via Michael Addition

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ABSTRACT: Vinyl-sulfone-functionalized dextran (Dex-VS) was readily prepared by a single-batch synthetic procedure at room temperature. The thiol molecule, 1,4-dithioerythritol (DTE, commercially available) with two terminal thiol groups was used as crosslinking agent. Hydrogels were rapidly prepared *in situ* by Michael addition under mild physiological conditions. Properties including gelation duration, mechanical modulus, and degradation behavior could be expediently controlled. Moreover, the hydrogels had comparatively low cytotoxicity, and the *in vivo* tests demonstrated an effective delivery of growth factor. This study might provide a powerful material to be used in tissue regeneration. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: hydrogel; biomaterials; biodegradable; rheology; biocompatibility

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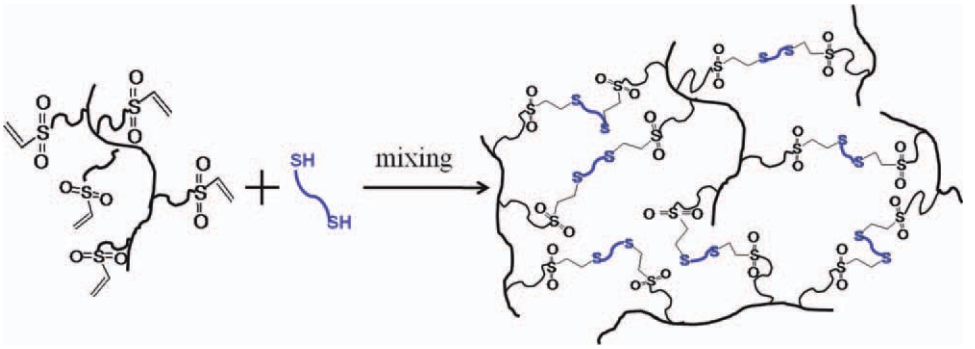
INTRODUCTION

Three-dimensional hydrogels are hydrate networks of crosslinked hydrophilic polymers, with physical properties similar to the native extracellular matrix (ECM), which can be *in situ* prepared under physiological conditions and display some key characteristics of the provisional ECM. Therefore, these hydrogels have been extensively studied as drug delivery where they serve as degradable scaffold for tissue engineering.[1] Crosslinked hydrogels are generally classified into two categories: physically crosslinked[2,3] and chemically crosslinked.[4] Physically crosslinked hydrogels are usually prepared via self-assembly of amphiphilic copolymers, stereo-complexation, hydrophobic, and ionic interactions; however, the weak mechanical strength restraints some practical applications. Chemically crosslinked hydrogels are comparatively stronger, but the risk of cytotoxicity may arise from the crosslinking agents.

As alternatives, several novel chemically crosslinking approaches, such as photopolymerization, Schiff-base formation, and Michael-type addition, have been employed to prepare biocompatible and injectable hydrogels in recent years. Therein, the most common method was based on UV radiation of methylacrylate-functionalized polymers.[5,6] For example, Phelps et al. prepared PEG hydrogel using UV light to initiate photopolymerization.[7] The same method was used by Yang et al. to *in situ* prepare cell-encapsulated PEG diacrylate (PEGDA) hydrogels.[8] However, the *in situ* formation *in vivo* is usually limited because of the low penetration depth of UV light, which was mostly absorbed by skin. Michael type addition reaction or Schiff-base formation may overcome this limitation, and the hydrogels can be rapidly shaped via reactions between the functional groups within several minutes under mild conditions. In addition, these hydrogels display good biocompatibility to support cell survival and differentiation. Tan et al. reported hydrogels prepared via Schiff-base formation between water-soluble chitosan and oxidized hyaluronic acid, which showed that chondrocytes survived in these hydrogels and retained their round morphology.[9] Hubbell's research group also successfully prepared PEG-based hydrogels from PEG-SH and MMP-2 sensitive peptides which had two terminal thiol groups, using Michael addition reaction.[10–13] Kraehenbuehl et al. found that the Young's modulus of the hydrogel could be tailored by altering the molar ratio between PEG and peptides.[14]



Scheme 1. Schematic representation of the gelation mechanism of Michael addition between Dex-VS and DTE. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Dextran-based materials are highly hydrophilic and well biocompatible, which are particularly compelling as scaffolds for tissue engineering applications. Modifications can be achieved by substitution of multiple hydroxyl groups with a broad range of functional groups. Jan et al. prepared hydrogels with modified dextran[15–17] using Michael addition between mercapto-PEG and vinyl sulfones. Moreover, biomimetic scaffolds could be easily obtained by incorporation of thiol-bearing molecules. However, most of this works focused on the synthesis and characterization of the hydrogels.

Enlightened from the crosslink occurring between reactive vinyl sulfone and mercapto groups, we hypothesized that DTE, a kind of commonly used mercapto agent, could form a crosslink network. Herein, in this study, we developed a novel degradable hydrogel that was rapidly formed via Michael addition between dextran vinyl sulfones (Dex-VS) and mercapto molecules thiols, 1, 4-Dithioerythritol (DTE) as the crosslinking agent (Scheme 1). Structure characterization and properties were systematically investigated in terms of gelation duration, mechanical properties, rheological behavior, swelling, and *in vitro* degradation. In addition, the cytocompatibility of the hydrogel was evaluated with C2C12 mouse myoblase cells. A representative growth factor, recombinant human bone morphogenetic protein-2 (rhBMP-2), was loaded on the hydrogels and the effect of ectopic bone formation was evaluated. To the best of our knowledge, no effort has been made on the fabrication of hydrogels integrated with DTE as well as its application in tissue regeneration. The study suggested that the hydrogel was well-synthesized and exhibited good biocompatibility, and thus could be used as bioscaffolds in tissue engineering.

EXPERIMENTAL

Materials and Reagents

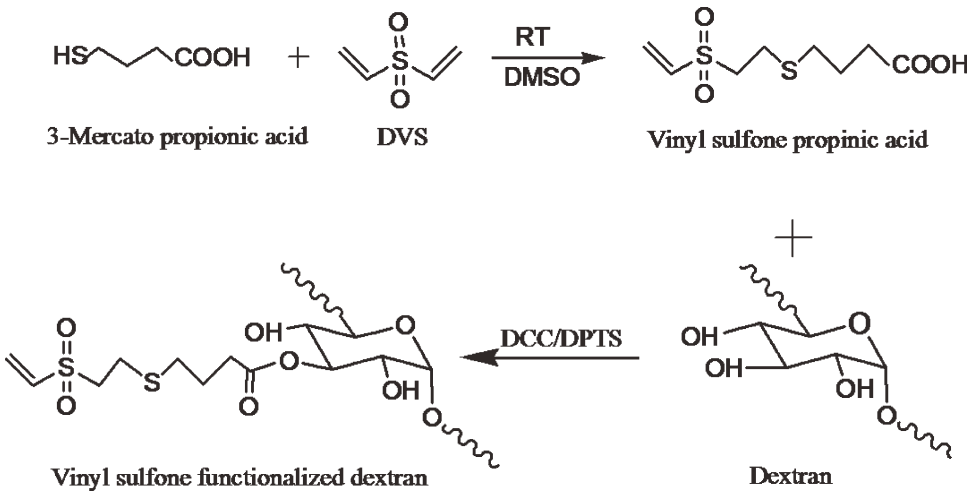
Dextran (M_n = 4W) and 4-dimethylaminopyridine (DMAP) were purchased from Sinopharm Chemical Reagent (Shanghai, China), divinyl sulfone (DVS) from Shanghai Jiachen Chemical Reagent (Shanghai, China), and dithioerythritol (DTE) and *N, N'*-Dicyclohexylcarbodiimide (DCC) were acquired from Shanghai Yuanju Biotechnology (Shanghai, China). Hydrated *p*-toluenesulfonic acid (PTSA), benzene and dimethyl sulfoxide (DMSO) were obtained from Shanghai Lingfeng Chemical Reagent (Shanghai, China), dichloromethane (DCM) from Shanghai Chemical Reagent (Shanghai, China), and 3-Mercaptopropionic acid (3-MPA) from A Johnson Matthey Company (Alfa Aesar). The catalyst, 4-Dimethylaminopyridinium 4-toluenesulfonate (DPTS) was obtained from the reaction between DMAP and PTSA.[18] Briefly, the hydrated PTSA was dried by azeotropic distillation in a benzene solution using a Dean-Stark trap. The warm benzene solution with equimolar DMAP dissolved inside was then added to the anhydrous benzene solution of PTSA and mixed thoroughly. The resulting suspension was cooled down to room temperature and the solid was collected by suction filtration. The crude product was purified by recrystallization from DCM, yielding white needle-like substance. rhBMP-2 was kindly donated by Shanghai Rebone Biomaterials. (Shanghai, China). DCM and DMSO were dried with calcium hydride. Benzene was dried with sodium wire under inert atmosphere. All other solvents were distilled prior to use.

Synthesis of Dex-VS

Dex-VS was synthesized by one-batch synthetic procedures at room temperature, as reported elsewhere[1] (Scheme 2). First, DVS (32.85 g, 278 mmol) was dissolved in DMSO (60 mL) and 3-MPA (1.476 g, 13.9 mmol) was added dropwise, and the solution was stirred for 4 h. Dextran dried by azeotropic distillation in toluene (5.0 g, 30.9 mmol anhydroglucosidic (AHG), 5.0% w/v concentration), DPTS (0.62 g, 2.1 mmol) and DCC (4.346 g, 21.1 mmol) were dissolved in DMSO and then added to the DVS/3-MPA mixture. This solution was stirred for another 24 h. The generated *N, N*-dicyclohexylurea (DCU) salt was removed by filtration. The product was precipitated from cold ethanol and purified by dialysis in water. The final product (with weight of 3.65 g) was obtained by lyophilization. Different degrees of substitutions (DS) were achieved by varying molar ratios of 3-MPA to AHG of dextran and varieties of catalyst (Table I).

Characterizations

^{1}H-NMR spectra were acquired on an Avance DRX-500 (Bruker BioSpin, Germany) spectrometer operating at 500 MHz, using D_2O as solvent. Degree of substitution (DS) was determined from ^{1}H-NMR spectra by comparing the integral areas between signal corresponding to vinyl sulfone protons at δ 6.5 and 6.9 and that to dextran glucosidic protons at δ 3.1-4.1, 5.2, and 5.4 ppm.



Scheme 2. Schematic representation of synthetic procedures of Dex-VS.

Dex-VS/DTE Hydrogel Preparation

Mercapto molecule DTE was selected to bridge Dex-VS which has two terminal thiol groups. Solutions of the Dex-VS with various degrees of substitution (DS = 9, 12.5) and concentrations (7.5 wt %, 10 wt %, 12.5 wt %) and the DTE (the molar ratio of thiol groups to vinyl sulfone groups is kept at 1.1) in 500 μL of phosphate buffered saline (PBS, pH = 7.6) were mixed at room temperature by vortexing. Gelation, while the sample stops flowing, was observed within minutes.

Rheology Analysis

Rheological behavior during gelation was inspected on a stress-controlled rheometer, Physical MCR-301 (Anton Paar Co. Inc. Austria). For all the measurements, a plate-plate measuring geometry with 25 mm diameter was used. Solutions of Dex-VS and DTE in PBS (pH = 7.6) were mixed and quickly placed to the rheometer with adjusting gap to 0.5 mm. A thin layer of low-viscosity silicon oil was placed around the borders of the measuring cell to prevent evaporation of water. Subsequently, under a frequency of 1 Hz and the strain of 1%, the kinetic of the gelation was studied by monitoring the storage modulus (G') and loss modulus (G'') with time.

Degradation Investigation

For the degradation tests, Dex-VS/DTE hydrogels (0.05 mL) were prepared as described above, followed by freeze-drying and weight measurement (W_0). We denoted them as gel scaffolds. Subsequently, each gel scaffold was immersed into 2 mL PBS solution for incubation at 37°C. In regular intervals, the buffer solution was removed from the samples and the remaining gel scaffolds were weighed. The buffer was refreshed everyday. The remaining gel content (%) was calculated from the original dry gel weight (W_0) and remaining gel weight (W_t) expressed as $W_t/W_0 - 100\%$. At least three parallel replicates were carried out and the average value was calculated for each sample.

Fabrication of Dex-VS/DTE Scaffold and Morphological Observation

The hydrogels were lyophilized overnight to obtain scaffolds. Subsequently, the scaffolds were immersed into liquid nitrogen and cut off to observe the cross-section. The morphology of the scaffolds was studied using a Hitachi S-4800 scanning electron microscope (SEM) after being gold-coated in a sputter coater.

Cytotoxicity Assay

C2C12 cells [purchased from the American Type Culture Collection (ATCC)], a myoblastic precursor cell derived from adult mouse skeletal muscle with osteoblastic potential,[19] were selected for cytotoxicity evaluation. C2C12 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2, and were seeded onto a 96-well tissue culture plate at a seeding density of 5000/well. Dex-VS/DTE scaffolds with different Dex-VS concentrations (7.5, 10, and 12.5 wt %) and DS (9 and 12.5) were prepared as described above and sterilized prior to use. Sample preparation and cytotoxicity experiments were performed using extraction method according to ISO 10993. C2C12 cells were cultured in 37.5 cm^2 flasks with leaching liquor for 1 day. After this, the leaching liquor was transferred into each cell-seeded culture plate and determined by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay using a microplate spectrophotometer at the absorbance of 492 nm. Blank culture media was used as control. All tests were performed in triplicate.

In Vivo Ectopic Bone Formation

The hydrogels with 10 wt % and DS = 9, DS = 12.5 were lyophilized overnight to obtain the scaffolds (6 × 20 × 5 mm^3).

Table I. Reaction Parameters in Synthesizing Dex-VS

	Reaction conditions					
Group	Dextran (g)	DVS (g)	3-MPA (g)	DMSO (mL)	Catalyst	DS
1	5	32.85	1.476	150	DMAP	2.35
2	5	32.85	1.476	15	DPTS	5.68
3	5	32.85	1.476	100	DPTS	7.5
4	5	32.85	1.968	150	DPTS	9
5	5	32.85	1.968	100	DPTS	12.5

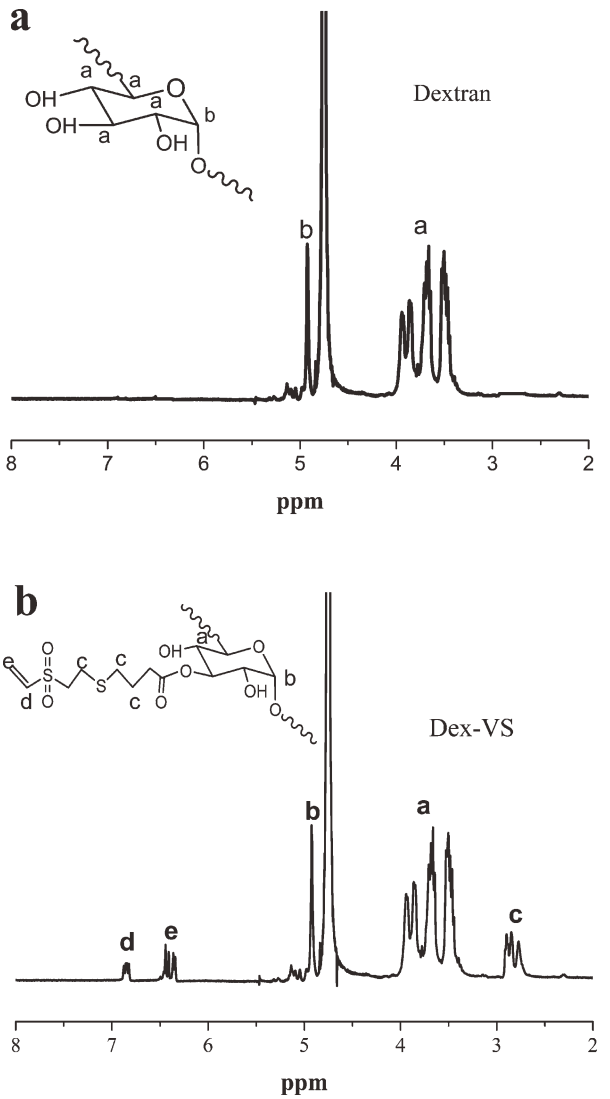


Figure 1. ^{1}H-NMR (D_2O) of (a) dextran and (b) dextran-VS.

For the rhBMP-2 loaded scaffold, the same sheet was impregnated with 100 μg rhBMP-2, and lyophilized.

All procedures were performed in accordance with the Institutional Animal Care and Use Committee. Eight 2-week-old female Kunming mice (20–30 g) were divided into two groups ($n = 4$) with different DS (A: DS=9, B: DS = 12.5), and anesthetized with sodium pentobarbital by intraperitoneal injection. RhBMP-2 loaded scaffolds were implanted into the right hind limb muscle. After shaving and disinfecting, a 5-mm-longitudinal skin incision was made and the implants were inserted into the muscle pouch. Then the wound was finally closed in layers with sutures. Four weeks after the implantation, the mice were sacrificed. One of the implants were retrieved together with surrounding tissues, and immediately fixed in 4% formaldehyde solution. The others were harvested and the wet weight was measured. Then the samples were incinerated at 600°C for 6 h in a muffle furnace and measured again for ash weight. The fixed implants were washed with phosphate buffer solution, dehydrated in gradients of alcohol, decalcified, and paraffin-embedded. Thin slices (10–20 μm) were prepared and stained using hematoxylin-eosin (HE) for histological examination.

Statistical Analysis

Results are presented as means ± SD from values of triplicate independent experiments. Comparisons between two samples were performed with the students' *t*-test. Differences were considered to be statistically significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Synthesis of Dextran Derivatives

Dextran vinyl sulfone, with varied degrees of substitution, was generated by one-batch synthetic procedures as reported by previous literature. The structure was confirmed by ^{1}H-NMR. Figure 1(a) shows the ^{1}H-NMR of the Dex-VS in D_2O: δ 5.2 ppm and 5.4 ppm (m, glucosidic protons linked to vinyl sulfone substituents), 6.5 ppm (m, $-SO_2CH{=}CH_2$), 6.9 ppm (m, $-SO_2CH{=}CH_2$). As presented in Figure 1(b), distinct peaks appeared at δ 6.5 ppm and 6.9 ppm, in addition to the original

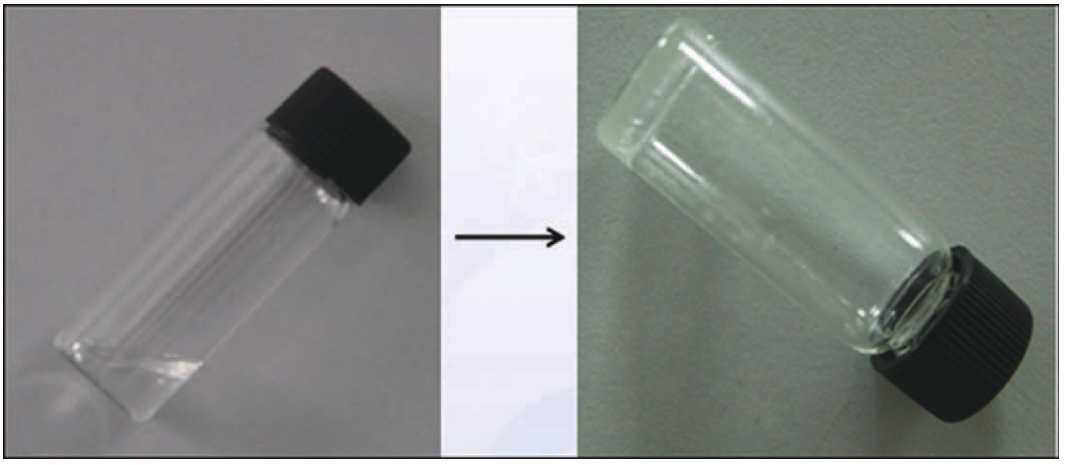


Figure 2. Representative process of gelation of Dex-VS (0.05 g) and DTE (0.002 g) in 0.5 mL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

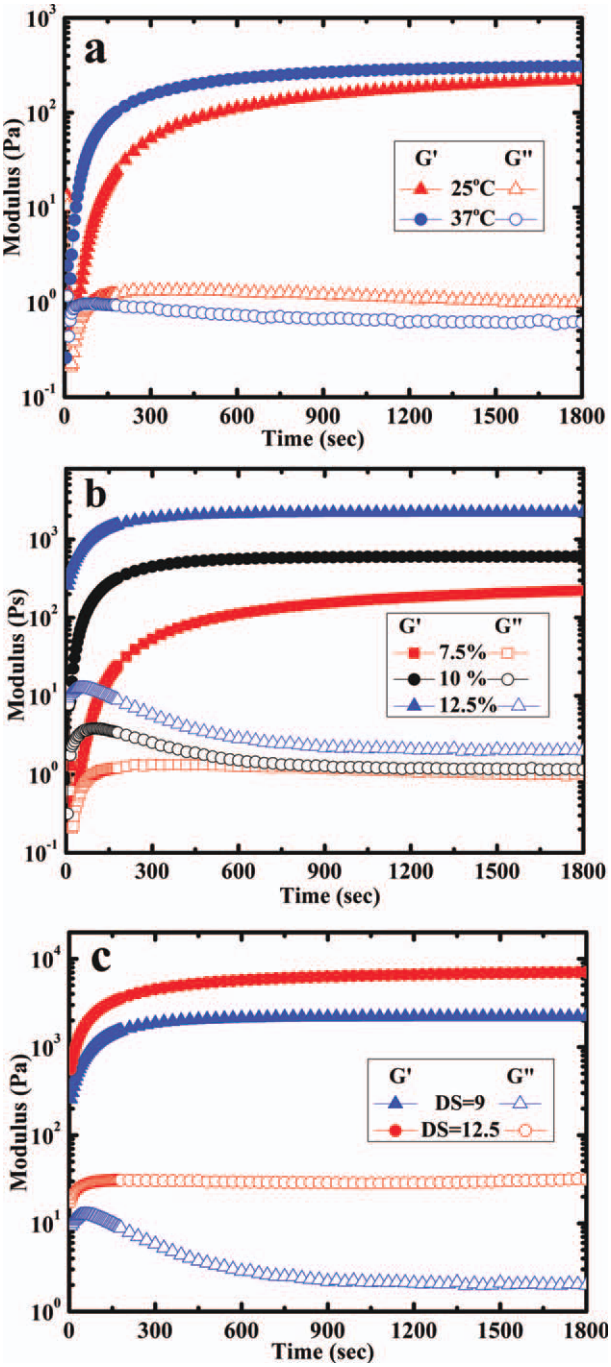


Figure 3. Time sweep of Dex-VS/DTE mixture: storage modulus (G') and loss modulus (G'') as function of time: (a) gelation of 7.5 wt% Dex-VS/DTE (DS = 9) at different temperature; (b) gelation of Dex-VS/DTE (DS = 9) under different precursor concentrations; (c) gelation of Dex-VS/DTE (12.5% w/v) with different DS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

signals of dextran, which was attributed to the vinyl sulfone protons. Moreover, small signal trace at δ 5.2 ppm and 5.4 ppm was observed due to the shift of glucosidic protons of the anhydroglucose unit after reaction with the vinyl sulfone acid. Therefore, we speculated that the sulfuryl groups had been grafted onto the dextran.

Degree of substitution (DS) was defined as the number of the sulfuryl groups per 100 anhydroglucosidic rings (AHG) of Dextran, which could be calculated from the sulfuryl based on the glucosidic protons of dextran (δ = 3.4–4.1, 5.2, and 5.4) and the protons of the sulfuryl (δ = 6.5 and 6.9). Different DS could be controlled by variable molar ratio of 3-MPA to AHG of dextran or alterable Dextran concentration (Table I).

Gel Formation and Rheological Studies

A common mercapto agent, DTE, was used in this study to crosslink the hydrogel. Hydrogels could be formed *in situ* via Michael addition between Dex-VS and mercapto molecules DTE in PBS with pH = 7.4 at 37°C. The dextran solution turned into transparent hydrogels in short duration, as shown in Figure 2.

The gelation behavior and mechanical properties of the hydrogels were investigated by oscillatory rheology experiments as shown in Figure 3. Figure 3(a) showed the storage modulus (G') and loss modulus (G'') as a function of the gelation time at the constant Dex-VS concentration of 7.5% and DS of 9. Fast gel formation was observed and the gel points within 5 min were obtained. In addition, it seemed that both the gelation rate and the storage modulus at 25 and 37°C shared highly similar values, indicating that temperature had little affect on neither the kinetic of gelation nor the mechanical properties. For convenience, investigations were carried out at 25°C thereafter. Figure 3(b) revealed the effects of Dex-VS concentration on the gel formation and the storage modulus. It is Obvious that the gelation time decreased from 5 min to 2 min with increasing concentration from 7.5 to 12.5% w/v. Similarly, the improvement of the storage modulus was also achieved by increasing precursor concentration. For example, the storage modulus of Dex-VS hydrogel (DS = 9) with concentration of 12.5% w/v was 4 kPa, while that of 7.5% w/v was only 221 Pa. It was rationalized that a higher crosslinking density (the number of thiol groups and vinyl sulfone groups) was achieved under elevated precursor concentration, therefore resulting in a shorter time for formation of the steady gel network and improvement of the

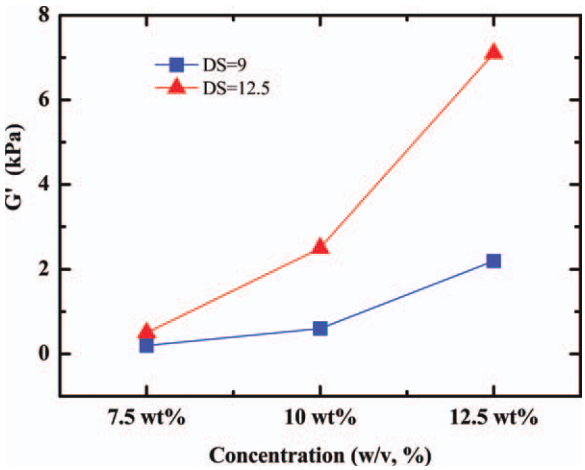


Figure 4. Storage modulus plateau values of the hydrogels as a function of concentration at two different DS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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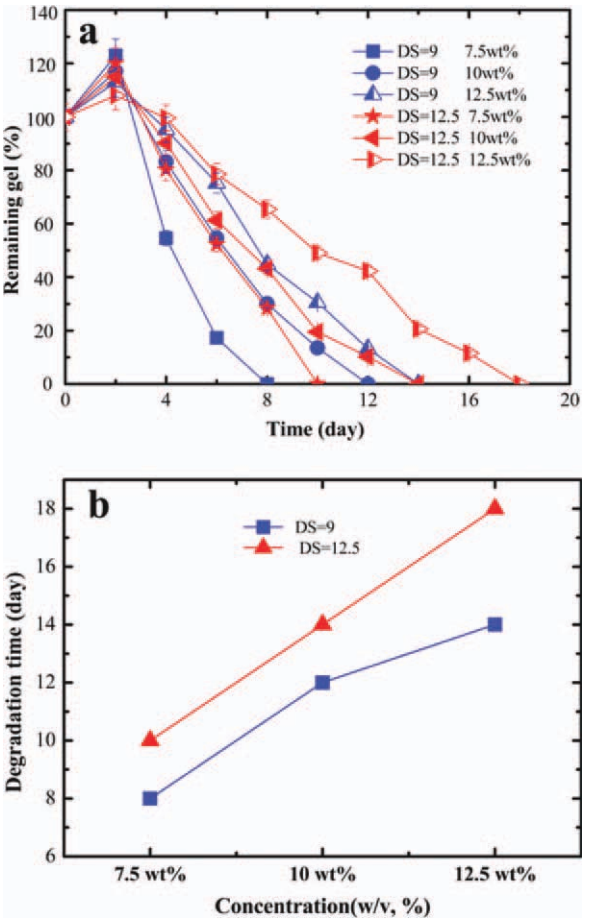

Figure 5. Plots of degradation time at different DS and concentration in PBS at pH = 7.6 and 37°C: (a) the remaining gel content (%) at regular intervals at different DS and precursor concentration; (b) the degradation time as a function of precursor concentration at different DS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

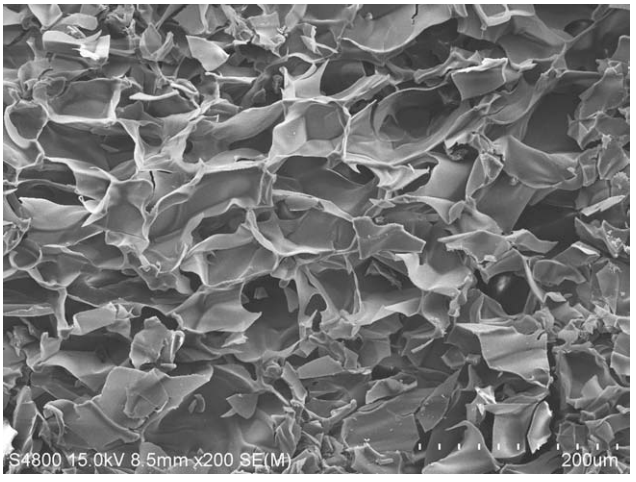

Figure 6. SEM images of freeze-dried scaffold from Dex-VS crosslinked with DTE.

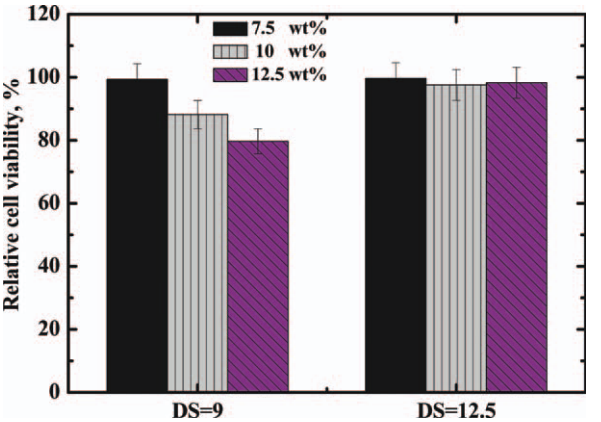

Figure 7. *In vitro* cytotoxicity of the hydrogel towards C2C12 cells, determined by the MTT assay at 492 nm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mechanical properties. In Figure 3(c), the influence of DS on gelation time and modulus were examined at a constant precursor concentration of 12.5% w/v. The storage modulus increased considerably with the higher DS value. This should be attributed to a higher crosslinking density of the network.

Further investigation on the DS with varied Dex-VS concentrations was presented in Figure 4. Analogously, the storage modulus increased with either increasing DS or precursor concentration. In brief, Dex-VS hydrogels could be rapidly generated *in situ*, and their storage modulus could be controlled conveniently by varying the precursor concentration and DS.

Degradation Behavior

Figure 5(a) showed the degradation behavior of the hydrogels. They exhibited swelling behavior at the first 2 days, and then gradually dissolved. The swelling ratios of the scaffolds at higher DS and concentration were relatively low than those at lower DS and concentration. For example, the swelling ratio was 1.23 under the condition of 7.5 wt % precursor concentration and DS of 9, while it decreased to 1.13 under the condition of 12.5 wt % concentration and the same DS, and decreased to 1.2

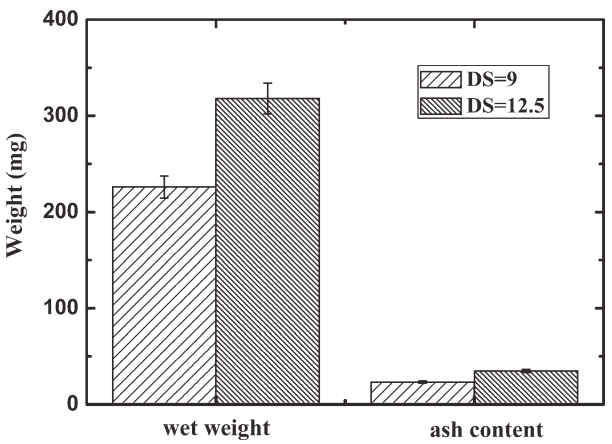

Figure 8. Wet weights and ash content of ectopic bone induced by rhBMP-2 *in vivo* at 4 weeks.

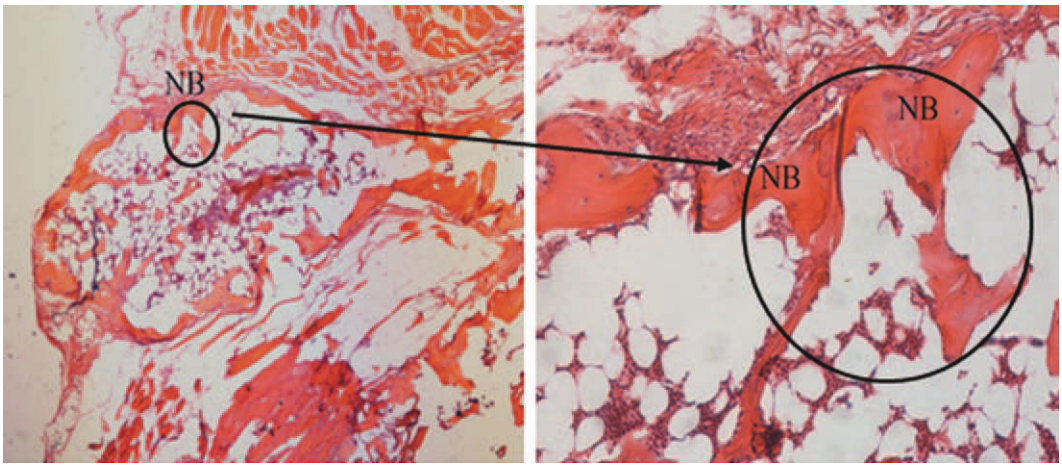

Figure 9. Representative histological evaluation of the harvested implants at 4 weeks (DS = 12.5, HE staining). NB: new ectopic bone. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

under the condition of 7.5 wt % concentration and DS of 12.5. This could be rationalized that higher DS and precursor concentration led to elevate the crosslinking density and the compact network. Thus smaller internal space could embed less water-content than that with the lower DS and concentration. After 2 days, the gel reached a swelling-deswelling equilibrium, and the weight of remaining gel began to decrease gradually. All the hydrogels completely degraded within 8–18 days due to the hydrolytic cleavage of the ester bonds between the dextran backbone and the thioether group.[1]

A plot of the degradation time(defined as the duration after which the hydrogel was completely dissolved in aqueous environment) revealed that the degradation time increased with increasing DS or concentration [Figure 5(b)]. As a function of DS, the degradation time of the scaffolds with concentration of 10 wt % (DS = 9) was 12 days, while 14 days were required for that with DS of 12.5. Thus we concluded that the gels with higher DS were more stable than those with lower DS. On the other hand, influence of precursor concentration on the degradation time shared a similar trend, which showed a prolonged degradation behavior at higher Dex-VS concentration. For instance, under a condition of constant DS (DS = 9), the gel with 12.5 wt % concentration was completely degraded after 14 days, while the disentangle duration were 12 days and 8 days, respectively, under lower concentrations (corresponding to 10 and 7.5 wt %). Thus it could be concluded that the higher precursor concentration led to slower degradation.

The hydrolysis of the ester bonds between the dextran backbone and the thioether group might be involved in the degradation mechanism. It was rationalized that both higher density of vinyl sulfone groups and higher dextran concentration contributed to the formation of denser crosslinking network, and thus led to longer degradation time.

Morphology of the Scaffold

The morphology of the freeze-dried Dextran-VS hydrogel was studied by SEM. As displayed in Figure 6, the scaffolds exhibited porous and well-interconnected microstructure, which was presumed in favor of the nutrition transmission.

Cytotoxicity Evaluation

To assess the biocompatibility of hydrogels, the cytotoxicity of the extraction was tested via MTT methods. Blank culture media served as a control group. The relative cell viability (%) was denoted as $OD_{hydogel}/OD_{control}$, The results were plotted in Figure 7, which showed that the hydrogels did not exhibited obvious cytotoxicity *in vitro* with all the values above 80%. We concluded that the scaffolds had good biocompatibility *in vitro*.

Ectopic Bone Formation

BMP-2 has been proved to be one of the most potent cytokines for inducing bone formation. In our study, the *in vivo* implantation showed obviously new bone formation in the muscle porch, demonstrating that the dextran-based hydrogel scaffold was an efficient carrier for rhBMP-2 delivery. After implantation for 4 weeks, the wet weights of new ectopic bone induced by dextran/BMP-2 with different DS were 226 mg (DS = 9) and 318 mg (DS = 12.5), respectively, as shown in Figure 8. The results of ash content had the similar tendency (23.2 mg vs. 34.8 mg). These consequences were complied with the degradation behavior. We concluded that higher DS possessed longer degradation period, which led to slow down the release of rhBMP-2, and thus gave rise to more extensive ectopic bone formation than a short-term delivery in rat models.

The histological analysis was performed in order to provide more details on the new ectopic bone formation induced by Dex/BMP-2 (Figure 9). After 4 weeks, trabeculae appeared to be obviously mature, and normal bone histology with lots of bone marrow and bony trabeculae was present observed in an ectopic site. Seldom residual materials were observed, confirming the complete degradation of the scaffold *in vivo*.

CONCLUSIONS

A novel dextran-based hydrogel was prepared by facile crosslinking the vinylsulfone-grafted dextran with mercapto molecules DTE via Michael addition. The hydrogel could rapidly

form *in situ* under mild physiological conditions. Both the gelation time and rheological behavior could be controlled readily by varying the concentrations and DS. The hydrogel could degrade via hydrolysis mechanism. Furthermore, cytotoxicity tests had shown the good biocompatibility *in vitro* of the hydrogels. The new bony tissue emerged in the *in vivo* animal tests, confirming the efficiently sustained delivery of the growth factor. In addition, better osteogenesis effect was found with higher DS, indicating that the sustained delivery increases the bone regenerative efficacy of BMP-2. This study suggested the hydrogel based on dextran-VS and DTE had promising applications in tissue engineering and drug delivery. Researches on the growth factor-loading and application in tissue regeneration are undergoing.

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