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Graphical Abstract

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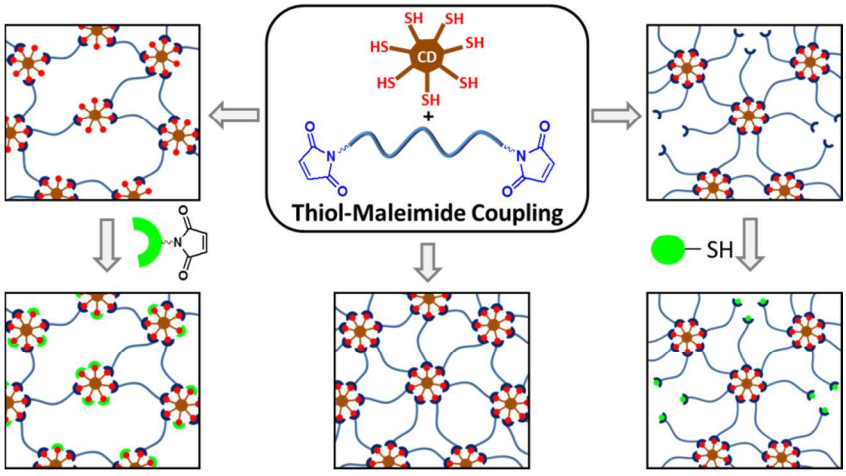

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Cyclodextrin mediated polymer coupling via thiol-maleimide conjugation: facile access to functionalizable hydrogels

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Fabrication of well-defined chemically cross-linked poly(ethylene glycol) (PEG)-based hydrogels using the thiol-maleimide addition reaction is reported. Maleimide containing homobifunctional PEGs with different molecular weights were synthesized and reacted with thiol functionalized β-cyclodextrin (β-CD(SH)$_7$) to yield hydrogels with high efficiency under mild conditions. The resulting hydrogels were characterized by their water uptake properties, surface morphologies and rheological behaviours. In order to introduce reactive functional groups into the hydrogels, the stoichiometry of the thiol and maleimide groups was varied to obtain either thiol or maleimide functionalized hydrogels. Efficient functionalization of these reactive hydrogels in a tailored fashion was demonstrated through conjugation of appropriately functionalized fluorescent dye molecules.

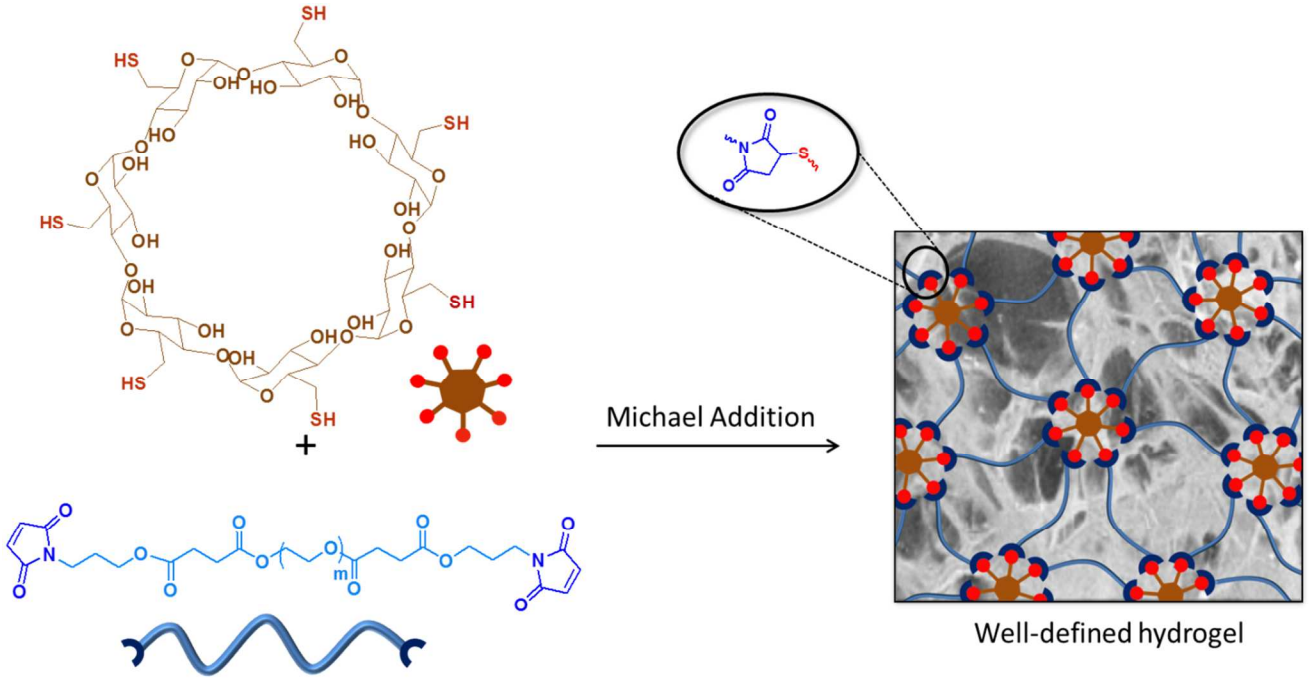
Introduction

Attributes such as biocompatibility, tunable water uptake and physical characteristics similar to soft tissues make hydrogels attractive materials for various biomedical applications. Hydrogels have been intensely explored for fabricating controlled release systems for drug delivery, platforms for biomolecular immobilization, implant coatings, contact lenses and wound healing dressings.[1-7] Their structural similarities to the natural extracellular matrix (ECM), enables mimicking natural systems for tissue engineering.[8-9] Oftentimes, biological cues such as peptides and growth factors are incorporated onto structural scaffolds to interface with biological materials in a desired fashion e.g. promote cellular functions to enable tissue formation.[10-12] Among various polymeric materials investigated for fabrication of hydrogels, poly(ethylene glycol) (PEG) based systems have been extensively explored since this class of materials possess desirable characteristics such as anti-biofouling, biocompatibility, rapid clearance from the body, non-toxicity and resistance towards recognition by the immune system.[13-15]

Increasing demand of hydrogels for abovementioned applications has necessitated development of efficient and benign synthetic methods for fabrication and functionalization of these materials. In recent years, many different efficient conjugation chemistries grouped as "click" methodologies have been widely used to fabricate and functionalize hydrogels. Although majority of the chemically crosslinked hydrogels studied until recently possess random cross-linking, advances in efficient conjugation chemistry has attracted attention in creating well-defined network structures.[16]

Among the various 'click' reactions available at hand, the thiol-maleimide Michael addition reaction is attractive since it proceeds under metal-free conditions with high conversions and has been utilized to fabricate and functionalize polymeric materials.[17] Recently, Nguyen, Du Prez and coworkers screened a variety of thiol-based coupling reactions and deduced that the thiol-maleimide conjugation proceeds with excellent efficiency and rapid kinetics.[18] Although several hydrogel systems based on thiol-maleimide chemistry have been reported in recent years,[19-20] utilization of this chemistry to obtain well-defined functional networks has been limited. In a recent example, Bowman and coworkers reacted thiol and maleimide containing PEG-based macromonomers in an off-stoichiometric manner to obtain maleimide containing hydrogels that were later functionalized with furan-containing peptides to obtain a controlled release system.[21] Notably, thiol-based conjugation has been extensively utilized to modify various polymeric materials in the realm of bioconjugations.[22]

Well-defined hydrogels can be obtained using building blocks that contain the reactive units responsible for effective crosslinking at precise positions. Efficient crosslinking of telechelic polymers containing reactive groups at chain termini with small multivalent cross-linkers can yield hydrogels with near ideal structures. A seminal contribution in this area was reported by Hawker and coworkers where they outlined the synthesis of PEG-based hydrogels by crosslinking acetylene terminated polymer with a tetra-azide crosslinker.[23] It can be envisioned that a variety of 'click' reactions utilizing multivalent crosslinkers can yield well-defined hydrogels.



Scheme 1. Schematic illustration of fabrication of hydrogels via thiol-maleimide conjugation.

A readily available versatile multivalent molecule such as cyclodextrins (CDs), cyclic oligosaccharides with six to eight glucopyranose units can be utilized as a crosslinker to obtain hydrogels. CDs possess a toroidal structure with primary hydroxyl groups at the narrow bottom rim and secondary hydroxyl groups at the wider upper side. CDs are readily available materials and possess a well-defined rigid structure. The inclusion complexation ability of CDs with various guest molecules makes them attractive for drug delivery systems. The chemical structure of cyclodextrins also allows them to be used as multifunctional crosslinking reagents and to date several CD-containing randomly cross-linked functional hydrogel networks have been prepared.[24-28]

Herein, we report the facile preparation of poly(ethylene glycol) (PEG) based chemically cross-linked hydrogels using the thiol-maleimide conjugate addition reaction (Scheme 1). The synthetic approach employs utilization of maleimide end-functionalized poly(ethylene glycol)s as the matrix and thiol functionalized β-cyclodextrin (β-CD(SH)$_7$) as crosslinker. High reaction rate between the sulfhydryl groups and maleimide functionality provides rapid gelation. In order to allow post-modification reactions enabling further introduction of functionality, crosslinker ratio were tailored. Preparation of hydrogels using off stoichiometric amounts of thiol-maleimide functional groups in the feed provides direct access to either thiol or maleimide functionalized hydrogels amenable to facile post-gelation modifications. The strategy also allows the incorporation of CD units into hydrogel network in a controllable fashion that would allow potential host-guest interactions based applications.

Experimental Section

Materials and methods

Poly(ethylene glycol)s (PEG 2K, PEG 6K, PEG 10K), 4-dimethylaminopyridine (DMAP), (5,5'-dithio-*bis*-(2-nitrobenzoic acid), *N*-(5-fluorosceinyl)maleimide and fluoresceinamine, isomer I were obtained from Aldrich Chemical Co. 1-Ethyl-3-(3-dimethylamino propyl) carbodiimide (EDCI) and β-cyclodextrin hydrate were obtained from Alfa Aesar. 5-((2-(and-3)-S-(acetylmercapto)succinoyl)amino)fluorescein (SAMSA fluorescein) dye was obtained from Invitrogen (Carlsbad, CA). Other chemicals and solvents were purchased from Merck and used as obtained without further purification unless otherwise noted. Synthesis of alcohol exo-3a,4,7,7a-tetrahydro-2-(3-hydroxypropyl)-4,7-epoxy-1H-isoindole-1,3(2H)-dione,[29] thiol functionalized β-cyclodextrin[30] and PEG diacids[31] were conducted according to reported procedures. Syntheses and characterization of PEG-bismaleimides are described in the Supporting Information. Proton NMR spectral data were obtained using a Varian INOVA400 at 400 MHz. Thermogravimetric analyses (TGA) were carried out using a TA Instruments at a 10 ^{o}C/min heating rate under nitrogen atmosphere. Dye functionalized hydrogels were visualized with Zeiss Observer.Z1 inverted fluorescent microscope.

Representative hydrogel formation

Hydrogels were prepared by multiple Michael type addition of thiol-functionalized β-cyclodextrin (β-CD(SH)$_7$) onto PEG-bismaleimide (PEG-*bm*) polymers. Typical procedure is as follows: The polymer (50 mg, 0.02 mmol for PEG 2K-*bm*) was placed in a vial and dissolved in DMF (100 µl). Desired amount of β-CD(SH)$_7$ and catalytic amount of triethylamine (1/20 eq. per

thiol) were dissolved in DMF (100 µl) and then added to polymer solution. The stoichiometry of thiol to maleimide group was adjusted to obtain hydrogels with desired residual reactive functional groups. The mixture was sonicated to assist homogenous gelation. Hydrogel formation was rapid and in about one minute there was no flow of sample upon inversion of vial. Gelation was continued for 6h to ensure complete conjugation. After hydrogel formation, unreacted species were removed by washing with DMF followed by distilled water. Swollen hydrogel sample was freeze-dried in *vacuo* to yield dried hydrogel.

Swelling studies

Swelling studies were performed by immersing a known amount of hydrogel in distilled water and monitoring the increase in its mass as a function of time until equilibration was reached. The percentage weight change was obtained from fractional weight change using the equation:

Percentage of Swelling (%) = $(W_{wet} - W_{dry}) / W_{dry} \times 100$,

where W_{wet} and W_{dry} refer to the weight of wet and dry hydrogels respectively. All measurements were performed in triplicate and average data was plotted to obtain swelling curves.

Scanning electron microscopy (SEM)

The surface morphologies of hydrogels were analyzed with scanning electron microscopy (SEM). SEM images of dried hydrogels were acquired using an ESEM-FEG/EDAX Philips XL-30 (Philips, Eindhoven, The Netherlands) instrument operating at an accelerating voltage of 10 kV.

Rheological measurements

Hydrogel rheological behaviors were evaluated by measuring the loss (G″) and storage (G′) moduli of the swollen hydrogel as a function of angular frequency using an Anton Paar MCR 302 rheometer. Tests were carried out in triplicate at 25 °C by applying a 0.5 % strain between 0.05–100 rad/s. The samples prepared as disks were analyzed using a parallel plate (15 mm diameter) with a gap of 2.0 mm.

Determination of sulfhydryl content

Free sulfhydryl group content in hydrogels was determined using Ellman's method.[32] Firstly, a reaction medium of 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA buffer was prepared. Ellman's reagent solution was prepared by dissolving of 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) (4 mg/ml). A sample of freshly prepared hydrogel (5 mg) was placed in a vial and 2.5 mL of buffer and corresponding Ellman's reagent solution were added onto the solution. The resultant mixture was incubated at 37 °C for 2 hours. The absorbance at 412 nm was measured to calculate the total sulfhydryl group content in the sample using the molar extinction coefficient of 2-nitro-5-thiobenzoic acid (TNB) (14,150 $M^{-1}cm^{-1}$).[33]

Functionalization of hydrogels with maleimide-containing fluorescent dye

Functionalization of hydrogels containing free sulfhydryl groups were performed using *N*-(5-Fluorosceinyl)maleimide. First, the amount of free sulfhydryl groups in 5 mg hydrogel sample was calculated and ten equivalents of *N*-(5-fluorosceinyl)maleimide in PBS (pH 7.4, 0.5 mL) solution was added. After incubation at room temperature for 12 h, hydrogel was washed several times with organic solvents and deionized water before analysis of fluorescence microscopy. As a control experiment, a sample of $CDP6_{(2:1)}$ hydrogel was incubated with fluoresceinamine dye.

Functionalization of hydrogels with thiol-containing fluorescent dye

Hydrogels containing free maleimide groups were functionalized via thiol-containing SAMSA fluorescein dye conjugation. Firstly, protecting group of the dye was removed by treatment with 0.1 M NaOH at room temperature. Then, the solution was neutralized with concentrated HCl and buffered to pH 7.0 with 0.5 M sodium phosphate. The amount of free maleimide groups in 5 mg hydrogel sample was calculated and ten-fold excess of activated SAMSA fluorescein thiol solution was added. After incubation at room temperature for 12 hours, hydrogel was washed several times with organic solvents and deionized water before analysis using fluorescence microscopy. As a control experiment, a sample of $CDP6_{(1:2)}$ hydrogel was incubated with maleimide-functionalized fluorescein dye.

Results and discussion

Synthesis and characterization of hydrogels

Commercially available PEG-diols of varying molecular weights were appended with reactive maleimide functional groups at both ends. Briefly, the PEG-diols were converted to respective diacids by reacting with succinic anhydride, followed by coupling with a furan-protected maleimide containing alcohol. Removal of the furan protecting groups via the retro Diels-Alder reaction yields the maleimide-containing telechelic PEG polymers (Supporting Information Scheme S1). The quantitative nature of this deprotection step was determined using ^{1}H NMR spectroscopy. Thiol functionalized β-cyclodextrin crosslinker was synthesized by converting primary hydroxyl groups of β-CD molecule to thiol groups using a two-step procedure.[30] The primary hydroxyl groups were first converted to iodo groups using iodine and triphenylphosphine, followed by their conversion to thiol functional groups by treatment with thiourea. Ellman's assay of thus obtained heptavalent CD-crosslinker revealed a thiol content of 98 %.

Hydrogels were prepared by simply mixing the solutions of PEG-bismaleimide polymers and β-$CD(SH)_7$. Rapid crosslinking occurs through multiple Michael additions between maleimide groups and thiols. Gel formation occurs within a minute in the presence of Et_3N as a catalyst. In order to compare the effect of polymer chain length and crosslinker ratio on physical and morphological properties, a library of hydrogels was prepared (Table 1). PEG contents were varied in molecular weight as 2000, 6000 and 10000 $gmol^{-1}$. The ratio of β-$CD(SH)_7$ crosslinker and PEG-bismaleimide was adjusted to obtain hydrogels with varying crosslinking density and reactive functional group composition. In the first series of gels an equimolar stoichiometry of thiol-maleimide was used in the feed.

Table 1 Series of hydrogels having different crosslinker ratio and gel conversions of synthesized hydrogels.

Item	Hydrogel	Polymer	Feed Ratio [SH] : [Maleimide]	Gel Conv. (%)	Thiol Content[a] (mmol x10^{-4})	Thiol Consumed (%)
1	$CDP2_{(1:1)}$	PEG 2K-*bm*	1 : 1	89	36.10 / 4.62 (±1.07)	87.20
2	$CDP6_{(1:1)}$	PEG 6K-*bm*	1 : 1	87	14.70 / 2.26 (±0.83)	84.60
3	$CDP10_{(1:1)}$	PEG 10K-*bm*	1 : 1	86	9.28 / 1.73 (±0.67)	81.30
4	$CDP6_{(2:1)}$	PEG 6K-*bm*	2 : 1	92	28.07 / 16.92 (±3.39)	39.70[b]
5	$CDP6_{(1.5:1)}$	PEG 6K-*bm*	1.5 : 1	88	21.60 / 9.63 (±3.64)	55.40[c]
6	$CDP6_{(1:2)}$	PEG 6K-*bm*	1 : 2	80	7.59 / 0.59 (±0.24)	92.20
7	$CDP6_{(1:1.5)}$	PEG 6K-*bm*	1 : 1.5	82	10.02 / 1.14 (±0.22)	88.60

[a] Thiol amount used for synthesis of 5 mg hydrogel / Determined thiol amount in 5 mg hydrogel sample (Data in triplicate). [b] Maximum expected value is 50 %. [c] Maximum expected value is 66.67 %.

Ideally, upon complete consumption of all reactive functional groups this should provide and hydrogels cannot undergo effective post-polymerization functionalization. To obtain hydrogels that would allow covalent functionalization, a second series of hydrogels was synthesized where the amount of crosslinker was doubled so that all the maleimide groups would combine with thiols and free thiol functionalities will be present in the gel network. A third series of hydrogels were designed to contain free maleimide groups in the network. Such hydrogels were obtained by adjusting the stoichiometry in the feed to contain excess maleimide functional group. The main advantage of this approach is the simplicity for obtaining either thiol or maleimide functionalized hydrogels.

Hydrogels reported here are obtained using well-defined building blocks with specific crosslinking chemistry. A high degree of both physical and chemical homogeneity can be expected for these hydrogels when compared to traditional gels obtained through random crosslinking of polymer chains. The extent of near-ideal network formation can be probed by determination of residual functional groups within the hydrogel. Quantification of residual thiol groups in these hydrogels will provide information about the deviation from ideal network structure expected when equimolar stoichiometry of thiol and maleimide functional groups is employed for gel formation. In order to gain a quantitative information about the number of sulfhydryl groups, hydrogels prepared using PEGs with varying chain length were treated with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent). The reaction of DTNB with

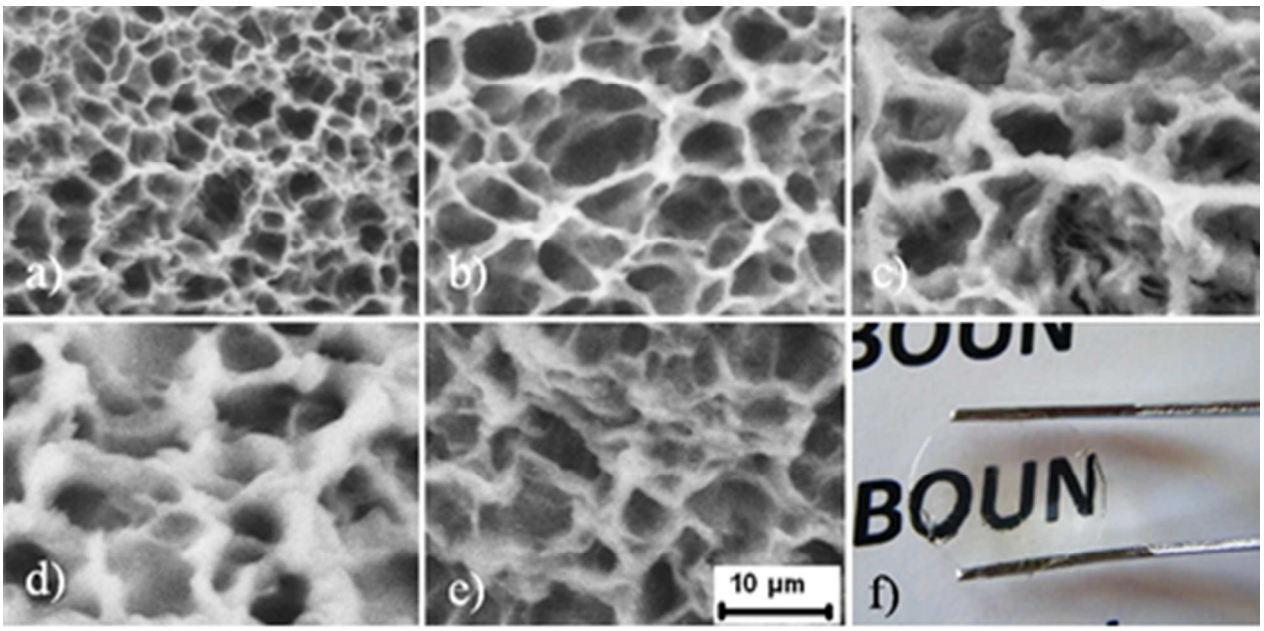


Figure 1. Representative ESEM images of freeze-dried hydrogels: (a) $CDP2_{(1:1)}$, (b) $CDP6_{(1:1)}$, (c) $CDP10_{(1:1)}$, (d) $CDP6_{(1:2)}$, (e) $CDP6_{(2:1)}$, (f) Photograph of transperent hydrogel $CDP6_{(1:1)}$. Scale bar for all images is 10 μm.

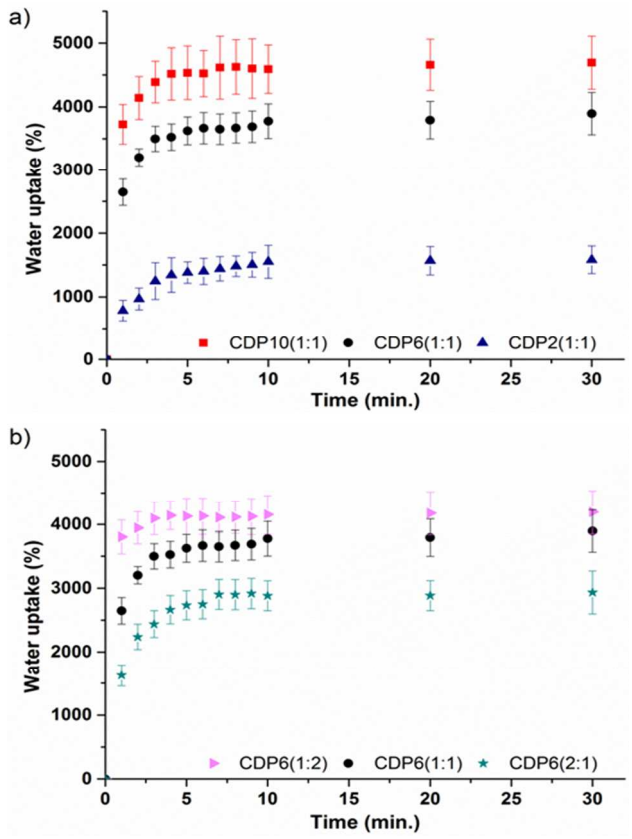


Figure 2. Water uptake of hydrogels in water versus time for comparison of hydrogels as a function of (a) PEG chain length (b) crosslinker ratio. All data are in triplicate, and error bars indicate one standard deviation.

sulfhydryl groups yields a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB). The coloured TNB species has a high molar extinction coefficient in the visible range (412 nm); thus sulfhydryl groups can be quantified by reference to the extinction coefficient of TNB. First, samples of hydrogels $CDP2_{(1:1)}$, $CDP6_{(1:1)}$ and $CDP10_{(1:1)}$ were evaluated in terms of free thiol groups in the gel networks. Since stoichiometrically equivalent amount of maleimide and thiol groups were utilized for hydrogel formation, ideally, complete consumption of sulfhydryl groups is expected. However, due to the steric bulk of the polymer chains and steric crowding around the crosslinking sites, some of thiol groups may remain unreacted and the amount of these thiols can provide information about the deviation from ideal network structure (assuming that formation of loops and cycles of polymer chains with crosslinker is low). Equal weights of freshly prepared hydrogel samples were treated with corresponding DTNB in 2.5 mL of PBS buffer and after incubation the absorbance at 412 nm was measured using a spectrophotometer. Calculation of residual thiol content within the hydrogels indicated that 80-90 % of thiol groups were consumed during network formation (Table 1). It was noted that increasing the polymer chain length caused a decrease in overall thiol consumption. The hydrogels $CDP6_{(2:1)}$ and $CDP6_{(1:2)}$ with different β-CD crosslinker ratio were also treated with DTNB to gain information about the amount of free thiol groups. A higher thiol consumption was observed in $CDP6_{(1:2)}$ hydrogel than $CDP6_{(1:1)}$. This can be attributed to the presence of higher maleimide feed ratio compared to thiol groups which leads to increased thiol consumption.

Hydrogels were obtained as clear and transparent samples (Figure 1f). The microstructures of these hydrogels were examined with ESEM on freeze-dried hydrogel samples. As shown in Figure 1, the hydrogels were highly porous and possessed relatively uniform microstructure which suggests that gelation reactions were homogenous. Increase in apparent pore size was observed upon increase of the PEG chain length that results in decreased crosslinking density. It was also observed that decreasing the crosslinker ratio provides PEG-rich hydrogels as suggested by the fleshy textures along the network (Figure 1d).

Swelling properties of the hydrogels were probed gravimetrically by recording water uptake over time until they reached equilibrium. Firstly, water uptake of hydrogels prepared by PEGs of increasing molecular weights (ca. 2000, 6000 and 10000 $gmol^{-1}$) was investigated. Swelling profiles show that hydrogels reach equilibrium swelling rapidly and the highest swelling ratio was dependent on the chain length of the PEG polymer (Figure 2a). As expected, decreased crosslinking density and increased hydrophilicity due to the increased chain length results in higher water uptake. Comparative water uptakes of $CDP6_{(1:1)}$, $CDP6_{(2:1)}$ and $CDP6_{(1:2)}$ hydrogels with different

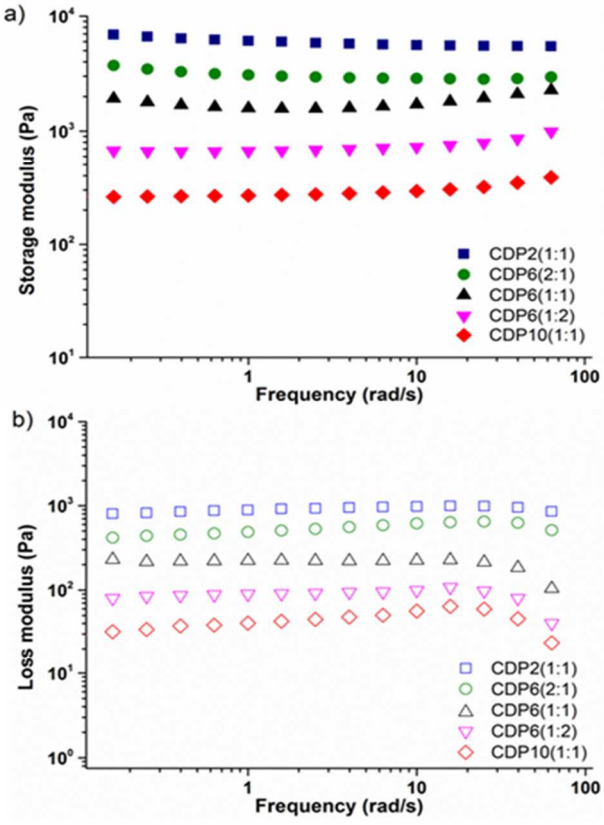


Figure 3. The frequency dependence of (a) storage moduli and (b) loss moduli for the hydrogels.

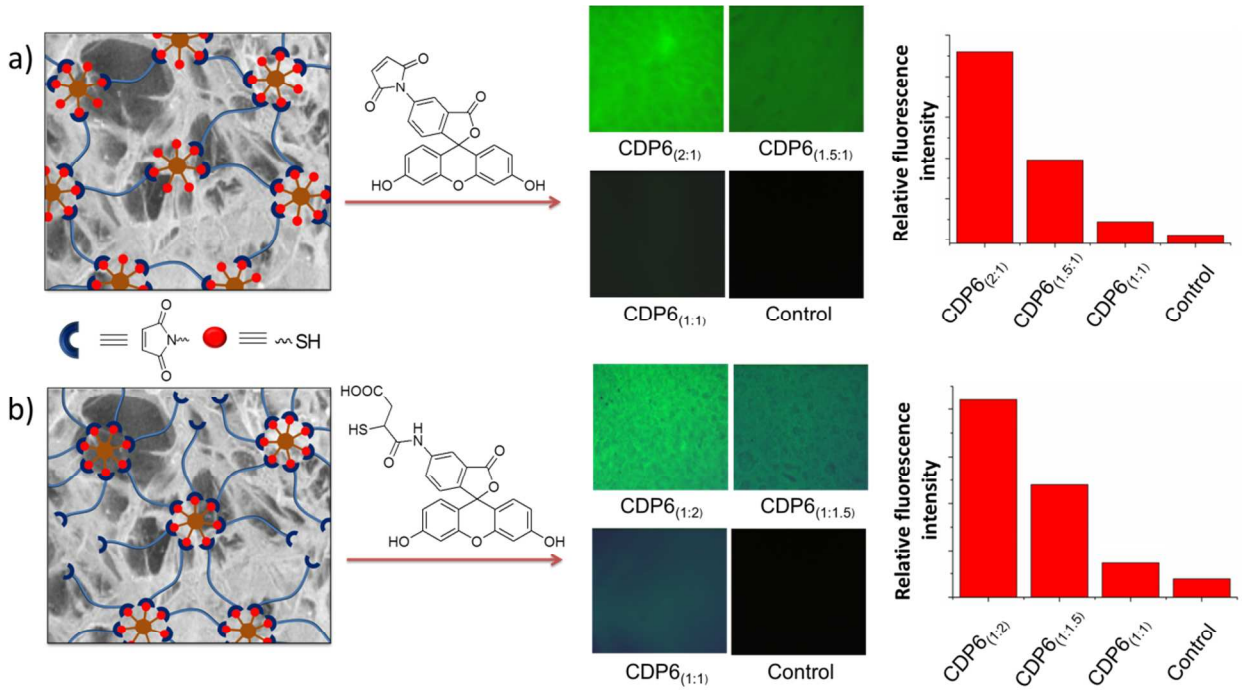


Figure 4. (a) Functionalization of thiol-containing hydrogels with maleimide-containing fluorescein dye (b) Functionalization of maleimide-containing hydrogels with thiol-based fluorescein dye.

crosslinking degrees demonstrate that the highest swelling ratio belongs to the hydrogel $CDP6_{(1:2)}$ which has lowest degree of crosslinking as well as the highest PEG content (Figure 2b).

Hydrogels were also evaluated in terms of their rheological properties via dynamic frequency scan measurements of water-swollen samples. It was found that the value of storage modulus (G′) reflecting the elastic properties and loss modulus (G″) reflecting viscous behaviour ranged from 10 to 10^3 Pa (Figure 3). In all cases, G′ values are higher than G″ values which is characteristic for hydrogels. The angular frequency change had relatively low effect on the storage and loss moduli indicating the elastic solid behaviour. A direct relationship between the polymer molecular weight and G′ values was observed. More enhanced viscoelastic properties were obtained in case of using lower molecular weight polymer. The viscoelastic properties of hydrogels were also modulated by changing the feed of β-CD crosslinker, whereby an increase in the β-CD crosslinker content resulted in increased storage moduli.

Functionalization of Hydrogels

Various applications of hydrogels such as providing platforms for biomolecular immobilization and scaffolds for tissue engineering requires incorporation of reactive units within the gel to enable their post-polymerization functionalization with functional (bio)molecules through efficient conjugation chemistry. Thus, functionalization efficiency of synthesized hydrogels was examined by attaching fluorescent dyes onto the reactive groups in the gel matrix. The main advantage of this approach is the simplicity for obtaining either thiol or maleimide functionalized hydrogels, since by changing of $\beta\text{-CD(SH)}_7$ ratio, desired amount of free functional groups can be incorporated in the network. This strategy of using "off stoichiometric" mixture of complementary reactive components in the network formation has recently been demonstrated by Bowman and co-workers.[34] A two-stage thiol-acrylate reaction based network forming strategy was utilized to fabricate shape memory polymers and impression materials. In our work, for the first series of hydrogels, crosslinker ratio was adjusted to yield hydrogels with residual thiol groups. The functionalization of the thiol groups embedded in the hydrogel matrix was probed by conjugation of maleimide functionalized fluorescein dye. The series of hydrogels $CDP6_{(1:1)}$, $CDP6_{(1.5:1)}$ and $CDP6_{(2:1)}$ with increasing crosslinker ratio (i.e. increasing free thiol content) were reacted with excess *N*-(5-fluorosceinyl)maleimide. After removal of unreacted dye with successive washings using organic solvents and water, the conjugation was investigated using fluorescence microscopy to obtain relative fluorescence intensities. As expected, hydrogels possessing higher amounts of free thiol groups were able to immobilize higher amounts of the fluorescein maleimide dye (Figure 4a). As a control experiment to ensure that dye was not trapped in the gel network, hydrogel $CDP6_{(2:1)}$ was incubated with fluoresceinamine, a dye devoid of the maleimide group, and sample was analyzed after removal of unbound dye. Analysis with fluorescence microscopy revealed that there was minimal attachment of dye thus indicating that dye conjugation was occurring via the thiol-maleimide coupling. To demonstrate the tunability of functionalization of the hydrogel using thiol containing molecules a series of $CDP6_{(1:2)}$, $CDP6_{(1:1,5)}$ and $CDP6_{(1:1)}$ hydrogels were synthesized and reacted with thiol-containing fluorescent dye. After incubation, hydrogels were thoroughly washed to remove excess dye and thereafter analysed using fluorescent microscopy. A direct correlation was observed

between free maleimide groups in the hydrogels and fluorescence intensity of functionalized hydrogels. As a control experiment, incubation of hydrogel $CDP6_{(1:2)}$ with *N*-(5-fluorosceinyl)maleimide dye exhibited significantly lower fluorescence. These results demonstrate that amount of free thiol and maleimide functional groups in the hydrogels can be varied as desired to tune the extent of their functionalization.

Conclusions

Functionalizable hydrogels with network structure were prepared using the thiol-maleimide conjugation reaction between telechelic maleimide functionalized linear PEGs and thiol functionalized β-cyclodextrin. Clear and transparent hydrogels are rapidly formed with high gel conversions. Water uptake properties of these hydrogels were found to be dependent on the molecular weight of PEG and the crosslinker ratio. The amount of CD-based crosslinker was varied to obtain hydrogels containing reactive maleimide or thiol functional groups within the hydrogel matrix. Maleimide containing hydrogels were efficiently functionalized with thiol-containing fluorescein dye and extent of immobilization onto the hydrogels was dependent on the amount of free maleimide groups in gel matrix. Likewise, hydrogels containing free thiol groups were functionalized with maleimide containing dye molecules with high efficiency. One can expect that functionalizable hydrogels thus obtained from easily accessible precursors using effective gelation under benign conditions will find usage in various areas of biomedical sciences. The hydrogel synthesis methodology depicted here allows incorporation of CD units that would allow host-guest interactions with hydrophobic molecules and is believed to find potential application in design of controlled drug release systems.

Acknowledgements

T. N. G. thanks to Bogazici University Foundation (BUVAK) for fellowship. The authors would like to thank the Scientific and Technological Research Council of Turkey (TUBITAK 211T036) and the State Planning Organization of Turkey (200909K120520) for financial support. R.S. thanks the Turkish Academy of Sciences for a TUBA-GEBIP fellowship.

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†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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