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A new biodegradable crosslinked polyethylene oxide sulfide (PEOS) hydrogel for controlled drug release

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ABSTRACT

We developed a polyethylene glycol (PEG)-based biodegradable hydrogel through disulfide crosslinking of polyethylene oxide sulfide (PEOS). The crosslinking rate was highly dependent on temperature, and incubation at about 40–50 °C was required for efficient crosslinking. The crosslinked PEOS hydrogel showed glutathione-dependent dissolution and corresponding controlled release of a model drug—fluorescein isothiocyanate (FITC)-labeled dextran—because the disulfide bond, the main linker, is selectively degraded in response to the high concentration of glutathione. The temperature-sensitive crosslinking and the hydrogel formation have the potential for use as an injectable biogel precursor, which was confirmed by in situ gel formation in mice.

1. Introduction

The need for biocompatible polymers as research or therapeutic materials has recently increased. Various biocompatible polymers such as poly(lactic-co-glycolic acid) (PLGA) (Kim et al., 2002), poly(methyl methacrylate) (PMMA) (Irzh et al., 2007), and poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) (Wada et al., 2007) are used in the fields of drug delivery, tissue engineering, and protein modification. Among them, poly(ethylene glycol) (PEG) is the most frequently and widely used. Along with its biocompatibility, which was approved by the FDA (Cai et al., 2007), other special characteristics contribute to the wide application of PEG. When PEG is conjugated to hydrophobic molecules, it adds hydrophilicity and increases the solubility of the conjugate (Y. Lee et al., 2005). Furthermore, the large hydrodynamic volume of PEG can protect the conjugate from adhesion, aggregation, and enzymatic attack under biological conditions (Manjula et al., 2003).

In addition to these characteristics, some researchers have made efforts to introduce selective biodegradability into PEG. The boundaries of PEG application could be further extended by biodegradability in response to the specific signals such as increase in temperature (Kaihara et al., 2008) or pH (Shenoy et al., 2005). The introduction of a disulfide bond is one of those efforts. Disulfide bonds are degraded in response to different glutathione concentrations. The disulfide is relatively stable in the cell exterior but is degraded rapidly in the cytosol because the glutathione concentration there is about 10 mM, which is more than 500 times higher than the extracellular concentration (Kakizawa et al., 2001). It was also reported that the concentration of free thiol groups near tumor tissue is seven times higher than that in normal tissue (Russo et al., 1986). Therefore, the use of disulfide bonds could be promising for controlled release in the cytosol or in tumors. Several successful introductions of disulfide bonds into PEG derivatives were reported. Kataoka's group introduced disulfide bonds between a PEG block and a poly(amino acid) block to construct an efficient gene delivery carrier (Oishi et al., 2005), and we also developed poly(ethylene oxide sulfide) (PEOS) by linking oligo ethylene oxide monomers with disulfide bonds to construct therapeutic nanoparticles encapsulating hydrophobic drugs (W.K. Lee et al., 2005).

Here, we report the crosslinking of PEOS with a trifunctional crosslinker. Interestingly, a rigid hydrogel was formed through temperature-dependent crosslinking. The crosslinking reaction was very slow at 25 °C, but the rate increased at 37 °C and increased

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sharply at 50 °C. Considering that hydrogels are useful tools in therapeutic fields including drug delivery (Sun et al., 2006; Li et al., 2003), gene delivery (Megeed et al., 2004), tissue engineering (Kirkland et al., 2008), ocular lenses (Aliyar et al., 2005; Kim et al., 2005), and so on, a PEG-based biodegradable hydrogel formed by crosslinking above human body temperature could have great potential. As an example of one application, we show the controlled release and in situ formation of a PEOS hydrogel.

2. Experimental

2.1. Materials

Poly(ethylene glycol), dichloromethane (DCM), p-toluenesulfonyl chloride, 4-(dimethylamino)-pyridine (DMAP), potassium thioacetate, triethylamine, S-acetylmercaptosuccinic anhydride, N,N-dimethylformamide (DMF), tris(2-aminoethyl)amine, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and fluorescein isothiocyanate (FITC)-dextran were purchased from Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) was used as received from LONZA (Walkersville, MD).

2.2. Synthesis of crosslinked PEOS hydrogel

2.2.1. Synthesis of the thioacetyled poly(ethylene glycol) monomers (**3**)

Poly(ethylene glycol) (**1**)(M_n = 600; 18.00 g, 30.00 mmol) was dissolved in dichloromethane (200 mL). 4-(Dimethylamino)-pyridine (1.833 g, 15.00 mmol), p-toluenesulfonyl chloride (12.58 g, 66.00 mmol), and triethylamine (19.24 mL) were added. After 3 h stirring at 0 °C, the solvent was removed by evaporation. The remaining mixture was dissolved in chloroform (200 mL) and washed with 0.1N HCl (×2) and distilled water (×3). The organic layer was dried with $MgSO_4$ and evaporated. The reaction yield was over 90%.

This tosylated poly(ethylene glycol) (**2**) (8.99 g, 10.00 mmol) were dissolved in DMF (200 mL). Potassium thioacetate (2.856 g, 25.00 mmol) was added and stirred overnight at room temperature. The mixture volume was reduced by evaporation to about 10 mL and diluted with chloroform (400 mL). It was then washed with distilled water (×3). After drying with $MgSO_4$, all solvent was removed by evaporation.

^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) of **2**: δ 2.45 (s, 6H, C***H*****$_3$**–C_6H_4–), 3.40–3.89 (m, 48H, –C***H*****$_2$**O(C***H*****$_2$**C***H*****$_2$**O)$_{11}$C***H*****$_2$**–), 4.15 (t, 4H, –SO_3–C***H*****$_2$**–), 7.35 (d, 8H, CH_3–C_6***H*****$_4$**–).

^{1}H NMR ($CDCl_3$) of **3**: δ 2.35 (s, 6H, C***H*****$_3$**CO–), 3.10 (t, 4H, –SC***H*****$_2$**–), 3.60–3.80 (m, 48H, –C***H*****$_2$**O(C***H*****$_2$**C***H*****$_2$**O)$_{11}$C***H*****$_2$**–).

2.2.2. Synthesis of the trifunctional crosslinker (**4**)

Tris(2-aminoethyl)amine (731.2 mg, 5.000 mmol) was dissolved in DMF (30 mL), and S-acetylmercaptosuccinic anhydride (5.225 g, 30.00 mmol) was dissolved in DMF (5 mL) and added dropwise to the Tris-DMF solution. After 3 h stirring at r.t., the mixture was precipitated in ethyl acetate. The precipitated product was washed several times with excess ethyl acetate and dried completely by evaporation.

^{1}H NMR ($CDCl_3$) of **4**: δ 2.35 (d, 9H, C***H*****$_3$**COS–), 2.55 (m, 6H, (–NHCH_2C***H*****$_2$**)$_3$–N), 3.30 (d of t, 6H, –SCHC***H*****$_2$**COOH), 3.53 (t, 6H, (–NHC***H*****$_2$**CH_2)$_3$–), 4.35 (t, 3H, –SC***H***CONH–).

2.2.3. Deprotection and synthesis of crosslinked PEOS (**5**)

Various ratios of the monomers (**3**) and crosslinker (**4**) were dissolved in 2 M NH_3/MeOH (30 mL) and stirred overnight at r.t. to deprotect the thioacetyl groups. After that, the solution was concentrated by evaporation.

For the initial crosslinking, the deacetylated mixture was dissolved in 30% DMSO aqueous solution (30 mL). After stirring for 2 days at r.t., this was dialyzed against distilled water. The initially crosslinked PEOS (**5**) was then collected after lyophilization and dissolved in water at a concentration of 30% (w/v). The solution was incubated at various temperatures for additional crosslinking and gelation.

^{1}H NMR ($CDCl_3$) of **5** (in case of monomer (**3**)/crosslinker (**4**) = 1.0): δ 2.55 (m, 6H, (–NHCH_2C***H*****$_2$**)$_3$–N), 2.71–2.83 (t, 4H, –SC***H*****$_2$**–), 2.90 (d of t, 6H, –SCHC***H*****$_2$**COOH), 3.53 (t, 6H, (–NHC***H*****$_2$**CH_2)$_3$–), 3.60–3.80 (m, 48H, –C***H*****$_2$**O(C***H*****$_2$**C***H*****$_2$**O)$_{11}$C***H*****$_2$**–), 3.90 (t, 3H, –SC***H***CONH–).

2.3. Rheometry measurement

A dynamic rheometer (Thermo Haake model RS 1) was used for confirmation of the temperature-dependent crosslinking. The solution of PEOS 12-1.2 (**5**), monomer/crosslinker ratio = 1.2 (30%, w/v), was placed between parallel plates of 25 mm in diameter and separated by a gap of 0.5 mm. The data were collected under a controlled stress (4.0 dyn/cm^2) and a frequency of 1.0 rad/s. The heating rate was 0.2 °C/min.

2.4. Ellman's assay for the degree of crosslinking

Ellman's reagent, 5,5′-dithio-bis-(2-nitrobenzoic acid), was dissolved in 0.1 M sodium phosphate buffer, pH 8, at a concentration of 4 mg/mL (Hermanson, 1996). The standard solutions were prepared by dissolving cysteine in the same buffer and serially diluting this solution at various concentrations. Crosslinked PEOS 12-1.2 (**5**) aqueous solution (10%, w/v) was kept in an incubator at several temperatures (25 °C, 37 °C, 50 °C), and at several time points 10 μl of each solution was taken and diluted 100-fold. Five microliters of Ellman's reagent solution was added to 100 μL of each standard and sample solution in a 96-well microplate. The absorbance of each solution was measured at 450 nm. The absorbance versus cysteine concentration was plotted for each of the standards and from this the concentration of crosslinked PEOS solution was calculated.

2.5. Scanning electron microscope observation

The crosslinked PEOS gel (**6**) was frozen using liquid nitrogen. After 48 h lyophilization, the dried sample was mounted onto an aluminum stud and sputter-coated with gold for 300 s. The surface and interior of the sample were observed by a scanning electron microscope (JEOL model JSM 5410LV).

2.6. Swelling ratio measurement

The swelling ability of crosslinked PEOS was determined gravimetrically. The dried weight (W_d) of the lyophilized sample of **6** (n = 3, initial weight was 100 mg) was measured. The sample was added to distilled water and PBS. The swollen weight (W_w) of the PEOS hydrogel was determined at a given time interval. The swelling ratio (q) of the PEOS hydrogel was calculated from the following expression $q = (W_w - W_d)/W_d$ (Xu et al., 2006).

2.7. Degradation of the crosslinked PEOS hydrogel

The lyophilized PEOS (**5**) (60 mg) was dissolved in distilled water at a concentration of 30% (w/v) and aged at 50 °C for 24 h (n = 3). The glutathione solution was then added and incubated at 37 °C. At a given time, the sample was collected and washed with

distilled water. The weight of the remaining sample was measured after lyophilization.

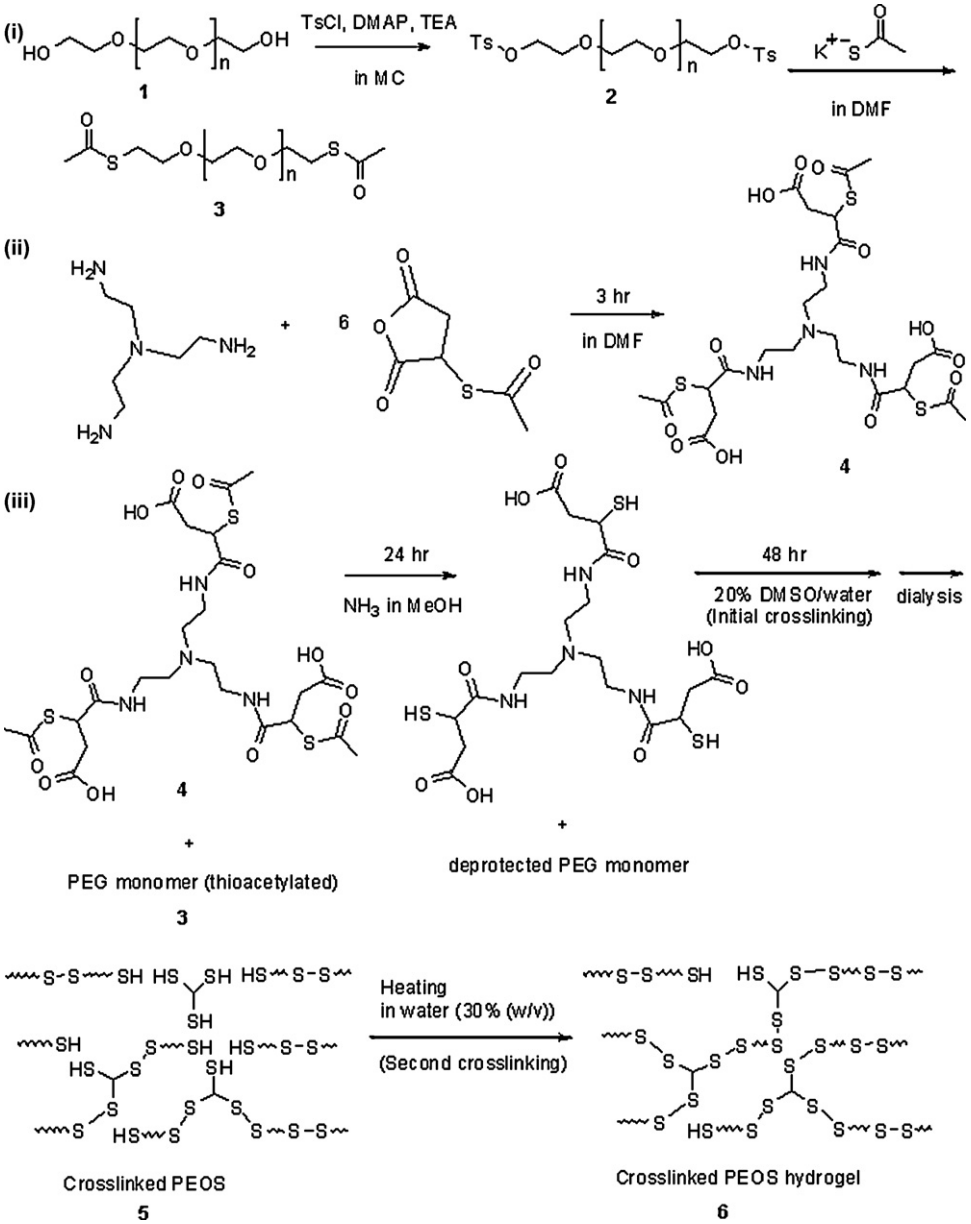
2.8. FITC-dextran release

Twenty microliters of fluorescein isothiocyanate (FITC)-dextran (average M_w 70,000) solution (10 mg/mL) was added to the solution of initially crosslinked PEOS (**5**) (150 mg/500 μL, $n = 3$). The solution was incubated at 50 °C for 24 h to form a rigid hydrogel. One milliliter of distilled water containing various amounts of glutathione was added to the hydrogels and incubated at 37 °C with mild rotation (hybridization incubator, FINEPCR model combi-H). At a given time interval, the 0.5 mL of supernatant was collected and replaced with the same volume of fresh glutathione solution.

The collected supernatant was measured fluorometrically (spectrofluorometer, Jasco FP-750) using 494 nm and 520 nm for the excitation and emission wavelengths, respectively. The fluorometer was calibrated using a known concentration of FITC-dextran (standard curve). The time course of the released FITC-dextran was plotted cumulatively.

2.9. Cytotoxicity assay

The cytotoxicity of the crosslinked PEOS hydrogel was measured by MTT assay (Kim et al., 2007). Human cervical carcinoma (HeLa) cells were seeded in a 96-well tissue culture plate at 7000 cells per well in 90 μL DMEM medium containing 10% FBS. Cells achieving 70–80% confluence after 24 h were exposed to 10 μL of the crosslinked PEOS solution for 48 h. Then, 26 μL of MTT stock solution (2 mg/mL in PBS) was added to each well. After 4 h of incubation at 37 °C, each medium was removed by micropipette and 150 μL of DMSO was added to each well to dissolve the formazan crystals formed by proliferating cells. Absorbance was measured at 570 nm



Scheme 1. Synthesis scheme of branched PEOS (i) Thioacetyl poly(ethylene glycol) synthesis, 0 °C, 3 h; (ii) Crosslinker synthesis, room temperature, 3 h; (iii) Deprotection and hydrogelation.

using a microplate reader (Molecular Devices Co., Menlo Park, CA) and recorded as a percentage relative to that of untreated control cells.

2.10. In vivo gel depot

C57BL/7 mice, each weighing 20–30 g, were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). 0.1 mL of the crosslinked PEOS solution (**5**) (30%, w/v in PBS) was subsequently injected subcutaneously into the abdomen using a syringe (1/2 cm^3 ultra-fine insulin syringe, Becton Dickinson, BD) with a 30 gauge needle. The mouse was placed on a heating pad and heated mildly at 43 °C for 30 min. In the alternative procedure, the crosslinked PEOS solution in the syringe was pre-heated at 43 °C for 10 min before the injection. The injected site was then heated with a heating pad at 43 °C for further 20 min. The results of both procedures were same. After 1 h, the injected site was cut open and the gel formed in situ was observed.

3. Results and discussion

3.1. Crosslinking and hydrogelation of PEOS

The crosslinking reaction of polyethylene oxide sulfide (PEOS) was performed using a trifunctional crosslinker (**4**). The trifunctional crosslinker was synthesized by the ring-opening reaction between tris (2-aminoethyl) amine and S-acetylmercaptosuccinic anhydride (Scheme 1) (Klotz and Heiney, 1962). For efficient crosslinking, the acetyl protecting groups of both monomer (**3**) and crosslinker (**4**) were simultaneously deprotected.

The initial polymerization and crosslinking of **3** and **4** were performed using DMSO (Koo et al., 2005). The initially crosslinked polymer was further crosslinked at various temperatures. The kinetics of the disulfide bond formation, shown in Fig. 1a, were measured using Ellman's assay. At 25 °C, the free thiol percentage was maintained even after 24 h, whereas it decreased promptly at 37 °C. The decrease rate was even faster at 50 °C. Fifty percent of the remaining thiol groups disappeared within 2 h. The activation energy of the crosslinking reaction could be calculated from

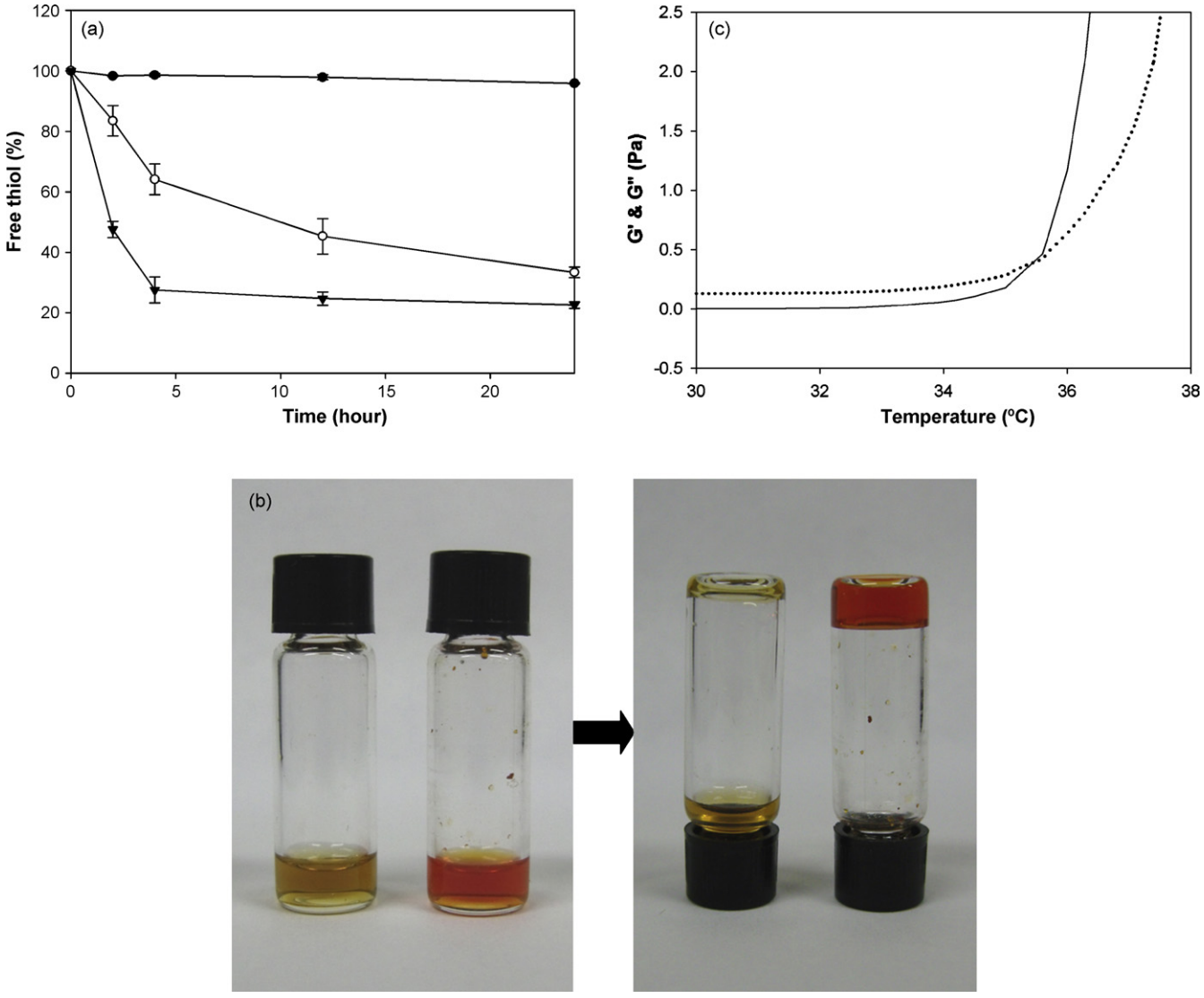


Fig. 1. Hydrogelation of crosslinked PEOS. (a) Free thiol quantity determination with Ellman's reagent in crosslinked PEOS 12-1.2. 25 °C (●), 37 °C (○), 50 °C (▼). (b) Crosslinked PEOS 12-1.2 hydrogel. The left vial contains fluid PEOS 12-1.2 (**5**) solution before heating, and the right vial contains rigid PEOS hydrogel (**6**) after heating (50 °C, 12 h). (c) Storage and loss moduli of crosslinked PEOS 12-1.2. Solid line is G′ and dotted line is G″.

the temperature dependence of the reaction rate. The detailed calculation procedure was described in supporting information and the calculated activation energy was 81.6 kJ/mol, which was similar to that of the disulfide crosslining reaction in the previous report (Miyauchi et al., 1969).

The temperature-sensitive crosslinking induced hydrogelation. The 30% (w/v) solution of the crosslinked PEOS formed a hydrogel (Fig. 1b) and the gelation was analyzed by rheometry. The storage (G′) and loss (G″) moduli are low and G′ is smaller than G″ at low initial temperatures (Weng et al., 2007). Because the increase rate of G′ is larger than that of G″, there is a crossover between G′ and G″ at a particular temperature. This temperature is the gelation temperature (T_g), at which the solution phase changes from sol to gel (Hiemstra et al., 2007). The gelation of PEOS started at about 37 °C (Fig. 1c). Comparing Fig. 1a and c, we concluded that the hydrogelation was highly dependent on the crosslinking of the remaining disulfide bonds at a high temperature.

3.2. Morphology of crosslinked PEOS gel

The morphology of the crosslinked PEOS gel was observed by scanning electron microscope (SEM) (Nederberg et al., 2007). The hydrogel was frozen rapidly by liquid nitrogen and lyophilized instantly. Although the water inside the gel was sublimed to make cavities, the structure of the hydrogel was preserved. The SEM image is shown in Fig. 2. The structure of the PEOS gel is very porous, and it could help to form a high-water-content hydrogel, as is generally shown in other hydrogels (Crescenzi et al., 2007). Average pore diameter is about 10 μm.

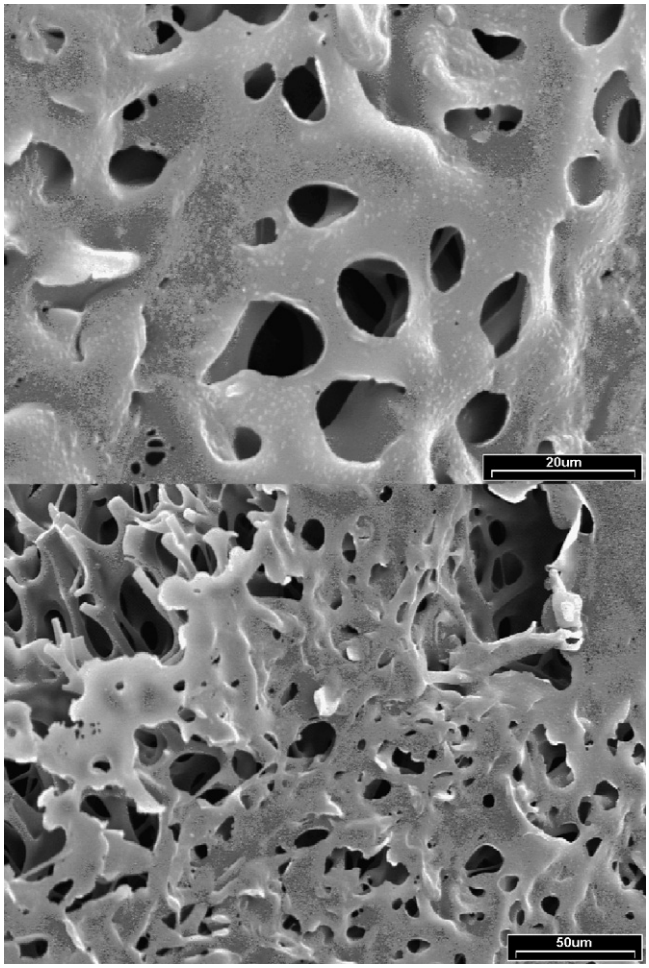


Fig. 2. SEM images of crosslinked PEOS 12-1.2 hydrogel. White bar is 20 μm (top) and 50 μm (bottom).

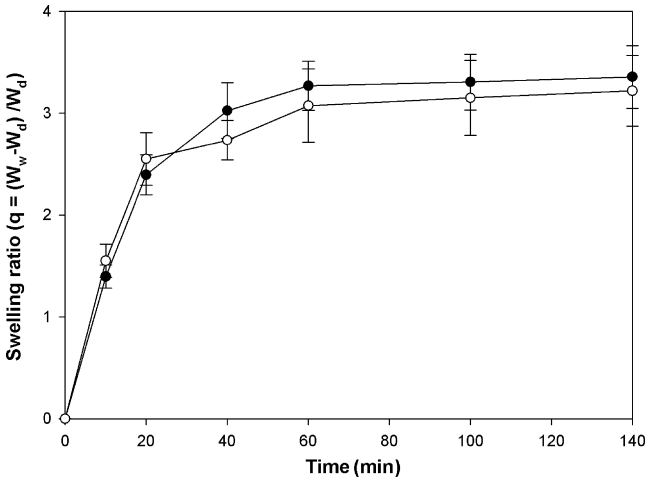


Fig. 3. Swelling ratio graph of crosslinked PEOS 12-1.2 hydrogel. Swelling in distilled water (●) and in PBS (○).

3.3. Swelling and destabilization of crosslinked PEOS gel

The crosslinked PEOS hydrogel has porous structure so it can interact with the aqueous medium. The swelling kinetics profile of the crosslinked PEOS hydrogel is shown in Fig. 3. The crosslinked PEOS showed high water-swelling ability in both distilled water and PBS. After 1 h, crosslinked PEOS can absorb about 3.2 times its mass of water and the amount was 3.1 times in PBS. The small difference of final ratio is probably due to the change of the osmotic pressure in PBS (Pitarresi et al., 2007). But there is not significant difference of swelling property in between water and PBS.

Because the crosslinking of the PEOS was based on the disulfide bond, it was expected that the hydrogel would destabilize under reductive conditions (Lee et al., 2007). The destabilization profile of the crosslinked PEOS hydrogel is shown in Fig. 4. Without the glutathione, several days were required for the final dissolution of the hydrogel in an excess amount of water. However, when the hydrogel was incubated in the presence of 10 mM glutathione, the half life of the dissolution was around 4 h. In 50 mM, the half life was less than 2 h. Based on these results, we concluded that

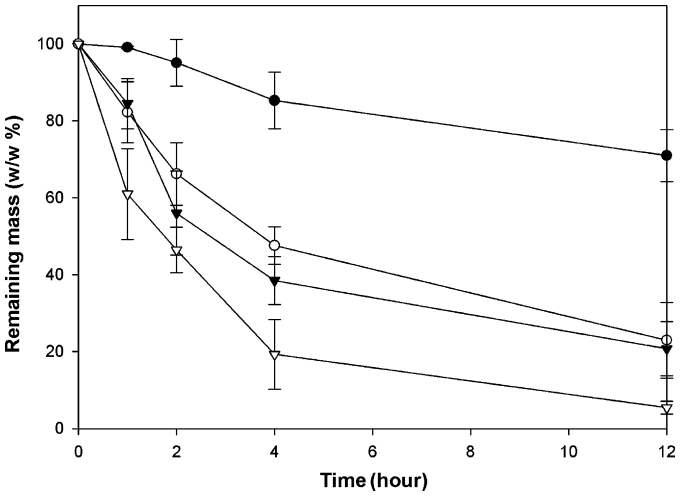


Fig. 4. Degradation of crosslinked PEOS 12-1.2 hydrogel. The concentration of glutathione solution is 0 mM (●), 10 mM (○), 20 mM (▼), and 50 mM (▽). 0 mM glutathione solution means pure distilled water.

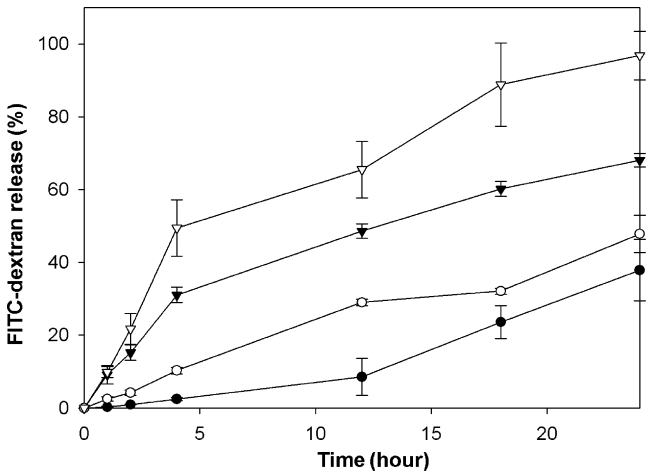


Fig. 5. FITC-dextran release from crosslinked PEOS 12-1.2 hydrogel. The concentration of glutathione solution is 0 mM (●), 10 mM (○), 20 mM (▼), and 50 mM (▽). 0 mM glutathione solution means pure distilled water.

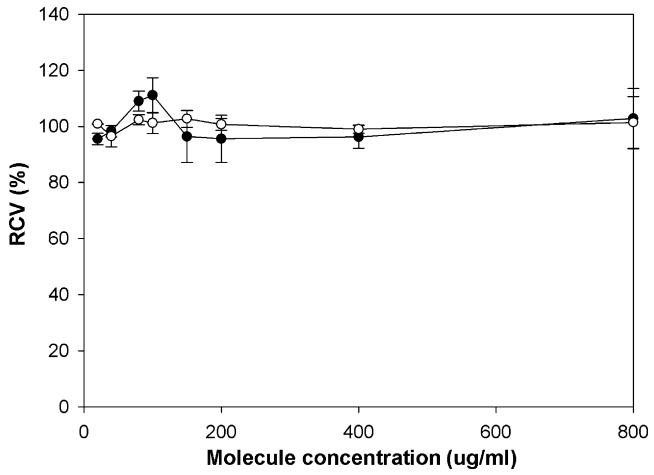


Fig. 6. Cytotoxicity of PEOS gel solution in HeLa cells by MTT assay. RCV: relative cell viability. Data were expressed as mean standard ($n = 4$). Crosslinked PEOS solution (●) and degraded molecules (○).

the fast dissolution of the crosslinked PEOS hydrogel was strongly dependent on the degradation of the disulfide bonds in its structure.

3.4. In vitro model of drug release

The swelling and degradation properties of the crosslinked PEOS hydrogel can be used in controlled drug release. Crosslinked PEOS hydrogel containing a model drug, FITC-labeled dextran (Torres-Lugo et al., 2002), was formed and the concentrations of the released FITC-labeled dextran were determined by fluorometry (Fig. 5).

Because the disulfide bond is degraded in the reductive environment, the release rate of the drug from the hydrogel is strongly dependent on the concentration of glutathione. Without glutathione, over 60% of the FITC-dextran still remained in the hydrogel. However, when the hydrogel was incubated in the glutathione buffer, the release rate increased according to the concentration of glutathione. The time required for 50% release was around 24 h, 12 h, and 4 h at the concentrations of 10 mM, 20 mM, and 50 mM glutathione, respectively. The release characteristics of the crosslinked PEOS hydrogel in response to reduction potential could help with the application of anticancer drugs to tumors and in controlled release systems.

3.5. Cytotoxicity test

For biological applications, toxicity or biocompatibility is also an important problem. The cytotoxicity of the crosslinked PEOS was examined in HeLa cell lines by MTT assay. As shown in Fig. 6, the crosslinked PEOS has negligible cytotoxicity even at the concentration of 800 μg/mL. High biocompatibility of the crosslinked PEOS

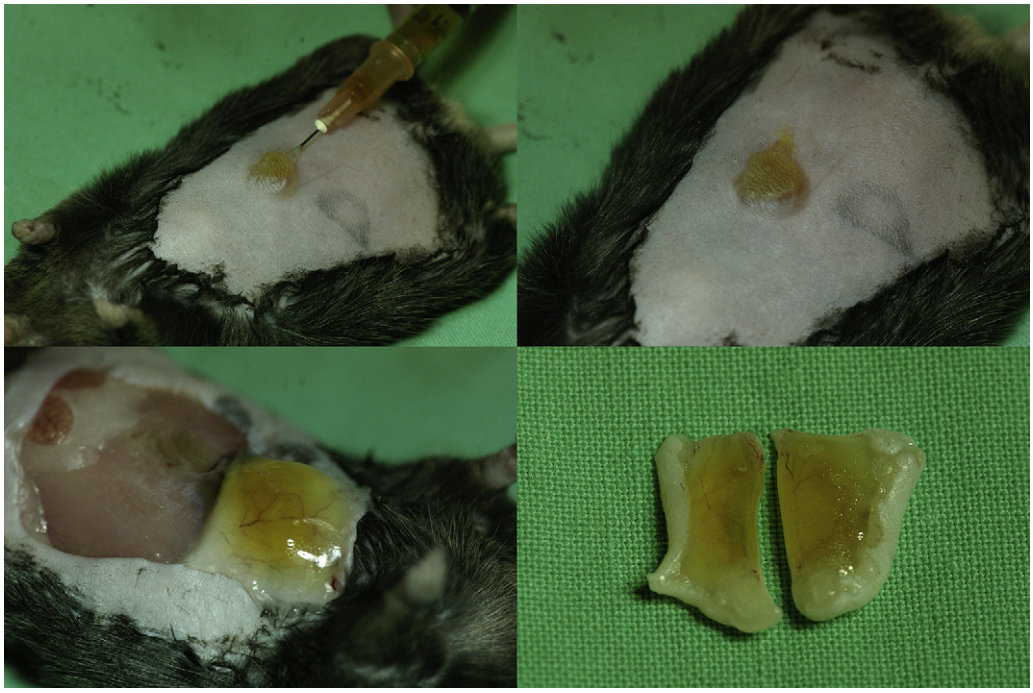


Fig. 7. Photographs of crosslinked PEOS hydrogel in mouse. The injected site was cut open after 1 h.

was expected because its main backbone consists of biodegradable disulfide bonds.

The degradation products of crosslinked PEOS were also tested for biocompatibility. Crosslinked PEOS was degraded completely by incubation in 10 mM glutathione buffer for 48 h, and the cytotoxicity of the resulting product was analyzed via a similar method. The degradation product also showed no toxicity. The resulting biocompatibility supports the high potential of crosslinked PEOS for applications in biological fields.

3.6. In situ gel formation

Because the crosslinking and gelation of PEOS was dependent on temperature, the possibility of in situ hydrogelation was expected. A mouse was injected subcutaneously with initially crosslinked PEOS solution (30%, w/v). For faster gelation, the injected site was contacted with a heating pad, and heated mildly at 43 °C for 30 min. After 1 h, the injected site was opened and the gel formation was observed (Fig. 7). The hydrogel maintained its shape after detachment from the mouse and radial cutting.

The in situ gelation time could be reduced by pre-heating of the initially crosslinked PEOS solution. Because the viscosity of the solution increased sharply, we injected the crosslinked PEOS solution after 10 min-pre-heating. After heating the injected site by a heating pad for further 20 min, almost the same PEOS hydrogel was obtained (data not shown). This experiment showed that PEOS hydrogel could be used as an in situ gel-forming material.

4. Conclusions

We have developed a new hydrogel based on the disulfide crosslinking of a PEG derivative, PEOS. The crosslinking reaction of PEOS was strongly dependent on temperature. Because the threshold temperature for the crosslinking was above 37 °C, it could have high potential as an in situ gelation material in the human body (Chung et al., 2002; Qiu et al., 2003). The resulting hydrogel showed sustained drug release properties and high biocompatibility. Moreover, the hydrogel exhibited selective degradation activity in a reductive environment due to its internal disulfide bonds, so it could exhibit fast degradation around tumors or in the cell cytosol. These results show that this material could be applied to various biological and medical fields.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2009.03.010.

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