Hydrogels of Modified Ethylenediaminetetraacetic Dianhydride Gelatin Conjugated with Poly(ethylene glycol) Dialdehyde as a Drug-Release Matrix

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ABSTRACT: Hydrogels have been recognized as versatile biomaterials in biomedical applications. This article describes the synthesis and characterization of a poly(ethylene glycol) (PEG) dialdehyde derivative, the modification of gelatin with ethylenediaminetetraacetic dianhydride (EDTAD), and the conjugation of PEG dialdehyde for enhanced hydrophilicity, biocompatibility, and flexibility. Hydrogels of gelatin conjugated with various percentages of PEG dialdehyde (10–30%), 35% EDTAD-modified gelatin, and 12% PEG dialdehyde conjugated with 31% EDTAD-modified gelatin with or without 1% chlorhexidine were prepared. For all the synthesized gel formulations, the swelling kinetics and drug release in pH 7.4 and pH 4.5 buffers at 37°C were studied. Gels of PEG-conjugated gelatin, 35% EDTAD-modified gelatin, and 12% PEG conjugated with 31% EDTAD-modified gelatin, with or without 1% chlorhexidine, showed significantly improved swelling ratios in comparison with gelatin. The drug release was unaffected by an increase in the percentage of PEG conjugation with gelatin. Complete drug release was recorded within 48 h in the pH 4.5 buffer, whereas in the pH 7.4 buffer, the drug release was accomplished within 128 h. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 91: 1059–1067, 2004

Key words: hydrogels; biodegradable; swelling

INTRODUCTION

Hydrogels are versatile biomaterials. Acrylic-based hydrogels have a very good water uptake capacity, but most acrylic polymers are nondegradable and are very toxic because of unreacted toxic monomers;[1–10] therefore, synthetic polymers have limited applications as biomaterials. Although polyurethanes provide good mechanical strength and biocompatiblity,[11–17] the prolonged use of polyurethanes can lead to inflammation because of interactions of the material with proteins that lead to an adverse host reaction. Hence, biomaterial researchers have focused on biodegradable, biocompatible, natural-polymer-based hydrogels such as gelatin, collagen, amylase, cellulose, chitosan, hylauronic acid, fish, soy, agarose, and alginate.[18–27]

However, natural-polymer-based hydrogels are less hydrophilic and provide low mechanical strength; unlike synthetic polymers, natural polymers are unique, having various functional groups. Therefore, by modifying the functional groups, we can tailor the properties as we desire. We propose developing a protein-based hydrogel with improved swelling and mechanical properties with drug-release capability. For our studies, we have chosen gelatin, a denatured protein derived from collagen, because of its unique gelling properties, abundance, solubility, biocompatibility, and biodegradability and because of the functional groups that are present on the gelatin backbone, which can be chemically modified for increased hydrophilicity and biocompatibility along with improved physicochemical properties. Lysyl residues of gelatin have been modified with ethylenediaminetetraacetic dianhydride (EDTAD) to incorporate carboxylic groups in order to increase the hydrophilicity and conjugated with poly(ethylene glycol) (PEG) dialdehyde to increase the flexibility and biocompatibility.

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EXPERIMENTAL

Materials and methods

Type A porcine skin gelatin (Bloom 300) and trinitrobenzenesulfonic acid (TNBS; 20% chlorhexidine digluconate) were obtained from Sigma Chemical Co. (St. Louis, MO). PEG (2000 Da), sodium cynoborohydride, EDTAD, and acetic anhydride were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were analytical-grade.

Modification of gelatin with EDTAD

The EDTAD-modified gelatin was synthesized as previously reported.[18,28] A 1% gelatin solution in 100 mL

of water was preheated to 65°C at pH 12 for 30 min. The preheated gelatin solution was cooled to room temperature, and EDTAD was added incrementally at a 1:0.035 protein/EDTAD ratio while the solution was stirred and maintained at pH 12. After the complete addition of EDTAD, the stirring was continued for 3 h at pH 12. The reaction mixture was dialyzed overnight with a 10,000-Da cutoff membrane and freeze-dried.

Derivatization of PEG into PEG dialdehyde

To 1.9 mL (20 m*M*) of acetic anhydride, 2.0 g (1 m*M*) of PEG dissolved in 10 mL of dry dimethyl sulfoxide was added, and the mixture was stirred at room temperature for 2 h and precipitated with 100 mL of ethyl ether.[29,30] The precipitate was redissolved in the minimum amount of methylene chloride and was again precipitated with 100 mL of ethyl ether and vacuum-dried. The percentage of PEG dialdehyde obtained after the reaction of PEG with acetic anhydride was determined with reverse-phase high-performance liquid chromatography (HPLC) coupled to an ultraviolet–visible detector at $\lambda = 200$ and $\lambda = 220$ and to an evaporating light scattering detector. Acetonitrile (10–100%) was used for eluting the samples at a flow rate of 1 mL/min in 30 min with a Jordi 500-Å column (Milwaukee, WI). An infrared spectroscopy examination for PEG and PEG dialdehyde was performed with a PerkinElmer 599 B infrared spectroscope (Boston, MA). KBr pellets (100 mg), each containing 1 mg of a sample, were used for the spectral analysis. The spectrum was scanned from 4000 to 200 cm^{-1}. ^{1}H-NMR and ^{13}C-NMR data for PEG and PEG dialdehyde dissolved in chloroform ($CDCl_3$) were obtained with a Bruker AM 300-MHz FT-NMR spectrometer (Billerica, MA).

Conjugation of gelatin with PEG dialdehyde

Gelatin was conjugated with PEG dialdehyde according to Harris et al.[30] To a 1 m*M* gelatin solution, an aqueous solution of PEG dialdehyde (85% purity) in 10 mL and 7 mmol of $NaCNBH_3$ in a 10-mL aqueous solution were added simultaneously with two separate dropping funnels at 60–70°C. The stirring was continued at 70°C for 24 h and was followed by dialysis and freeze drying. Various percentages of PEG dialdehyde conjugation with gelatin were achieved by the concentration of PEG dialdehyde being increased from 1.0 to 2.5 m*M*. The conjugation of 31% modified EDTAD gelatin with PEG dialdehyde at a 1.0 m*M*/1.0 m*M* ratio was also performed as discussed previously. The percentages of PEG dialdehyde conjugation with gelatin and EDTAD-modified gelatin were estimated by the TNBS method.[31]

Quantification of the modified lysyl residues

The lysyl content for the modified and unmodified gelatin with EDTAD and PEG dialdehyde was determined by the TNBS method in triplicate, as described by Hall et al.[31] To 1 mL of 4% $NaHCO_3$, 0.5 mL of protein was added, and this followed by the addition of a 0.2-mL TNBS solution (12.5 mg/mL). The solution mixture was incubated at 40°C for 2 h, 3.5 mL of concentrated hydrochloric acid was added, and the solution mixture was left at 110°C for 3 h. The solution was cooled and was increased to 10 mL; it was extracted twice with equal amounts of ether, and the aqueous solution was separated and left at 40°C for the removal of the traces of ether. The absorbance of the resulting yellow solution was measured at $\lambda = 415$ nm against a blank. The amounts of reactive lysyl residues for the modified and unmodified gelatin were determined as follows: optical density $\times$ number of dilutions/1.5×10^7/mol of protein = amount of modified lysyl residues.

Preparation of the gels with or without the drug loaded

Ten-percent hydrogels of gelatin and conjugated gelatin with EDTAD and PEG dialdehyde were made by the dissolution of the calculated amounts of the samples in deionized water at 100°C. The dissolved homogeneous solutions were poured onto petri dishes and allowed to set overnight; the gels were punched into small, circular discs and crosslinked with 1% glutaraldehye solutions for 6 h. The crosslinked gels were washed thoroughly to eliminate surface glutaraldehyde and were dried to a constant weight. The gels loaded with the drug were prepared by the dispersion of 1% chlorhexidine into the gel solutions after they cooled to room temperature. The gels were allowed to set and were cut into discs, and this was followed by crosslinking and drying, as previously explained. The various gel compositions with or without the drug loaded are listed in Table I.

TABLE I
Definitions of Formulations with or without Loaded 1% Chlorhexidine

Abbreviation	Type of conjugation with gelatin
G	Gelatin
DG	Gelatin with 1% drug
10PEG-G	10% PEG
D10PEG-G	10% PEG with 1% drug
14PEG-G	14% PEG
22PEG-G	22% PEG
D22PEG-G	22% PEG with 1% drug
30PEG-G	30% PEG
35EDTAD-G	35% EDTAD
D35EDTAD-G	35% PEG with 1% drug
12PEG+31EDTAD-G	12% PEG and 31% EDTAD
D12PEG+31EDTAD-G	12% PEG and 31% EDTAD with 1% drug

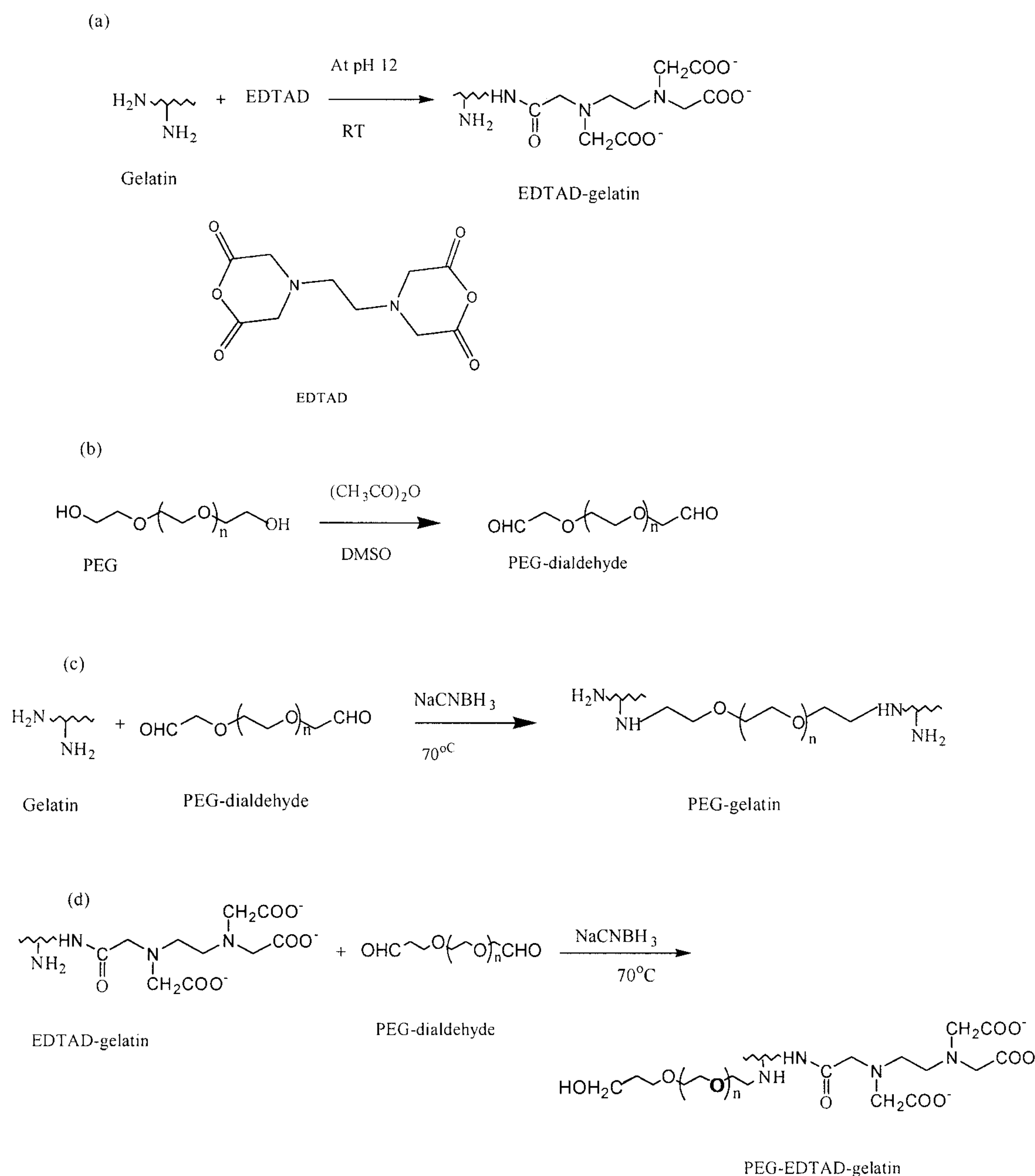


Figure 1 Schematic representation of the reaction methods: (a) the modification of gelatin with EDTAD, (b) the derivatization of PEG into PEG dialdehyde, (c) the conjugation of PEG dialdehyde with gelatin, and (d) the conjugation of PEG dialdehyde with EDTAD-modified gelatin.

Swelling kinetics

The swelling studies for all the gels with or without the drug loaded were carried out in deionized water and phosphate buffers of pH 7.4 or 4.5, respectively, at 37°C. The dried, circular discs were weighed and transferred into the swelling medium, and changes in the swelling were recorded at regular intervals until the gels reached equilibrium swelling. During the weighing, the excess medium on the disc surface was removed gently with Kim Wipes (Milwaukee, WI).[20] The swelling ratio was calculated from the weight ratio of the wet polymer. Swelling studies were performed twice for all the gels.

Drug-release studies

The drug-release studies were performed twice for the gels with and without the drug loaded in buffers of pH 7.4 or 4.5. The exact amount of chlorhexidine released into the buffer was monitored with an ultraviolet–visible spectrophotometer (Spectronic 21, Bausch & Lomb) at λ = 285 nm. The dried, drug-loaded samples were transferred into 5-mL buffer solutions; at time intervals, the swollen gels were pressed gently with Kim Wipes and transferred into fresh buffer solutions. This process was repeated until the gels achieved a zero amount of release. The gels without the drug loaded were used as controls to observe the

TABLE II
^{13}Carbon δ (ppm) Assignments for PEG and PEG Dialdehyde

Carbon assignment	δ (ppm)
HO—$C^{\alpha1}H_2$—$C^{\beta1}H_2$—$(CH_2$—CH_2—$O)_n$—$C^{\beta2}H_2$—$C^{\alpha2}H_2$—OH (PEG)	
—$C^{\alpha1}$ H_2	61.3
—$C^{\beta1}$ H_2	72.4
—$(CH_2$—CH_2—$O)_n$	70.4
—$C^{\beta2}$ H_2	72.4
—$C^{\alpha2}$ H_2	61.3
$OHC^{\alpha1}$—$C^{\beta1}H_2$—$(CH_2$—CH_2—$O)_n$—$C^{\beta2}H_2$—$C^{\alpha2}HO$ (PEG dialdehyde)	
—$C^{\alpha1}$ HO	200.9
—$C^{\beta1}$ H_2	73.5
—$(CH_2$—CH_2—$O)_n$	70.9
—$C^{\beta2}H_2$	73.5
—$C^{\alpha2}HO$	200.9

interference of the optical density readout as a result of any unreacted glutaraldehyde and soluble gelatin.

Statistical analysis

A statistical analysis was performed for data with a Student *t* test. Each experiment was repeated twice in duplicate or triplicate. The statistical significance was determined at $p \leq 0.05$ for all data analysis.

RESULTS AND DISCUSSION

Modification of gelatin with EDTAD

From the experimental analysis, we determined the lysyl residues of the medical-grade gelatin consisted of 3.28 residues per 10,000 molecular weight units. The modification of gelatin with EDTAD was limited to 30–35%, for which a protein/EDTAD ratio of 1.0:0.034 was required. On the basis of the reaction conditions of EDTAD with gelatin, about three carboxylic groups were introduced for each lysine residue.[18,19] The reaction scheme is shown in Figure 1(a).

Derivatization of PEG into PEG dialdehyde

The hydroxyl group of PEG was oxidized into an aldehyde to immobilize the PEG molecule by conjugation with the lysyl residues of gelatin or EDTAD-modified gelatin. The amino groups of gelatin were reacted with the aldehyde groups of PEG. The derivatization of PEG into PEG dialdehyde was accomplished by the reaction of PEG with acetic anhydride at a 1.0 m*M*:20 m*M* ratio for 2 h[27] [Fig. 1(b)]. However, from the HPLC results, we determined that only 85% of PEG was derivatized into PEG dialdehyde and that the remainder was PEG monoaldehyde; 100% derivatization to PEG dialdehyde was not achieved by changes in the molar concentration of acetic anhydride or by changes in the reaction time. From an infrared analysis, a band due to C═O of the —CHO functional group was observed at 1740 cm^{-1}. ^{1}H-NMR spectra showed a characteristic peak at 9.2 ppm for the proton due to —CHO of PEG dialdehyde. Table II shows the ^{13}C assignments for PEG and PEG dialdehyde. A characteristic ^{13}C peak for —CHO was observed at 200.9 ppm.

Conjugation of PEG dialdehyde onto gelatin

The conjugation of PEG dialdehyde onto gelatin was performed by the reaction of amino groups of gelatin with the aldehyde functional groups of PEG dialdehyde in the presence of a reducing agent, sodium cyanoborohydride [Fig. 1(c)]. For various percentages of PEG conjugation with gelatin to be achieved, the molar ratio of gelatin was kept constant, and that of PEG dialdehyde was increased from 1.0 to 2.5 m*M*. However, the conjugation of PEG dialdehyde onto 31% EDTAD-modified gelatin was achieved at a 1.0 m*M*:1.0 m*M* ratio [Fig. 1(d)]. Our experimental analysis indicated that with an increase in the PEG dialdehyde molar ratio, there was an increase in the percentage of conjugation. The extent of the conjugation of gelatin with PEG and EDTAD-modified gelatin was estimated with the TNBS method, as described by Hall et al.[31] Table I shows various gel formulations with or without the drug loaded.

Swelling studies

Figure 2 shows the swelling ratio for various gel formulations with or without the loaded drug, in water and buffers of pH 7.4 or 4.5 at 37°C as a function of time. A significant increase in the rate of the swelling ratio was reported for gels with an increase in the percentage of the modification and conjugation of gelatin with EDTAD/PEG with or without the drug loaded in their respective media. The increase in the swelling ratio was due to the enhanced hydrophilicity of the network with the incorporation of carboxyl and

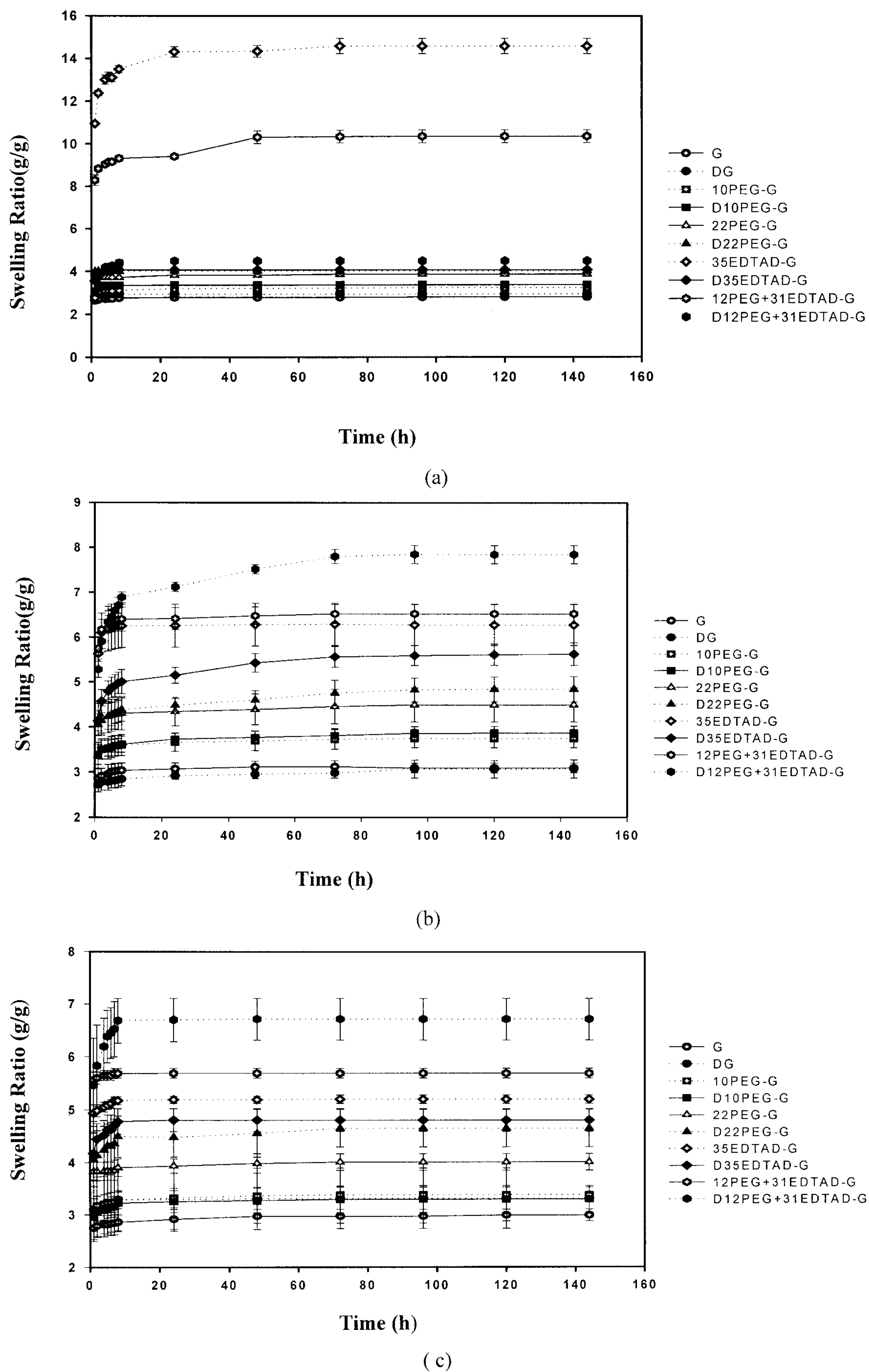
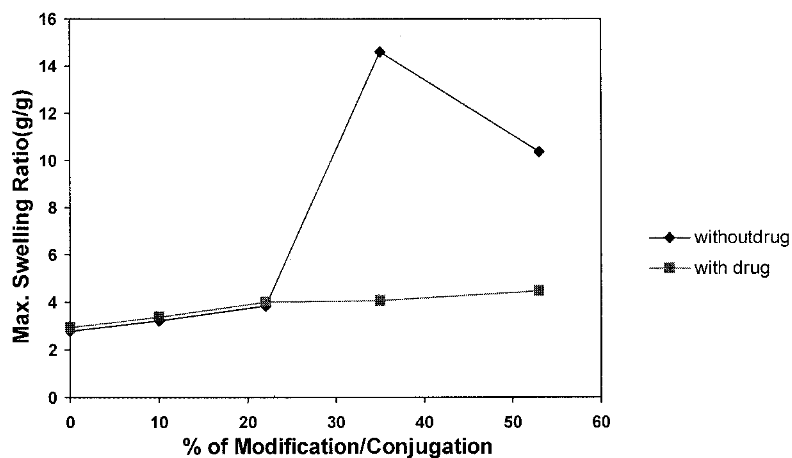
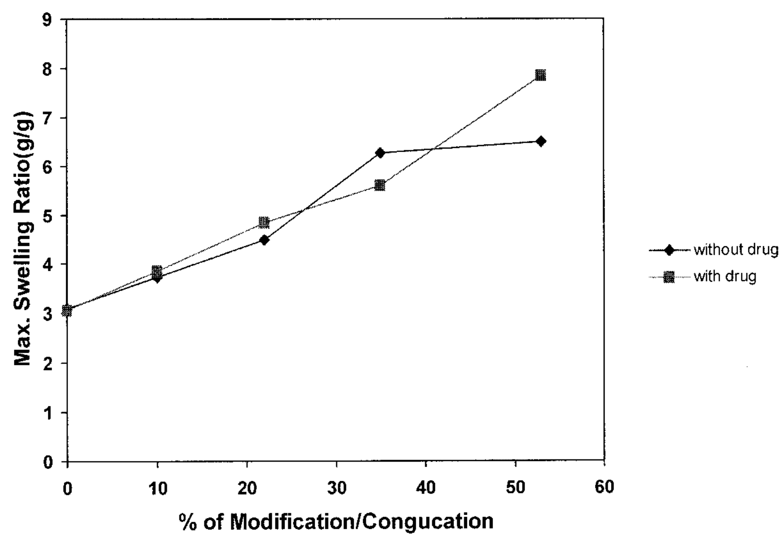


Figure 2 Swelling ratios for gels with or without the drug loaded at 37°C in (a) water, (b) a pH 7.4 buffer, and (c) a pH 4.5 buffer.





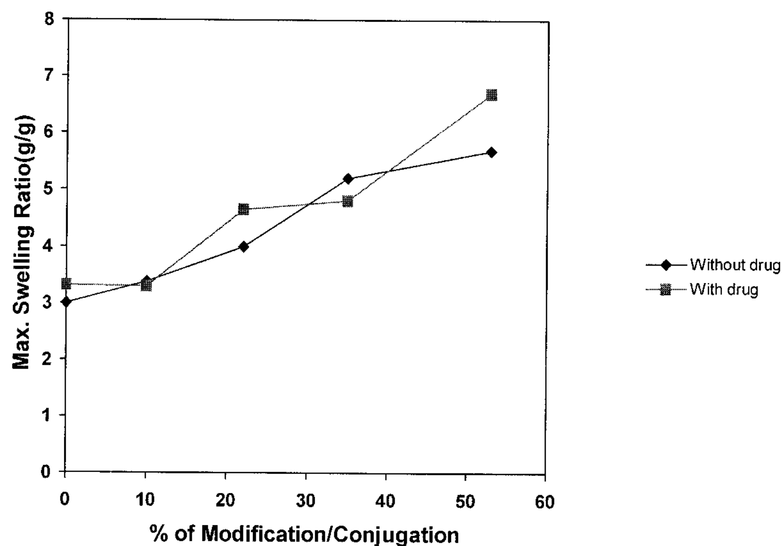


Figure 3 Maximum swelling ratios for gels with or without the drug loaded at 37°C as a function of the modification and conjugation of gelatin with EDTAD/PEG in (a) water, (b) a pH 7.4 buffer, and (c) a pH 4.5 buffer.

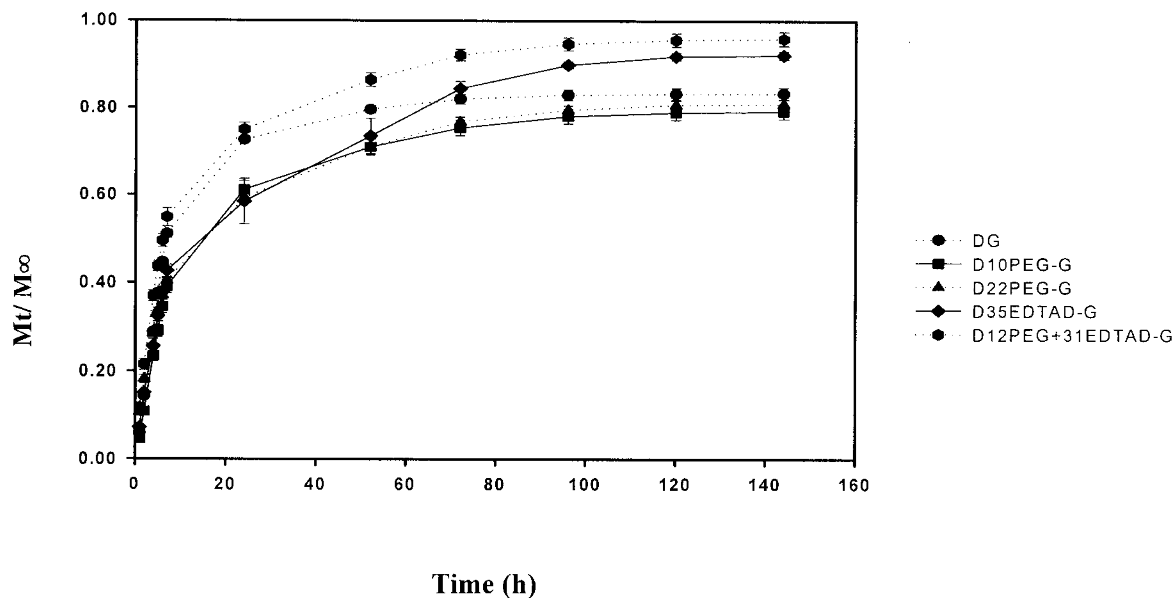


Figure 4 Cumulative drug released from gels with the drug loaded in a pH 7.4 buffer at 37°C.

hydroxyl groups of EDTAD and PEG, respectively. The responses in the swelling ratios for all the gel formulations remained unaltered with a change in the surrounding medium from water to buffers of pH 7.4 or 4.5, except for the gels modified with EDTAD; this is clearly evident in Figure 3(a–c), which shows the maximum swelling ratio as a function of the increased percentage of modification and conjugation of gelatin with EDTAD/PEG in various surrounding media. Figure 3(a–c) shows that 53% was contributed to both 12% PEG conjugation and 31% EDTAD modification of gelatin. In Figure 3(a), we can observe a tremendous increase in the swelling behavior in water for gels of 35EDTAD-G and 12PEG+31EDTAD-G without the drug loaded (ca. 15 and 10 g/g, respectively) in comparison with other gel formulations (which lie between 3 and 5 g/g). The reason for the higher swelling is the increase in the number of carboxylic groups with the EDTAD modification of gelatin. As a result of the modification, the net ionic charge was enhanced, and this induced a higher osmotic pressure in the network than in the surrounding medium, creating an osmotic pressure difference; to balance the pressure, a large amount of water rushed into the gel network, increasing the swelling ratio. However, the swelling for 53% modification and conjugation of gelatin with EDTAD/PEG (12PEG+31EDTAD-G) decreased because the net ionic charge in the network dropped from 35 to 31% EDTAD. The swelling ratio for the gels with increased PEG conjugation of gelatin with or without the drug loaded showed a gradual increase in the swelling ratio, regardless of the surrounding medium, whereas in water, the gels with increased EDTAD-modified gelatin with the drug loaded showed an abrupt shrink in the swelling ratio from 10 to 4.6 g/g and from 14 to 4 g/g, respectively, in comparison with gels without the drug loaded. The transition in swelling from swollen to shrunken for EDTAD-modified gels with the drug loaded was due to a strong interaction of the drug chlorhexidine with the EDTAD-modified network; this created less relaxation of the gel network. An insignificant change in the swelling ratio was recorded for gels of gelatin and the respective gelatin conjugated with PEG with or without the drug loaded as a function of pH because the gels, being nonionic, did not respond to changes in pH. Gels modified with EDTAD responded with a greater increase in the swelling ratio in a buffer of pH 7.4 than in a buffer pH 4.5 because of the ionization of —COOH groups at the higher pH.

The cumulative drug release from the loaded drug gels in buffers of pH 7.4 or 4.5 is depicted in Figures 4 and 5, respectively. M_t/M_∞ indicates the fractional drug release, where M_t is the mass of the drug released at time t and M_∞ is the mass of the drug released at infinity. In individual buffer conditions, we observed a sustained type of release. About 98% of the drug release was recorded from D12PEG+31EDTAD-G and D35EDTAD-G gels. However, about 80% of the drug release was observed from DG, 10PEG-G, and 20PEG-G gels, regardless of the buffer solutions even after 148 h. One possible reason could be that the drug entrapment in the polymer networks was due to less relaxation in comparison with the EDTAD-G gels. The rate of drug diffusion was also concomitant with the swelling behavior of the network and the concentration of the drug present on the outer surface. In our earlier reports, we observed that the swelling was much faster

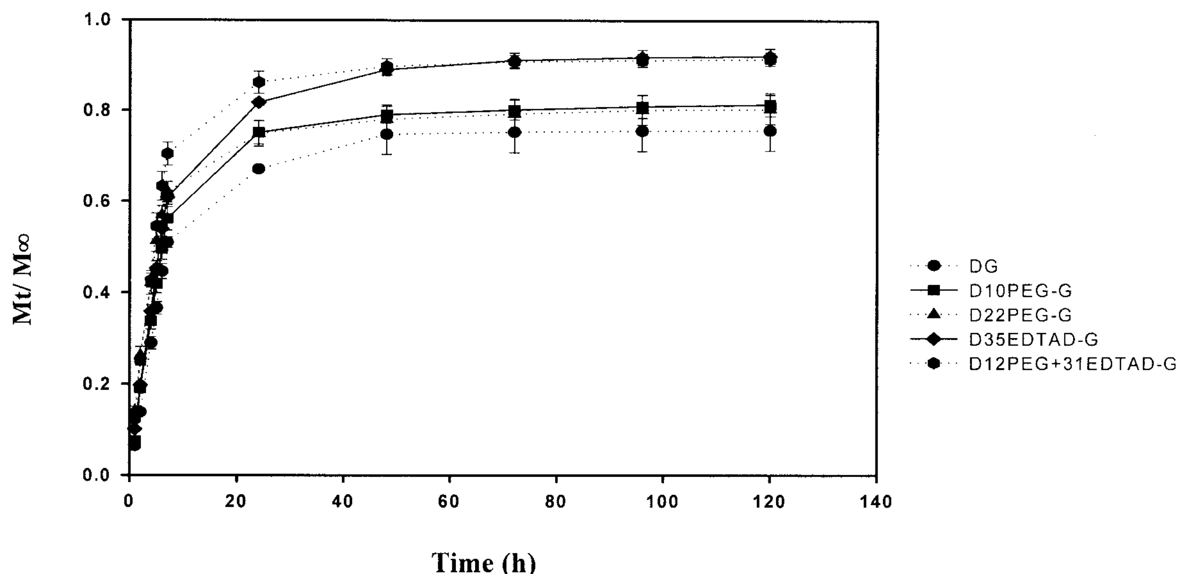


Figure 5 Cumulative drug released from gels with the drug loaded in a pH 4.5 buffer at 37°C.

in the initial 6 h and thereafter slowed and became steady until the gels reached equilibrium swelling.[32] The rationale for the faster swelling behavior was that initially the outer surface of the network came into contact with the surrounding medium and swelled immediately, whereas the inner core of the gel network required a longer period for the medium to diffuse for swelling. On the basis of these assumptions, we observed that the rate of drug diffusion was faster in the initial few hours (6–8 h) and thereafter declined with time. All gel formulations in a buffer of pH 7.4 continued to release the drug up to 128 h, whereas in a buffer of pH 4.5, the maximum drug was released within 48 h. The reason was that chlorhexidine at a physiological pH (7.4) behaved as a dication and interacted with the negatively charged polymer networks, encountering a slow release. At an acidic pH (4.5), no such interactions were possible, and so the drug release was faster.

CONCLUSIONS

The studies indicated that modified and conjugated gelatin gels with EDTAD/PEG had improved swelling behavior in comparison with gelatin gels. The swelling ratio was enhanced by an increase in the percentage of the modification and conjugation of gelatin with EDTAD/PEG, and this was attributed to an increased ionic charge and hydrophilicity of the network, respectively. According to their physical appearance, the gels containing PEG were more elastic and flexible than the gels without PEG. The cumulative drug release was unaffected for the gels with the conjugation of PEG (80%), but gels modified with EDTAD showed 98% release. The effect of pH variation on the swelling ratio was insignificant for all gel formulations, except for EDTAD-modified gelatin gels. However, the rate of drug release was affected by the change in pH, pH 4.5 being more favorable for faster release than pH 7.4.

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