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Enzymatically cross-linked hydrogels and their adhesive strength to biosurfaces

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Structured Abstract

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Objectives – To design an *in-situ* gelling hydrogel capable of solidifying rapidly under physiologic conditions into a hydrogel capable of adhering tissue surfaces together.

Design – Multifunctional polymers containing covalently bound peptide substrates of transglutaminase were designed.

Experimental Variable – Enzyme cross-linked hydrogels were compared with commercial fibrin tissue adhesive.

Outcome Measure – The shear strength between tissue surfaces or type 1 collagen membranes bonded with hydrogel was measured.

Results – The shear adhesive strength of transglutaminase cross-linked hydrogels was found to be equal to or better than fibrin sealant for tissue and collagen surfaces, respectively.

Conclusion – Transglutaminase cross-linked hydrogels are injectable, *in-situ* formed, biodegradable, and expected to be useful in a variety of applications including sustained drug delivery, medical and dental adhesives, tissue repair and engineering as polymeric scaffolds, and gene therapy.

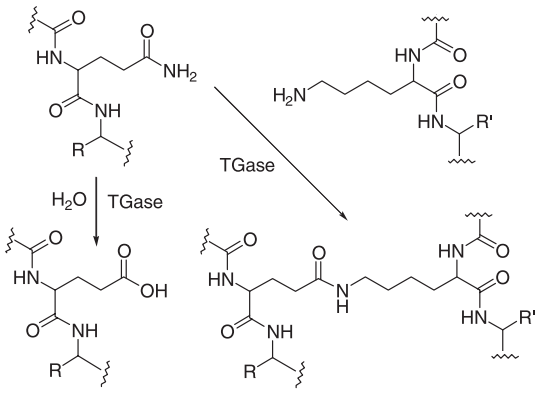
Key words: adhesive; hydrogel; polymer; tissue; transglutaminase

Introduction

Hydrogels are hydrophilic polymeric networks, which can absorb and retain large amounts of water and drastically increase in volume (1). Since they usually have structural similarity to the macromolecule-based components in the human body, hydrogels are generally considered biocompatible. Hydrogels have been widely used in controlled release systems for drug delivery (2) and tissue engineering (3), and as surgical sealants and adhesives (4–6). An emerging approach to formation of hydrogels relies on enzymatic cross-linking to form

polymer networks. As a mild approach under physiological conditions, hydrogels formed by enzymatic cross-linking should have the advantages of controllable gel formation and biocompatibility.

Transglutaminases (protein-glutamine: amine γ-glutamyltransferase, EC 2.3.2.13) are calcium-dependent enzymes that catalyse a post-translational acyl-transfer reaction between the γ-carboxamide groups of peptide-bound glutamine residues and the ε-amino groups of lysine residues in proteins, or certain primary amino groups (7). This reaction results in the cross-linking of proteins through the formation of ε-(γ-glutamyl)lysine isopeptide side-chain bridges (scheme 1). When tissue transglutaminase was first explored as an enzymatic cross-linking approach for hydrogel formation, no proof of gel formation was given (8,9). Recently, factor XIII (plasma transglutaminase) was reported to catalyze hydrogel formation (10). However, because of the stringent substrate specificity of factor XIII, a 20 amino acid-long peptide from the γ-chain of fibrinogen was synthesized and conjugated to a branched polyethylene glycol (PEG) polymer. The complexity of preparation of that conjugate made a large-scale preparation difficult.

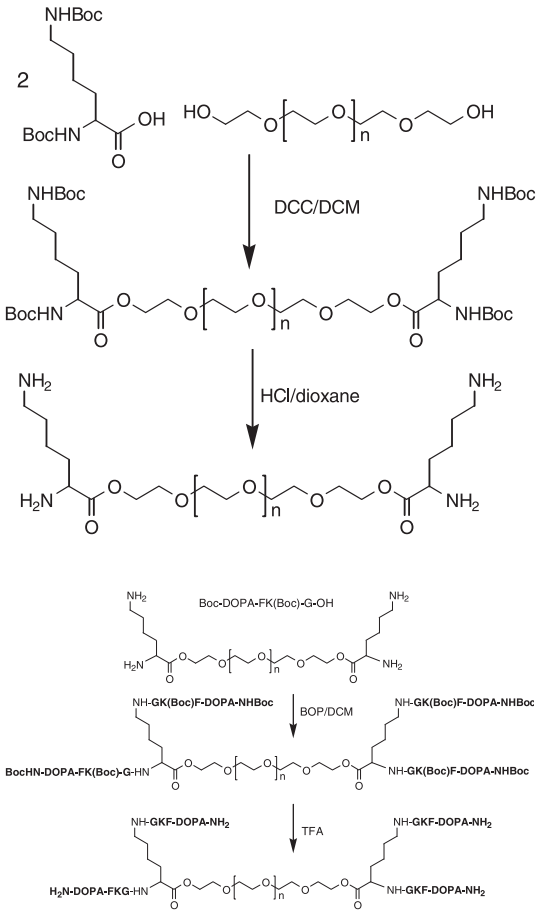


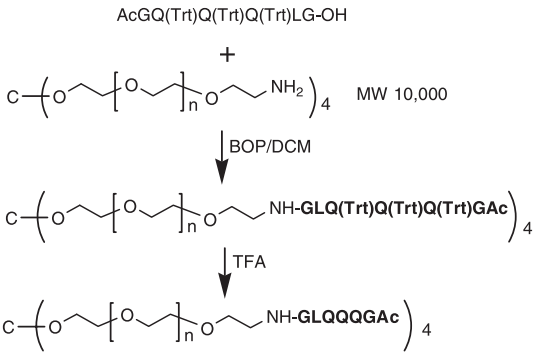
In this paper, we report the design and adhesive properties of peptide conjugated polymer hydrogels formed by tissue transglutaminase cross-linking. The peptide substrates were rationally designed and synthesized, coupled to polymer molecules, and the polymer-peptide conjugates were purified. These polymer-peptide conjugates were then cross-linked to rapidly form hydrogels by transglutaminase through a biomimetic approach under physiological conditions. These covalently cross-linked hydrogels are injectable, *in-situ* formed, and biodegradable. Adhesion experiments demonstrated that these hydrogels are equal to or better than fibrin glue in adhesion to skin tissue or extracellular matrix protein (collagen) membranes.

Materials and methods

Peptide synthesis

Peptide modified PEG was synthesized as illustrated in schemes 2–4 and using methods described previously (11). Boc-L-Lys(Boc)·DCHA (Advanced ChemTech, Louiseville, KY, USA) 7.92 g (15 mmol) was added to 100 ml of 2 M sulfuric acid aqueous solution and extracted with ethyl acetate 200 ml × 1, 100 ml × 2. The combined ethyl acetate solution was washed with 2 M sulfuric acid solution 100 ml, water 100 ml × 3, saturated NaCl aqueous solution 100 ml, dried over anhydrous magnesium sulfate for 30 min, and filtered. The filtrate was concentrated under reduced pressure, and the residue was dissolved in 50 ml of DCM. A solution of PEG 4000 (Fluka, Milwaukee, WI, USA) 20 g (10 mmol)





in DCM 50 ml and benzene 100 ml was azeotropically concentrated to dry the sample under reduced pressure, and the residue was dissolved in 50 ml of DCM. To PEG 4000 solution, Boc-L-Lys(Boc)-OH in DCM was added and stirred, followed by addition of DCC 3.1 g (15 mmol) in 20 ml of DCM, and stirred at room temperature overnight. The solution was filtered to remove the solid and concentrated under reduced pressure to afford the product. The product was purified by dissolving the product in 200 ml of MeOH, cooling it at −10°C overnight, and removing the solvent by centrifuge for three cycles. The purified product was dried in vacuum. Proton NMR confirmed the existence of Boc in the product. PEG 4000 di-Boc-L-Lys(Boc) was treated with 4 M HCl in dioxane 100 ml at room temperature for 2 h, and concentrated under reduced pressure. Addition of ether to the residual solution precipitated the product. The product PEG 4000 di-K was collected by filtration and dried in vacuum. MALDI–TOF MS confirmed the product structure.

A solution of Boc-DOPA-Phe-Lys(Boc)-Gly-OH 354 mg (0.48 mmol), PEG 4000 di-K 400 mg (0.4 mmol), BOP 212 mg (0.48 mmol), HOBt 74 mg (0.48 mmol) and DIEA 157 μl (0.9 mmol) in 3 ml of DCM was stirred for 2 h at room temperature, followed by addition of cold ether to precipitate the product. The product was collected through centrifuge, dried and treated with TFA–DCM (1:1) at room temperature for 60 min. The solution was dried, and the residue was dissolved in 20 ml of MeOH, and flowed by addition of 20 ml of ether. After cooled at −20°C overnight, the solid was collected by centrifuge and dried in vacuum. The product PEG4KK was further purified by preparative RP-HPLC and stored in a freezer.

A solution of Ac-Gly-Gln(Trt)-Gln(Trt)-Gln(Trt)-Leu-Gly-OH 0.66 g (0.48 mmol), (0.4 mmol), BOP 0.212 g (0.48 mmol), HOBt (0.48 mmol) 74 mg, and DIEA 250 μl (1.44 mmol) in 4 ml of DCM was stirred for 10 min at room temperature, followed by addition of PEG 4-armed amine (10 K) 1 g, and stirred overnight. The solution was dried, and the residue was dissolved in 5 ml of MeOH. To the MeOH solution, 45 ml of cold ether was added and cooled at −20°C overnight. The precipitate PEG4aQ(Trt)3 was collected through centrifuge, dried and further purified by passing through a Sephadex® LH-20 column (Pfizer, New York, NY, USA). PEG4aQ(Trt)3 was treated with TFA–TIS–H_2O (92:6:2) at room temperature 2 h. The solution was dried, and the residue was washed with cold ether 50 ml × 2, and dissolved in 50 ml of water. The insoluble solid was removed by centrifuge, and the solution was frozen and lyophilized. The lyophilized product PEG4aQ3 was further purified by preparative RP-HPLC and stored in a freezer.

Hydrogel formation and rheology

The following solutions were used: solution 1 – 4 mM PEG4aQ3 and 10 mM $CaCl_2$ in 75 mM Tris-HCl, pH 8.0, 25°C, 1.33 mM EDTA; solution 2 – 8 mM PEG4KK in

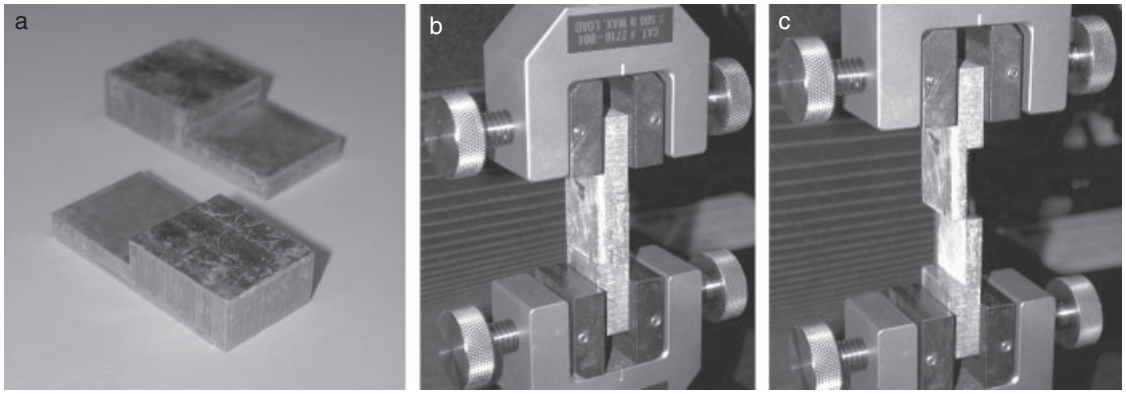


Fig. 1. Bioadhesion determination by Instron machine using fixtures: (a) Fixtures; (b) loaded fixtures before test; (c) after test.

water; solution 3 – transglutaminase from guinea pig liver (16 U/ml), 50 mM Tris-HCl, pH 8.0 25°C, 20 mM DTT, 1.33 mM EDTA in water. All solutions were cooled on ice for 10 min. 25 μl of solution 2, 25 μl of solution 3, and 50 μl of solution 1 were mixed on ice. After being vortexed, the solution was incubated at 37°C for 10 min. This experiment was performed before buffer system optimization. The reaction solution consisted of 2 mM PEG4aQ3 and 2 mM PEG4KK in 50 mM Tris-HCl, pH 8.0 (25°C), 5 mM $CaCl_2$, 5 mM DTT, 1 mM EDTA.

For rheology, all solutions were cooled on ice for 10 min. 125 μl of solution 2, 125 μl of solution 3, and 250 μl of solution 1 were added to a vial containing 2 U of tissue transglutaminase from guinea pig liver and mixed on ice. After being vortexed, 400 ml of the solution was loaded onto a rheometer (parallel plate 25 mm, gap 0.8 mm) at 37°C. The final reaction solution consisted of 4 mM PEG4aQ3 and 4 mM PEG4KK in 100 mM MOPS, pH 7.2, 10 mM $CaCl_2$, 5 mM DTT, and 1 mM EDTA.

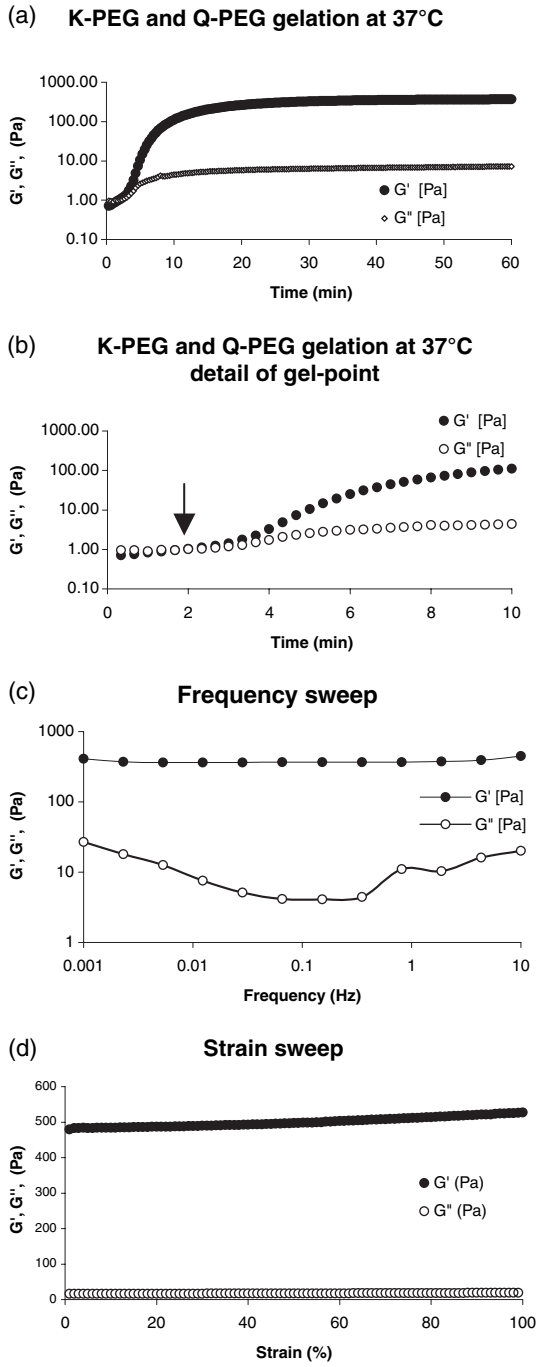


Fig. 2. Rheology of hydrogel formation.

Determination of hydrogel bioadhesion

The following solutions were used: solution 4 – 8 mM PEG4aQ3 and 20 mM $CaCl_2$ in 200 mM MOPS, pH 7.20; solution 5 – 16 mM PEG4KK and 2 mM EDTA in water; solution 6 – 20 mM DTT and 2 mM EDTA in water. Pieces of full thickness dorsal skin of guinea pig, or bovine collagen type I membrane (12) were glued to the flat surfaces of 2 × 2 cm test aluminum fixtures (Fig. 1) using cyanoacrylate glue. The skin or membrane was hydrated in 10 mM phosphate pH 7.4-buffered isotonic saline for 60 min. Just before applying adhesive solution, excess buffer was removed using a Kimwipes® (Kimberly Clark, Dallas, TX, USA) wiper. 125 μl of solution 5, 125 μl of solution 6 were added to a vial containing 2 U of tissue transglutaminase from guinea pig liver and mixed on ice, followed by addition of 250 μl of solution 4, and vortexed. 40 μl of the mixture

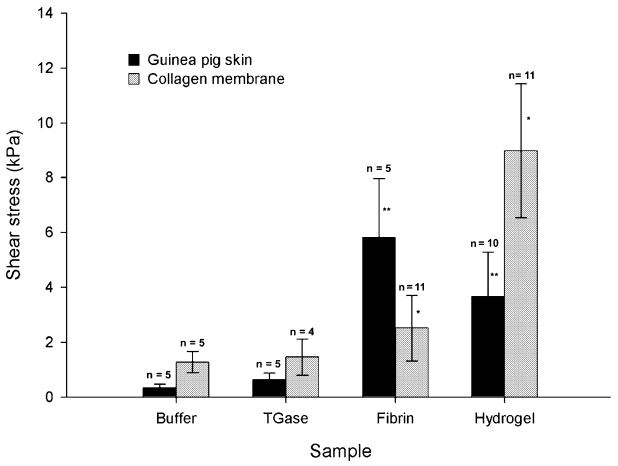


Fig. 3. Shear stress of hydrogel on guinea pig skin and collagen membrane ($*p < 0.01$; $**p > 0.05$).

was applied onto each hydrated surface, and the surfaces were pressed together using a constant force. The entire assembly was incubated at 37°C for 2 h under 100% humidity. The assembly was then mounted in an Instron machine (Fig. 1b) and tested using ASTM D1002 method (Fig. 1c; for results, see Fig. 3). Controls were performed in which the formulation described above was replaced with buffer only, enzyme only, and a commercial fibrin sealant purchased from Baxter Healthcare Corp. For the fibrin sealant test, 40 μl of the fibrinogen solution was applied onto one surface and 40 μl of the thrombin solution was applied onto a second surface, and the second surface was pressed onto the first surface.

Results and discussion

Since transglutaminases catalyze a cross-linking reaction between the γ-carboxamide groups of peptide-bound glutamine residues and the ε-amino groups of lysine residues in proteins, they involve two substrates: lysine substrate and glutamine substrate (scheme 1). The substrate specificity of lysine substrate and glutamine substrate were optimized using short peptides by rational design (11). Then, the optimized peptides were covalently conjugated to linear or branched polymers such as PEG, or linear polymers with multi-functional groups such as chitosan, gelatin, soluble collagens, hyaluronic acid, alginate, and albumins. The solutions of these polymer-peptide conjugates can be mixed with therapeutic agents or cells, which can be injected and triggered to form hydrogel and entrap the therapeutics or cells in the presence of tissue transglutaminase under physiological conditions *in vivo*. The solutions of these polymer-peptide conjugates can also be applied as surgical sealants and medical adhesives onto a tissue surface to be sealed.

In the presence of TGase, an aqueous fluid containing equimolar amounts of PEG-peptide conjugates formed a hydrogel within minutes under physiologic conditions. Rheological studies indicated that the hydrogel formed in less than 2 min, as indicated by the crossover of the storage (G') and loss (G") moduli in the gelation experiment (Fig. 2) (13). The elastic nature of the cross-linked gel was demonstrated by constant values of G' obtained over several decades of frequency in an oscillatory frequency sweep experiment, and the resulting hydrogel was found to be highly elastic, as indicated by constant values of G' at up to 100% strain during a strain sweep experiment.

This hydrogel was then tested as an adhesive using guinea pig skin and collagen membrane. The results (Fig. 3) demonstrate that the adhesive strength of guinea pig skin bonded using the experimental hydrogel was approximately similar to fibrin tissue adhesive. However, the adhesive strength of collagen membranes adhered using the experimental hydrogel surpassed that of fibrin tissue adhesives. These results suggested that transglutaminase cross-linked hydrogels may be useful as surgical tissue adhesives.

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