Synthesis of a New Photoreactive Gelatin with BTDA and HEMA Derivatives

Fan-Chun Ding,[1] Shan-hui Hsu,[2] Wen-Yen Chiang[1]

[1]*Department of Chemical Engineering, Tatung University, Taipei 10451, Taiwan*
[2]*Department of Chemical Engineering, National Chun Hsing University, Taichung 40227, Taiwan*

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ABSTRACT: A novel bio-affinitive, photocuring, and membrane-forming gelatin derivative was synthesized in this study. This process was based on the amide formation between carboxylic acid and the amine in methanol–water media using dicyclohexyl-carbodiimide (DCC) as a condenser. Gelatin and glycine were the sources of amine in the model reaction. Since there were two anhydride groups in each 3,3′,4,4′-benzophenone tetra-carboxylic dianhydride (BTDA) molecule, two 2-hydroxyethyl methacrylate (HEMA) molecules were used to induce the ring-opening reaction of BTDA and release two carboxylic acid groups. The resulting photoreactive gelatin was called GE-BTHE, of which the photoreactive component was the ketone groups of BTDA and HEMA that played the role of double bond supplier. This photoreactive gelatin could be converted from the transparent liquid phase into swollen membrane by a 6-min irradiation of high pressure mercury lamp. The most efficient irradiation was at 267 nm and the highest degree of swelling of the cured GE-BTHE membrane could reach 5.9. The elongation from the dried gel remained 5–10%, i.e., relatively elastic. The properties of this gelatin derivative were investigated using amide formation analysis, calculation of the gel content and the swelling ratio, and monitoring of the photocuring process. The GE-BTHE synthesized in this study should be very potential in applications such as protective wound dressings and hemostatic absorbents for minimally invasive surgery. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 589–596, 2008

Key words: BTDA; HEMA; gelatin; photoreactive; peptide reaction

INTRODUCTION

Photopolymerization is a fast and convenient way to produce high polymers. In recent years, an increasing number of materials that can be polymerized by ultraviolet (UV) irradiation have been developed. The technology has been used to generate many industrial products, in particular, the multifunctional acrylates and methacrylates.[1] With the advancement of biomedical technology, the need for high quality biomedical coating materials has evolved. Biological adhesives are widely used as tissue adhesion, hemostatic aids, and sealing materials against the leakage of air and body fluids during surgical procedures.[2] Various materials and methodologies, such as chemically crosslinkable gelatin[3] and cyanoacrylate polymers[4] have been developed as biological adhesives. However, toxic byproducts such as the resorcinol released, the carbodiimides generated by the crosslinking reaction of gelatin, and the formaldehyde produced by the degradation of cyanoacrylate, have made some of them unsuitable for biomedical applications.

Correspondence to: W.-Y. Chiang (chiang@ttu.edu.tw).

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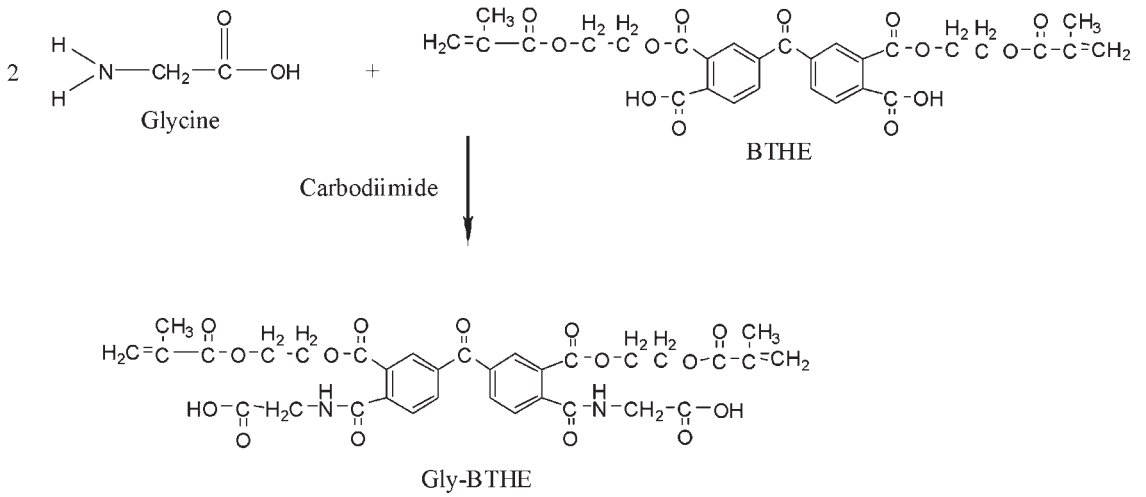
Derivatives of polyethylene glycol (PEG), hydrophilic bi-functional macromers capped with acrylate groups, have been developed and used as visible light curable wound healing aids by Matsuda[5] and Hubbell.[6] These derivatives of PEG are hydrophilic with low toxicity and good tissue compatibility.[7] Developments of photoscissable hydrogels incorporating branched PEG with cinnamyldiene acetyl,[8] *trans*-4-nitrocinnamate,[9] and anthracene moieties[10] have also been reported. Rapid photocrosslinking of these hydrogels occurred upon exposure to UV light of wavelength greater than 300 nm in the absence of photoinitiators or catalysts.[11]

Collagen and gelatin are common ingredients of hemostatic aids in surgical procedures.[12] Gelatin is a water-soluble denatured form of collagen and is a safe biodegradable carrier for drugs.[13] The making of photocurable gelatin is not a new idea, but there is still room for improvement in preparation. In this article, the synthesis of a novel gelatin derivative using photoreactive agent dimethacrylate dicarboxylic acid of BTDA[1] and HEMA[14] was reported, where gelatin was grafted with BTDA photocurable monomers bonded with HEMA hydrogel for the creation of a photocurable, bioadhering and water swellable multi-functional gel.



Scheme 1 The reaction of the preparation of Gly-BTHE.

MATERIALS AND METHODS

Materials

Gelatin (Food Grade, type A, 225 Bloom, 50,000 MW) was obtained from ς-Aldrich. Glycine (98%, m.p. 245°C), NaCl (99.5%), NaOH (98%), tetrahydrofuran (THF, 99%), acetone (99%), methyl alcohol (99.8%), 2-hydroxyethyl methacrylate (HEMA, 96%), 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC, water soluble carbodiimide, 98%), *N*,*N*′-dicyclohexyl carbodiimide (DCC, 99%), 4-dimethylaminopyridine (DMAP, 99%) were purchased from ACROS Organics. Poly(ethylene glycol) diacrylate (PEGDA, 200 MW) was purchased from Monomer Organics. Sodium lauryl sulfate (SDS, 90%) was purchased from SOWA Chemicals. The chemicals mentioned above were in reagent grade and used without further purification. *N*,*N*-dimethylformamide (DMF, 99%) was distilled before use and stored with molecular sieves; 3,3′,4,4′-benzophenone tetracarboxylic dianhydride (BTDA, 98.5%, 219–226°C) was purified by recrystallization from distilled acetic anhydride and dried overnight at 140°C under reduced pressure (m.p. 223°C). The 0.5*M* phosphate buffer saline (PBS, pH 7.4) was prepared by dissolving 1.7160 g of KH_2PO_4, 0.40 g of NaOH, 0.6510 g of NaCl in 220 mL deionized water.

Instruments

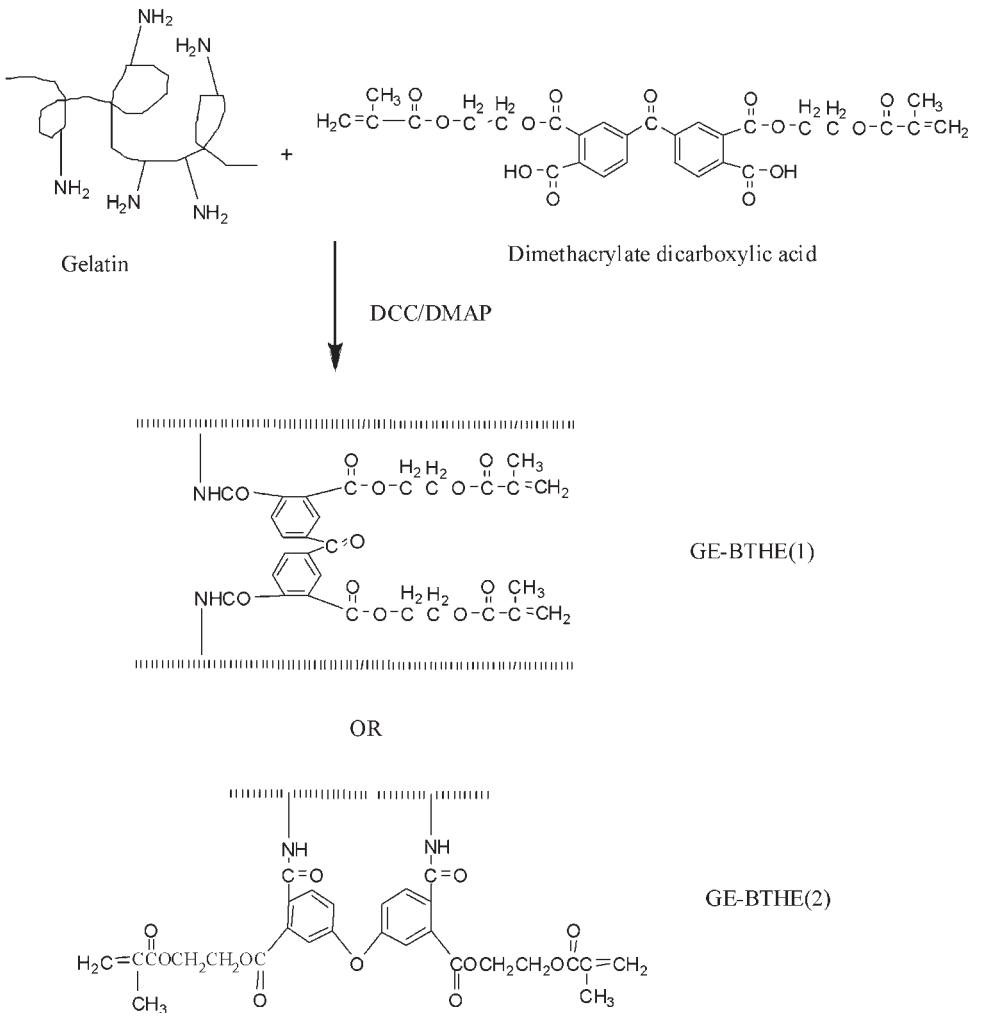
IR spectra of photoreacted monomers were obtained from Perkin–Elmer FTIR System Spectrum GX. UV–vis spectra were recorded on a UV-530 spectrometer/data system (Jasco). The UV-lamp (UVGL-25, 365/254 nm wavelength) was utilized for photocrosslinking and photocleavage studies. The mercury lamp with broad range of wavelength (Model USH-500D, Ushio Electric, Japan) was also used for photocuring. Atomic Force Microscope (AFM, Digital Instruments Dimension 3100) images were taken by Nanoscope IIIa Scanning Probe Microscope. The thermal properties were analyzed by a differential scanning calorimeter (DSC, Perkin–Elmer DSC 7, Waltham, MA) and thermogravimetric analyzer (TGA 2050, TA Instruments). The tensile property was measured by an Instron Testing Machine, Model 1130.

Model reaction

Gelatin is a macromolecule that is difficult to verify if a key functional group has reacted or not. A simple model reaction was adopted to check the possible reaction site. Glycine, the simplest but representative amino acid was used to replace gelatin in reacting with the derivatives of BTDA and HEMA. Meanwhile, the trinitrobenzene sulfonic acid (TNBSA) method was used to calculate the number of amino groups available for modification. Scheme 1 shows the complete reaction steps of Gly-BTHE.

Synthesis of Gly-Bthe

Glycine (0.75 g, 0.01 mol) was dissolved in 5 mL distilled water. BTDA (0.81 g, 0.005 mol) was dissolved in 5 mL methanol and heated at 70°C until being homogeneous. Once the BTDA solution was cooled to 30°C, 1.30 g (0.01 mol) of HEMA was added and the mixture was stirred for 15 min in an opaque device. By the ring-opening reaction, a BTDA derivative, dimethacrylate dicarboxylic acid monomer (BTHE), was generated.[14] DCC (0.001 mol) and DMAP (0.0001 mol) in 1 mL of methanol was then added to the transparent BTHE solution. After 15 min of stirring, the activated BTHE



Scheme 2 The reaction of the preparation of photoreactive gelatin.

solution was poured into the aforementioned glycine solution, stirred for 2 h and placed in the dark. After 24 h, the solid (urea) and the liquid phases were separated. Gly-BTHE was precipitated from the above filtrate (liquid part) by dropping acetone, and purified by a few cycles of dissolving and precipitation with water and methanol. After being rinsed with acetone for several times, Gly-BTHE was lifted with filter paper and dried for characterization by IR and DSC.

Synthesis of GE-Bthe

Gelatin (0.5 g) was dissolved in 5 mL distilled water and stirred at room temperature, while 1.61 g (0.005 mol) of BTDA was mixed in 5 mL methanol and stirred at 70°C for homogeneity. Once the BTDA solution was cooled to 30°C, 1.30 g (0.01 mol) HEMA was added to it and stirred together in an opaque device. The BTDA and HEMA mixture then turned into transparent photoreactive monomers, BTHE [see Ref. 15]. Water soluble carbodiimide (EDC) (0.2 g, 0.001 mol) or DCC/DMAP (0.001 mol/0.0001 mol) dissolved in 1 mL methanol was added with the BTHE solution and stirred for 15 min. The BTHE-EDC or BTHE-DCC mixture was then poured into the gelatin solution and stirred for 2 h. Precipitation of GE-BTHE was induced by adding 20 mL acetone to the mixture. Purified GE-BTHE was obtained through several cycles of dissolving/precipitation by water and methanol. The GE-BTHE was then rinsed with 30 mL of distilled water, 30 mL of methanol, and 30 mL of acetone and dried under reduced pressure. The yield of GE-BTHE was over 80%. This photoreactive modified gelatin was then characterized. The synthetic step of GE-BTHE is shown in Scheme 2.

Preparation of photocurable glue

The photocurable glue was prepared from photocurable gelatin (GE-BTHE, 8–30 wt %), PBS solution (70 wt %) and poly (ethylene glycol) diacrylate (PEGDA, 0–22 wt %). The total amount of solutes (GE-BTHE and PEGDA) was 30%[5]

Photocuring

The broad wavelength mercury lamp was used to photocrosslink the GE-BTHE. Crosslinking was performed at a distance of 10 cm.

Gel content determination

After irradiation and washing with distilled water, specimens of cured GE-BTHE were placed in vacuum and maintained at 40°C overnight. After the dry weights of all specimens were recorded, these specimens were extracted with distilled water for 12 h. The residuals were dried and weighted. The gel content was calculated based on the following equation:

$$\text{Gel content} = \frac{W}{W_0} \times 100\% \quad (1)$$

where W is the dry weight of the extracted specimen and W_0 is the dry weight of the original specimen

Swelling test

The degree of swelling of cured GE-BTHE specimens were defined as the ratio of the weight of water uptake upon immersion in distilled water for 24 h at room temperature against the dry weight of the gel, as follows:

$$\text{Degree of swelling (DS)} = \frac{W_w - W_d}{W_d} \quad (2)$$

where W_w is the weight of wet gel and W_d is the weight of dry gel.

Trinitrobenzene sulfonic acid

Gelatin specimens to be tested were dissolved in a buffer (sodium bicarbonate 0.1*M*, pH 8.5) at concentrations of 20–200 μg/mL. A 0.01% (w/v) working solution of TNBSA was added to each sample solution at 1:2 volume ratio and mixed thoroughly. The mixtures were incubated at 37°C for 2 h. Then, 0.25 mL of 10% SDS and 0.125 mL of 1*N* HCl were added to each sample mixture. The absorbance of each solution at 335 nm wavelength was measured by UV/VIS.

Tensile proterties

Specimens with the highest gel content (0.02 mol BTDA, 20% GE-BTHE, 60 min irradiation) were used in tensile property measurements that were performed according to ASTM-D882-91. After being conditioned for 24 h at 80°C to remove the water, cured membranes were cut into 50 × 5 mm^2 strips. The strips were prepared in two conditions: dried gels and partially swollen gels (~ 50% fully swollen based on DS data). Dried gels were about 0.1–0.2 mm in thickness and partially swollen gels were about 0.4–0.5 mm in thickness. All the strips were clamped in an Instron Testing Machine, Model 1130, with a jaw separation of 2 cm and extended at a rate of 5 cm/min to failure. The tensile strength, Young's modulus, and elongation at break were determined from the load extension diagram.

RESULTS

Synthesis and characterization of Gly-Bthe

A prototype of Gly-BTHE synthetic reaction was developed by inducing the reaction between carboxylic acid and amine using DCC as a condensation reagent.[15–17] This reaction scheme relied on the formation of peptide bond between the amino groups of glycine and the carboxylic acid groups of BTHE. The BTHE used in this study has been previously synthesized in our laboratory.[14] BTDA reacted with HEMA in ring-opening reaction. The resulting product was dimethacrylate dicarboxylic acid a four functional groups monomer. The characteristic anhydride absorption peaks of BTDA at 1858 and 1774 cm^{-1} completely disappeared from spectrum of the BTDA-HEMA half ester intermediate (BTHE) spectrum (Fig. 1). Once glycine was poured into the activated BTHE solution, it was observed that the transparent solution suddenly turned muddy and sediments appeared. These synthetic steps resulted in precipitates of urea. The peptide reaction in water solution using DCC reagent always generates byproduct precipitates (insoluble urea salt) at the same time. Urea precipitates was removed before the Gly-BTHE could be collected. The resulting Gly-BTHE was light yellow or white in color.

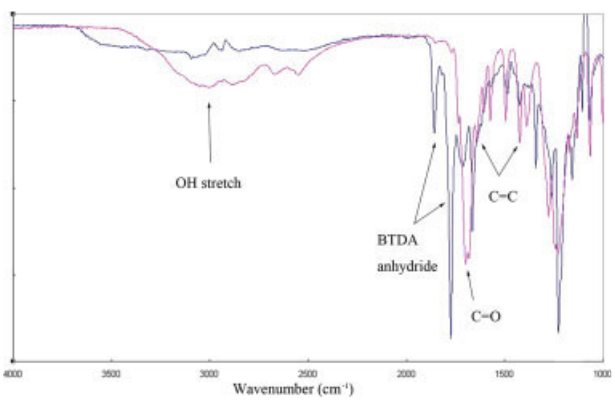


Figure 1 FTIR spectra of BTDA and BTDA-HEMA half ester intermediate (BTDA, BTDA-HEMA half ester intermediate). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

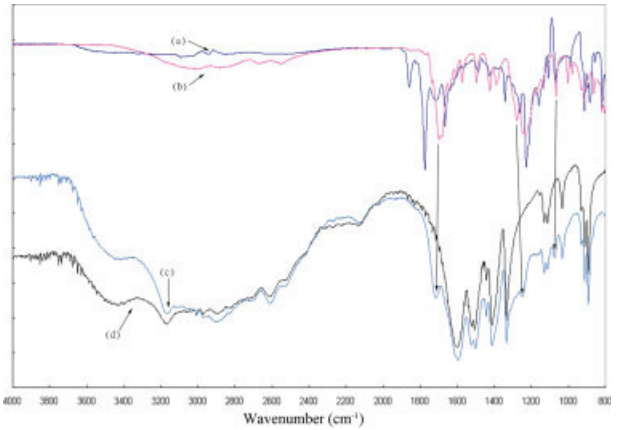


Figure 2 The comparison of FTIR spectra of (a) BTDA, (b) BTHE, (c) Gly-BTHE, and (d) glycine. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

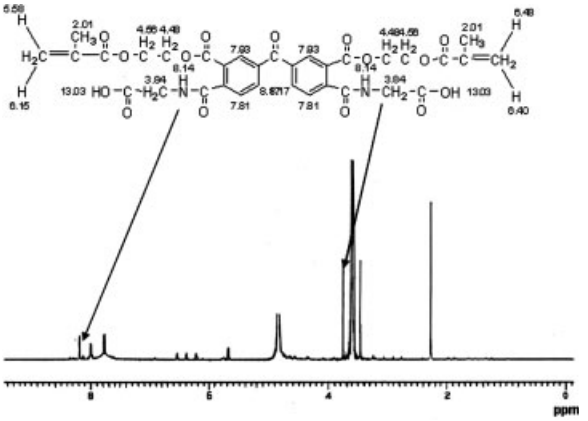


Figure 3 The estimated and actual ^{1}HNMR analysis of Gly-BTHE.

An examination of the IR spectra of Gly-BTHE (Fig. 2) revealed that the characteristic anhydride absorption peaks of BTDA at 1858 and 1774 cm^{-1} (a) disappeared completely while a strong peak appeared at 1710 cm^{-1} (b). In addition, there were peaks of OH stretching at 3000 cm^{-1}, C=C stretching at 1600 and 1400 cm^{-1}, C=O stretching of ester groups at 1680 cm^{-1} and C=O stretching of benzophenone groups at 1660 cm^{-1}. Figure 2(c,d) are IR spectra for glycine and Gly-BTHE. The spectrum of Gly-BTHE with characteristic BTHE peaks at 1712, 1247, and 1072 cm^{-1} provided a good evidence that Gly-BTHE was a reactive product of BTHE and glycine.

In ^{1}HNMR estimation, the BTHE showed three major absorptions of hydrogen; 5.58–6.48 ppm from the vinyl groups of HEMA, 7.75–8.20 ppm from the benzophenone, and 4.48–4.56 ppm from the $—CH_2—CH_2—$ groups of HEMA. The ^{1}HNMR absorptions of glycine was at 8.70 ppm ($—NH_2$), 4.24 ppm ($—CH_2—$), and 13.03 ppm (—COOH) originally. After being grafted with BTHE, the absorptions from $—CH_2—$ of glycine shifted to 3.62–3.75 ppm, and the absorption from amide appeared at 8.17–8.18 ppm (Fig. 3).

Differential scanning calorimeter analysis

The peak of DSC curve in Figure 4 represented an endothermic reaction and showed the melting point of Gly-BTHE. Since the melting points for BTDA and glycine were 223 and 245°C, respectively, the single sharp narrow peak indicated the extra-purity of Gly-BTHE.

Synthesis and characterization of GE-Bthe

According to the product information sheet (G-9382, ç-Aldrich; Catalog Number: 901771, MPBio), the gelatin had an average molecule weight of 50,000, and contained 4.1–5.2 g lysine as well as 1.04 g hydroxylysine residues per 100 g dry weight. Using the TNBSA method, the free amino residues available for reaction was 29.7 for each gelatin molecule. The IR spectrum was also evident for the appearance of OH stretching at 3000 cm^{-1}, C=C stretching at 1600 and 1400 cm^{-1}, C=O stretching of ester group at 1680 cm^{-1} and C=O stretching of benzophenone groups at 1660 cm^{-1}. This photoreaction gelatin was synthesized by coupling amino groups with carboxyl groups ring-opened from BTDA using EDC or DCC as a condensation reagent.

Figure 5 shows the IR spectra analyses of gelatin and the unexposed GE-BTHE. Newly generated amide functional groups were an evidence of the successful synthesis of GE-BTHE. Since GE-BTHE was a macromolecule, analysis by DSC was rather difficult. The TGA curve in Figure 6 illustrated that the photocuring process raised the decomposition temperature. An unexposed GE-BTHE specimen had a decomposition temperature (10% decomposed) at 167°C, while an exposed specimen had a higher decomposed temperature at 227°C.

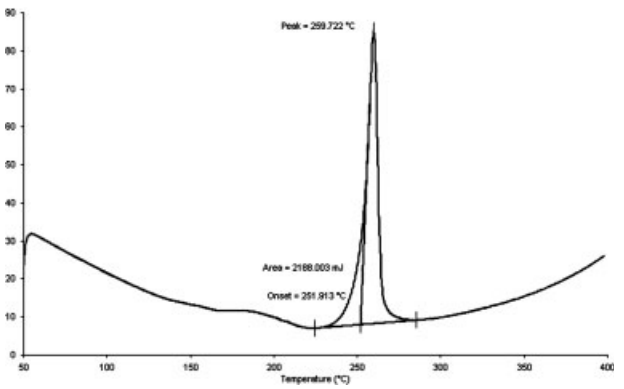


Figure 4 The DSC curve of Gly-BTHE.

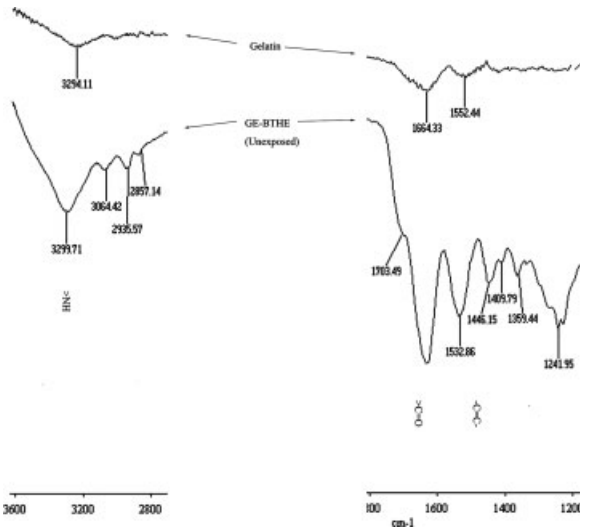


Figure 5 FTIR spectra of gelatin, unexposed GE-BTHE and exposed GE-BTHE.

Photosensitivity of GE-Bthe

The GE-BTHE had an obvious absorbance peak at 267 nm as determined by UV-530 spectrometer/data system. The wavelength of major absorbance was similar for the mixing mode (gelatin + BTHE) and the grafting mode (GE-BTHE). Within 10-min of irradiation, there was little difference of the intensity at the maximum absorbance (λ_{max}) and there was no blue shift resulted from rearrangements or isomers.

Effect of the concentration of GE-Bthe solution on photocuring

The concentration of GE-BTHE solution had an effect on the photoreaction process. Three different concentrations (13, 16.25, and 21.67 wt %) of GE-BTHE solution were tested. The 13 wt % solution was easily transformed into a swollen membrane after being irradiated with a mercury lamp for 6 min. The curing process was faster than that of the other two solutions given the same irradiation. The 16.25 wt % solution could not be completely transformed. Instead, it became slurry with some solid and mostly fluid. The 21.67 wt % solution could not be transformed into membrane at all, even after prolonged irradiation for 60 min. Only a color change from colorless to yellowish brown was observed.

These results indicated that, in the diluted solution of 13 wt %, photopolymerization slowly wrapped up water with polymers and the polymers became sponge-like. However, in the concentrated solution of 21.67 wt %, photopolymerization turned some parts of the solution into polymer vigorously and resulted in a phase separation between water and the polymer.

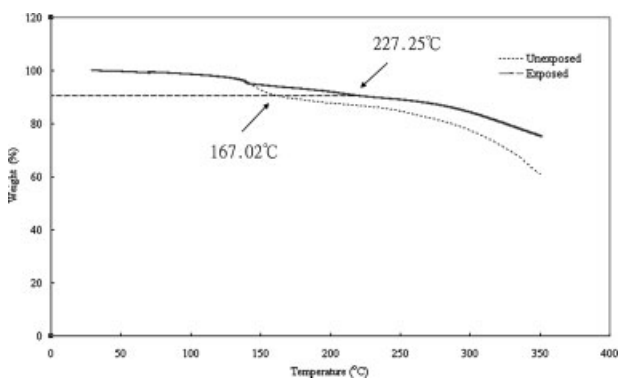


Figure 6 The TGA curve of GE-BTHE.

Gel and swelling behaviors of photoreactive gelatin (GE-Bthe)

The effect of irradiation time on the property of GE-BTHE is illustrated by the different irradiation time resulted in different gel content and degree of swelling (DS) (Fig. 7).

The ionic strength of a solution determined the swelling behavior of the gel after irradiation. The DS of GE-BTHE membranes in deionized water was higher than in saline (Fig. 7). The DS of GE-BTHE membranes after a certain period of irradiation (e.g., 1 h) also depended on the concentrations (Fig. 8).

Morphology of cured GE-Bthe

The surface topographies of GE-BTHE specimens were observed using AFM sized from 5 to 100 μm. The bright yellow areas were polymerized or crosslinked sections of the membrane. The dark areas were gaps or channels between two polymerized

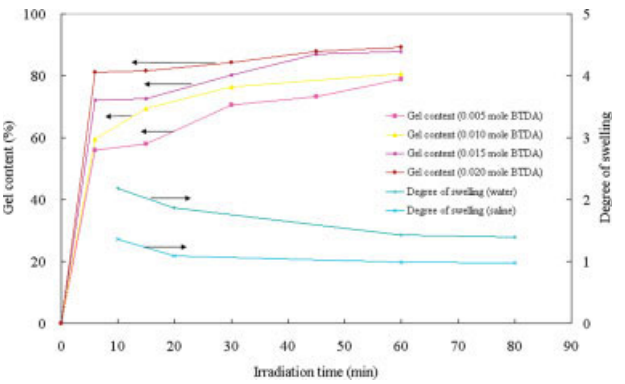


Figure 7 The gel content for different compositions of the GE-BTHE and the degree of swelling of 20% GE-BTHE (0.005 mol BTDA) in deionized and saline at different irradiation time. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

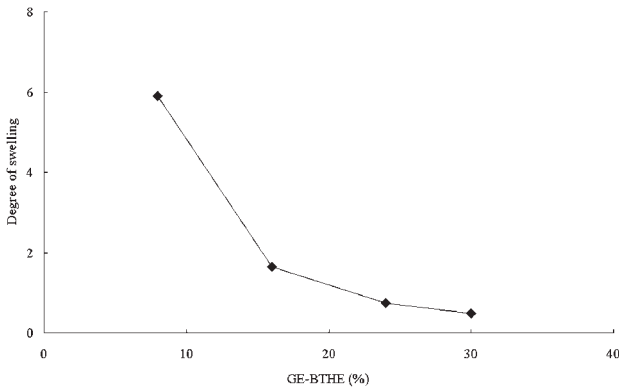


Figure 8 The degree of swelling at different concentrations of GE-BTHE. The irradiation time was 1 h.

areas. A section analysis for the level of flatness revealed that there was less than 10 nm difference in heights (Fig. 9). The macroscopic analysis showed that the surface of a 20% GE-BTHE membrane was quite flat and smooth; while in the microscopic analysis, a cross section with highlands, tunnels and valleys indicated that the membrane was expandable and permeable. The material may applicable as a protective layer over the skin, absorbance substrate for stopping bleeding, or temporary cover for tissue repair and regeneration.

Tensile properties of cured GE-Bthe

Specimens with the highest gel content (0.02 mol BTDA, 20% GE-BTHE, 60 min irradiation) were used in tensile property measurements. Gels were made into samples of two different moisture contents. Partially swollen strips had the averaged maximum strength of 1.595 MPa, break strength of 0.678 MPa, elongation of 56.44%, and modulus of 0.0091 GPa. Dried strips had the averaged maximum strength of 16.255 MPa, break strength of 16.020 MPa, elongation of 7.60%, and modulus of 0.5484 GPa. The data showed that the partially swollen strips were better in elongation but worse in strength (more elastic); dried strips were strong and brittle.

DISCUSSION

Bioglue plays an important role in endoscopic procedures widely used to replace the complex surgeries and reduced health care cost. In this study, to realize the possibilities of photocurable and water swellable while visible light minimizing hazards, BTDA, HEMA, and gelatin were used to prepare the bioglue. All the materials utilized in this synthesis are nontoxic to human according to the published reports.[6,7,12,14] Many related papers were focused in

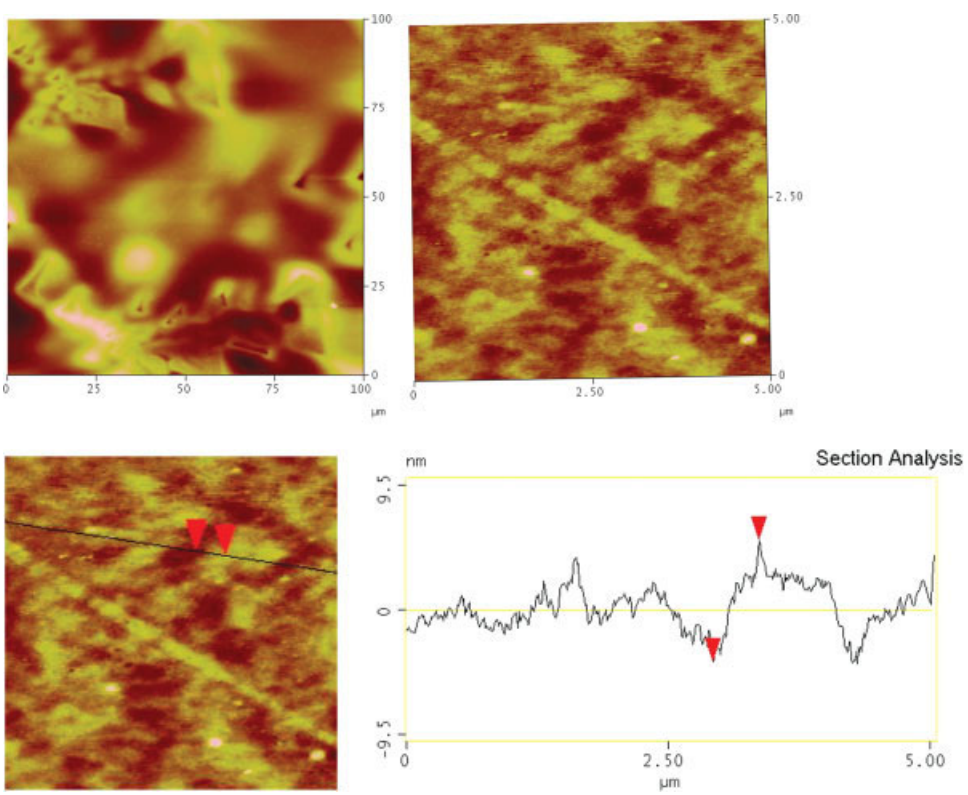


Figure 9 Tapping-mode AFM topography of 20% GE-BTHE irradiated membrane (6 min) (Up left: 100 μm, up right: 5 μm, down left: section line, down right, section analysis). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

this field to develop suitable bioglues for the substituents of suture, but mostly failed on achieving the adequate curing time and enough tensile strength. For confirmation of the reaction of GE-BTHE, a model reaction with simple amino-acid (Glycine) was used to forecast the possibility of reaction. The evidences for formation of Gly-BTHE were provided by IR, NMR and DSC. GE-BTHE was synthesized by DCC or EDC as the peptide reaction reagents. The use of EDC in preparation bioglues was reported no cytotoxicity to fibroblasts,[18] and DCC was relatively inexpensive. Most of the byproduct generated from the peptide reaction with carbodiimide reagent (e.g., DCC) could be easily removed by filtration.[19] With the generation and removal of urea precipitates, the amino groups and carboxylic groups should have been mostly reacted. The synthesis of GE-BTHE was confirmed by IR and NMR. GE-BTHE could be dissolved in water and conveniently transformed into a membrane by irradiation. It started as thin threads, quickly turned into fragments, and eventually formed a membrane. The photocuring time of GE-BTHE glue was 6 min without flipping under a 10-cm light source of mercury lamp. This indicated that GE-BTHE could be used in fluid system and cured without violent light sources. Depending on the applications, the concentration could be changed. It could also be swollen and applied as a hydrogel and/or drug delivery vehicle. Among all the concentrations, 20 wt % GE-BTHE with 0.005 mol BTDA content was a favorable one. This formula was most hydrophilic and had good photoreactivity. These characteristics are beneficial when used a biomaterial.

For the GE-BTHE membranes prepared in this study, the highest gel content was over 80%. The highest degree of swelling could reach 5.9. The tensile properties of the dried membranes could reach 5–10% elongation and 0.55 GPa average modulus. However, for the highly swollen membranes, the tensile property was still inadequate. This system should be further improved to possess hydrophilicity and mechanical strength all at once.

It has been reported[5] that PEGDA as an additive could enhance the membrane structure. A total of 20 wt % GE-BTHE and 10 wt % PEGDA was chosen to prepare for the GE-BTHE glue in this study. To obtain the best tensile strength, the BTDA content had to be increased to 0.02 mol (originally 0.005 mol). This was due to the fact that tensile strength was related to the gel content as well as the BTDA content. Partially swollen (about $\sim$ 50% fully swollen) strips had about eight times of elongation over the dried ones. However, they also had lower strength and modulus. The tensile strength under different moisture contents was rarely discussed in this field, but they may determine if the formula was suitable for the final applications.

The molecule weight of PEGDA used in this study was 200; and in Matsuda's previous work[5] it was 4000. This difference in molecular weight could affect the crosslinking rate and tensile strength of the cured membranes. PEGDA played a role of enhancement in the gel structure. It was possible that if PEGDA of a higher molecular weight was used, the concentration of GE-BTHE glue could be further modified, the tensile strength of cured membranes could be largely improved, and the curing time could be much reduced.

Finally, cytotoxicity as well as adhesive force *in vitro*, and performance in animal study should be conducted to verify the feasibility of the GE-BTHE photoreactive system as a potential bioadhesive.

CONCLUSIONS

A series of photoreactive, membrane-forming, BTDA and HEMA grafted gelatin (GE-BTHE) has been synthesized in this study. These membranes formed gels quickly (within a few minutes) upon irradiation and could keep abundant water in their structure. They may have potential to quickly stop bleeding and seal the wounds when used as a wound healing product.

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