

# Chapter 2

## Enzymatically Catalyzed Conjugation of a Biodegradable Polymer to Proteins and Small Molecules Using Microbial Transglutaminase

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### Abstract

Hydroxyethyl starch (HES) is a water-soluble, biodegradable derivative of starch that is widely used in biomedicine as a plasma volume expander. Due to its favorable properties, HES is currently being investigated at the industrial and academic levels as a biodegradable polymer substitute for polyethylene glycol. To date, only chemical methods have been suggested for HESylation; unfortunately, however, these may have negative effects on protein stability. To address this issue, we have developed an enzymatic method for protein HESylation using recombinant microbial transglutaminase (rMTG). rMTG enzyme is able to catalyze the replacement of the amide ammonia at the  $\gamma$ -position in glutamine residues (acyl donors) with a variety of primary amines (acyl acceptors), including the amino group of lysine (Lys). To convert HES into a suitable substrate for rMTG, the polymer was derivatized with either *N*-carbobenzyloxy glutaminyl glycine (Z-QG) or hexamethylenediamine to act as an acyl donor or acyl acceptor, respectively. Using SDS-PAGE, it was possible to show that the modified HES successfully coupled to test compounds, proving that it is accepted as a substrate by rMTG. Overall, the enzymatic approach described in this chapter provides a facile route to produce biodegradable polymer–drug and polymer–protein conjugates under relatively mild reaction conditions.

**Key words:** Hydroxyethyl starch, Biodegradable polymer, Recombinant microbial transglutaminase, Polymer–drug conjugates, Polymer–protein conjugates

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### 1. Introduction

The surface modification of therapeutic proteins by coupling them to water-soluble polymers imparts a number of advantages, such as increased water solubility, increased circulation time, reduced levels of aggregate formation, reduced immunogenicity,

and increased stability against proteolytic digestion. The gold standard for this approach is PEGylation (coupling to poly(ethylene glycol), PEG), and many types of PEGylated proteins are already commercially available (1). However, PEG is not biodegradable, raising concerns about its fate and effects after chronic use in large doses. This has motivated academia and industry to search for biodegradable substitutes, such as albumin and hydroxyethyl starch (HES). The latter is a semisynthetic biodegradable polymer that is currently used commercially as a blood plasma volume expander. Its favorable properties, such as high water solubility, low hypersensitivity, and the possibility to tailor its molar mass and biodegradation rate, have recently attracted much attention to HES as a promising substitute for PEG. Consequently, the polymer has been investigated for the stabilization of nanoparticles (2) and for protein conjugation in order to evaluate HESylation as a potential alternative to conventional PEGylation of proteins (3).

There are several chemical approaches to couple biomacromolecules to polymers; however, many of them can have detrimental effects on proteins, which are usually quite sensitive to their surrounding environment. Accordingly, enzymatic methods have been proposed and tested as gentle alternatives to chemical-based coupling strategies. Among these, enzymatic coupling using microbial transglutaminase (MTG) has earned a significant degree of attention in the development of site-specific conjugation strategies (4). MTG catalyzes the replacement of the amide ammonia at the  $\gamma$ -position in glutamine residues (acyl donors) with a variety of primary amines (acyl acceptors), including the  $\epsilon$ -amino group of lysine (5). Furthermore, MTG has a number of advantageous properties over eukaryotic TG – including being a calcium-independent enzyme and having fewer substrate specificity requirements (5) – and has thus found application in the food industry for crosslinking meat and fish products (5). In biomedical applications, Sato et al. provided a clear demonstration of the power of using MTG for site-specific protein–polymer conjugation when they used the enzyme to couple alkylamine derivatives of PEG selectively to a glutamine residue (Gln74) of recombinant human interleukin-2 (6). Fontana et al. have recently reviewed the relationship between the catalytic activity of MTG and the substrate structural characteristics, and concluded that both the primary structure (viz., the presence of nearby hydrophobic residues) and tertiary structure (viz., chain flexibility) of the substrate are important factors for site specificity (7).

In this chapter, we describe protocols for the modification of HES with hexamethylenediamine (HMDA) as well as with *N*-carbobenzyloxy glutaminyl glycine (Z-QG) to act as substrates for MTG (both as acyl acceptor and acyl donor, respectively). In addition, we present examples of the reaction of the modified

HES polymer with model compounds to demonstrate the feasibility of this conjugation strategy. To carry out these conjugation reactions, we utilize a highly purified recombinant MTG (rMTG) carrying a polyhistidine tag (His-tag) at the C terminus, which has recently been developed in our laboratory (8, 9). The incorporation of a His-tag into rMTG facilitates the workup and separation of the products, and further provides the possibility of immobilizing the enzyme without loss of activity (10).

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## 2. Materials

### **2.1. Modification of HES to Carry a Lysine-Like Residue (HES 70-Amine)**

1. HES (70 kDa) (HES 70) (Serumwerke Bernburg AG, Bernburg, Germany).
2. Tosyl chloride.
3. HMDA.
4. Triethylamine.
5. 50 mM borate buffer, pH 10.

### **2.2. Modification of HES to Carry a Glutamine Residue (HES 70-GQ-Z)**

1. HES 70 (Serumwerke Bernburg AG).
2. Dicyclohexylcarbodiimide (DCC).
3. *N*-Hydroxysuccinimide (NHS).
4. 4-(Dimethylamino)pyridine (DMAP).
5. *N*-Carbobenzyloxy glutaminyl glycine (Bachem AG, Bubendorf, Switzerland). Store at  $-20^{\circ}\text{C}$ .

### **2.3. Reaction of HES 70-Amine with Dimethylcasein**

1. Recombinant microbial transglutaminase (rMTG) (see Notes 1–3).
2. HES 70-Amine, as prepared in Subheading 3.2.
3. *N,N*-Dimethylcasein (DMC).
4. Human serum albumin (HSA), 20% (w/v). Store at  $4^{\circ}\text{C}$ .
5. Dithiothreitol (DTT).
6. 50 mM Tris-HCL buffer, pH 8.
7. Sample buffer (2 $\times$ ): Dissolve 1.21 g of Tris-HCl, 2.5 g of SDS, 50 mg of bromophenol blue, 10 g of glycerol, and 42 g urea in 95 ml of ultrapure water. Adjust the pH to 8.0 with HCl, and bring up to 100 ml with ultrapure water.

### **2.4. Reaction of HES 70-GQ-Z with Monodansyl Cadaverine**

1. Recombinant microbial transglutaminase (see Notes 1–3).
2. HES 70-GQ-Z, as prepared in Subheading 3.3.
3. MDC.
4. *N,N*-Dimethylcasein.

5. HES 70 (Serumwerke Bernburg AG).
6. 50 mM Tris-HCL buffer, pH 8.
7. Sample buffer (2×) with  $\beta$ -mercaptoethanol: Prepare as described in Subheading 2.3, item 7, and then add 10  $\mu$ l of  $\beta$ -mercaptoethanol/ml sample buffer before use.

## 2.5. SDS-Polyacrylamide Gel Electrophoresis

1. Mighty Small gel electrophoresis unit (Hoefer, Inc., Holliston, MA).
2. Separating gel buffer: Add 18.18 g of Tris-HCL, 0.4 g of SDS, and 100  $\mu$ l of 10% (w/v)  $\text{NaN}_3$  to 80 ml distilled water. Adjust the pH to 8.8 with 4N HCl (approximately 6 ml), and then bring up to 100 ml with water. Store at room temperature.
3. Stacking gel buffer: Add 6.06 g of Tris-HCL, 0.4 g of SDS, and 100  $\mu$ l of 10% (w/v)  $\text{NaN}_3$  to 70 ml distilled water. Adjust to pH 6.8 with 4 N HCl, and then bring up to 100 ml with water. Store at room temperature.
4. 30% (w/v) acrylamide/bisacrylamide solution: Dissolve 29.1 g of acrylamide and 0.9 g of bisacrylamide in distilled water and bring up to 100 ml. *CAUTION:* Acrylamide is a neurotoxin and a suspected carcinogen when unpolymerized, so use extreme care and wear gloves and goggles when handling.
5.  $N,N,N,N'$ -Tetramethylethylenediamine (TEMED).
6. 10% (w/v) Ammonium persulfate (APS): Dissolve 100 mg of APS in 1 ml of distilled water. Prepare immediately before use.
7. Water-saturated isobutanol: Shake equal volumes of water and isobutanol in a glass bottle and allow the phases to separate. Recover the organic (top) layer and store at room temperature.
8. Running buffer (10×): Mix 30.28 g of Tris-HCL, 144 g of glycine, 10 g of SDS, and 1 ml of 10% (w/v)  $\text{NaN}_3$  to distilled water and bring to 1 l. Store at room temperature.
9. Prestained molecular weight marker (Fermentas GmbH, Germany):  $\beta$ -Galactosidase (116.0 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), lactate (35.0 kDa), restriction endonuclease Bsp98I (25.0 kDa),  $\beta$ -lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa).
10. Staining solution: Dissolve one PhastGel™ Blue R (Coomassie R 350 stain) tablet (GE Healthcare) in a solution containing 50 ml of acetic acid, 100 ml of isopropanol, and 150 ml of distilled water. Accelerate the dissolution of the tablet by stirring. Filter the staining solution before use.
11. Destaining solution: Add 20 ml of isopropanol to 10 ml of acetic acid, and then bring up to 100 ml with distilled water.

### 3. Methods

#### **3.1. Modification of HES to Carry a Lysine-like Residue (HES 70-Amine) (see Note 4)**

1. Dry 1 g of HES 70 (5.4 mM of the anhydroglucose units, AGU) at 105°C for 2 h.
2. Dissolve the dried HES 70 in a solution containing 10 ml of dry DMF and 1 ml of triethylamine at 60°C.
3. Dissolve tosyl chloride (0.5 g, 2.6 mM) in 1 ml of dry DMF.
4. Cool both solutions on ice to 0°C and protect from light.
5. Add the tosyl chloride solution gradually to the HES 70 solution and stir at 0°C for 1 h.
6. Precipitate the polymer solution in 100 ml of cold acetone, filter, and wash with another 100 ml of acetone.
7. Dissolve the precipitate in water and dialyze against distilled water for 3 days (6–8 kDa MWCO), then lyophilize.
8. From the prepared HES tosylate, dissolve 200 mg in 30 ml DMF/borate buffer, pH 10 (1:2, v/v).
9. Add an excess of HMDA (500 mg, 4.3 mM) dissolved in 10 ml of DMF/borate buffer, pH 10 (1:2, v/v), and stir overnight.
10. Precipitate the polymer in 200 ml of isopropanol/methanol (1:1, v/v), filter, and wash with 100 ml of the precipitating solvent.
11. Dry the precipitate at room temperature for 2 days.
12. Characterize the HES 70-amine product by  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ ):  $\delta = 1.27$  (broad, 4H,  $-\text{NH}-(\text{CH}_2)_2-(\text{CH}_2)_2-(\text{CH}_2)_2-\text{NH}_2$ ), 1.5 (broad, 4H,  $-\text{NH}-\text{CH}_2-\text{CH}_2-(\text{CH}_2)_2-\text{CH}_2-\text{CH}_2-\text{NH}_2$ ), 5.1–5.7 (broad, 1H,  $\text{HC}$ —anomeric carbon of AGU).

#### **3.2. Modification of HES to Carry a Glutamine Residue (HES 70-GQ-Z) (see Note 5)**

1. Dissolve 185 mg (0.55 mM) of Z-QG, 114 mg (0.55 mM) of DCC, 66 mg (0.55 mM) of DMAP, and 64 mg (0.55 mM) of NHS in 2 ml of dry DMSO. Leave the mixture to react for 24 h under stirring at 400 rpm.
2. Dry 1 g of HES 70 (5.4 mM of the AGU) at 105°C for 2 h, and then dissolve the dried HES 70 in 10 ml of dry DMSO.
3. Filter the solution of activated Z-QG (Z-QG succinimidyl ester) to remove the insoluble byproduct of the reaction, dicyclohexylurea.
4. Add the filtrate from step 3 directly to the HES 70 solution (obtained from step 2) and stir for 6 h.
5. Dialyze the polymer solution against distilled water for 3 days (6–8 kDa MWCO), filter, and then lyophilize.
6. Characterize the HES 70-GQ-Z product by  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ ):  $\delta = 2.26$  (broad, 2H,  $-\text{CH}_2-\text{CO}-\text{NH}_2$ ), 5.03 (broad, 2H,  $-\text{O}-\text{CH}_2-\text{C}_6\text{H}_5$ ), (broad, 1H,  $\text{HC}$ —anomeric carbon of AGU), 7.31 (broad, 5H,  $-\text{O}-\text{CH}_2-\text{C}_6\text{H}_5$ ).

### **3.3. Reaction of HES 70-Amine with Dimethylcasein (see Note 6)**

The reaction of HES 70-amine with DMC (step 1) can be performed simultaneously with appropriate control experiments, such as those described in steps 2 and 3 below.

1. Add 100  $\mu$ l of HES 70-amine (5 mg/ml) to 100  $\mu$ l of DMC (5 mg/ml), both dissolved in 0.1 M Tris-HCL buffer (pH 8). Next, add 100  $\mu$ l of rMTG (15 U/ml) in glycerol to the mixture and incubate for 1 h at 37°C.
2. Positive control experiment (see Note 7): Add 200  $\mu$ l of 0.2% (w/v) HSA in 0.1 M Tris-HCL buffer (pH 8) with 10 mM DTT to 100  $\mu$ l of rMTG (15 U/ml) in glycerol. Incubate the reaction mixture at 37°C for 1 h.
3. Negative control experiment: Add 200  $\mu$ l of DMC (5 mg/ml) in 0.1 M Tris-HCL buffer (pH 8) to 100  $\mu$ l of rMTG (15 U/ml) in glycerol. Do not add HES 70-amine. Incubate the reaction mixture at 37°C for 1 h.
4. Withdraw samples of the reaction mixture at 10, 20, 30, and 60 min, and mix with an equal volume of 2 $\times$  sample buffer. Boil each sample at 99°C for 3 min. Analyze the samples by SDS-PAGE as described in Subheading 3.5 below (see Note 8). Stain the resulting gel with Coomassie Blue.

Figure 1 shows an example of an SDS-PAGE gel image obtained for the coupling of HES 70-amine to DMC using rMTG.

### **3.4. Reaction of HES 70-GQ-Z with MDC**

The reaction of HES 70-GQ-Z with MDC (step 1) can be performed simultaneously with appropriate control experiments, such as those described in steps 2, 3, and 4 below.

1. Add 25  $\mu$ l of 5 mM MDC in 0.1 M acetic acid to 75  $\mu$ l of a 1% (w/v) solution of HES 70-GQ-Z in 0.1 M Tris-HCL buffer (pH 8). Next, add 100  $\mu$ l of rMTG (15 U/ml) in glycerol to the mixture and incubate at 37°C for 1 h.
2. Positive control experiment: Add 25  $\mu$ l of 5 mM MDC in 0.1 M acetic acid to 75  $\mu$ l of DMC (5 mg/ml) in 0.1 M Tris-HCL buffer (pH 8). Next, add 100  $\mu$ l of rMTG (15 U/ml) in glycerol to the mixture and incubate at 37°C for 1 h.
3. Negative control experiment 1: Add 25  $\mu$ l of 5 mM MDC in 0.1 M acetic acid to 75  $\mu$ l of a 1% (w/v) solution of unmodified HES 70 in 0.1 M Tris-HCL buffer (pH 8). Next, add 100  $\mu$ l of rMTG (15 U/ml) in glycerol to the mixture and incubate at 37°C for 1 h.
4. Negative control experiment 2: Add 25  $\mu$ l of 5 mM MDC in 0.1 M acetic acid to 75  $\mu$ l of a 1% (w/v) solution of HES 70-GQ-Z in 0.1 M Tris-HCL buffer (pH 8). Next, add 100  $\mu$ l of glycerol buffer (do not add rMTG) to the mixture and incubate at 37°C for 1 h.

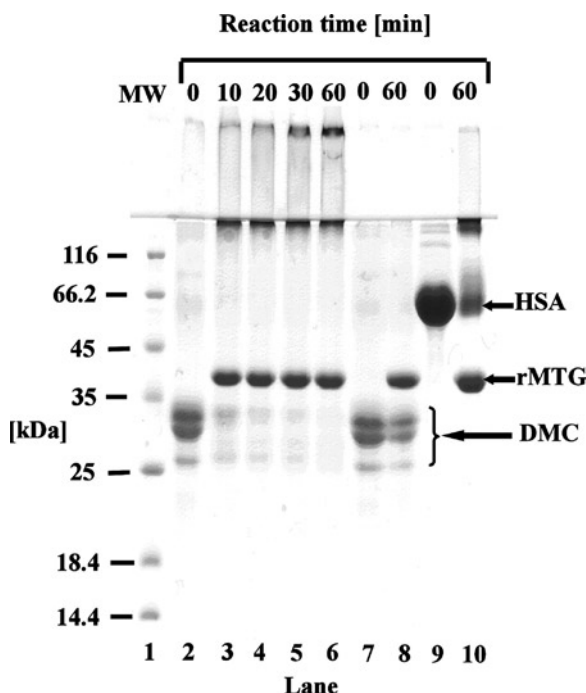


Fig. 1. SDS-PAGE analysis of the coupling of HES 70-amine to DMC using rMTG. *Lane 1*: Protein molecular weight markers, *Lane 2*: HES 70-amine + DMC (no rMTG), *Lane 3*: HES 70-amine + rMTG + DMC after 10 min, *Lane 4*: 20 min, *Lane 5*: 30 min, *Lane 6*: 60 min, *Lane 7*: DMC alone, *Lane 8*: DMC + rMTG after 60 min, *Lane 9*: HSA + DTT, and *Lane 10*: HSA + DTT + rMTG after 60 min. The gel was stained with Coomassie blue. Reproduced with permission from ref. 3 © 2009 John Wiley & Sons, Inc.

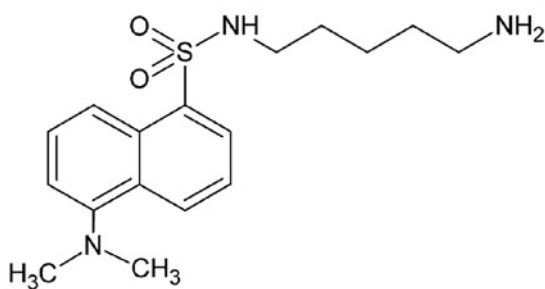
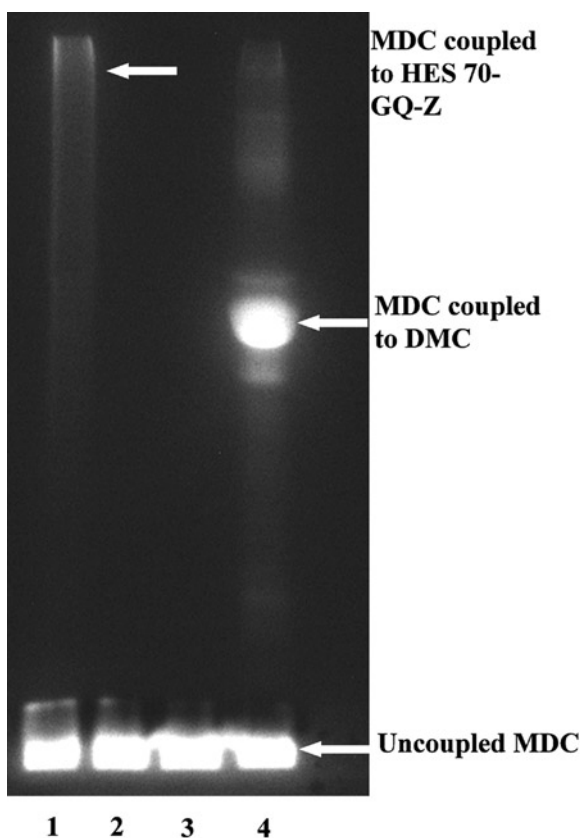
5. Withdraw samples (15  $\mu$ l) from each experiment and mix with 2 $\times$  sample buffer (15  $\mu$ l). Boil at 99°C for 3 min. Analyze the samples by SDS-PAGE as described in Subheading 3.5.
6. Examine the gels under UV light (365 nm excitation filter; 520 nm emission filter).

Figure 2 shows an example of a fluorescence gel image obtained for the coupling of HES 70-GQ-Z to MDC using rMTG.

### 3.5. SDS-Polyacrylamide Gel Electrophoresis

The following procedure describes the use of a Mighty Small electrophoresis unit from Hoefer, Inc., but can be easily modified for use with other mini-vertical gel electrophoresis systems.

1. Assemble the gel sandwich stack (comprised of one notched alumina or glass plate, one rectangular glass plate and two spacers) and slide it into the casting clamp assembly.
2. With the middle screws lightly tightened, align the plates and spacers of the gel sandwich stack so that they protrude slightly (~1 mm) from the bottom of the casting clamp assembly. Next, secure the gel sandwich stack in place by tightening all the remaining screws until they are finger-tight.



**Monodansyl cadaverine (MDC)**

Fig. 2. Fluorescence image of the SDS-PAGE gel obtained for the coupling of HES 70-GQ-Z to MDC using rMTG. *Lane 1*: HES 70-GQ-Z+rMTG+MDC, *Lane 2*: HES 70+rMTG+MDC, *Lane 3*: HES 70-GQ-Z+MDC (no rMTG), and *Lane 4*: DMC+rMTG+MDC. From top to bottom, the *arrows* point to HES 70-GQ-Z coupled to MDC; the band of DMC coupled to MDC; and uncoupled MDC. The chemical structure of MDC is also shown below the fluorescence gel image. Reproduced with permission from ref. 3 © 2009 John Wiley & Sons, Inc.

3. Place the clamp assembly in the casting cradle, with the screws facing outward. In this position, the gel will be visible through the rectangular glass plate. Ensure that there is a good seal between the bottom of the gel sandwich stack and the rubber gasket in the casting cradle.



4. Prepare the separating gel in the following order (stir the mixture before and after adding APS to ensure homogeneous polymerization): Distilled water (3.3 ml), separating gel buffer (2.5 ml), acrylamide/bisacrylamide (4.2 ml), TEMED (10  $\mu$ l), and APS (20  $\mu$ l).
5. Pour the separating gel into the sandwich stack. Fill the sandwich stack until the gel solution reaches a few centimeters below the top of the rectangular glass plate so that there is sufficient room left for pouring the stacking gel (step 9).
6. Overlay the separating gel with 500  $\mu$ l of isopropanol and allow the gel to polymerize for 30 min.
7. Remove the isopropanol by simply inverting the whole gel casting assembly and discarding the drained liquid into an organic solvent waste container.
8. Prepare the stacking gel in the following order (stir the mixture before and after adding APS to ensure homogeneous polymerization): Distilled water (3 ml), stacking gel buffer (1.25 ml), acrylamide/bisacrylamide (0.75 ml), TEMED (10  $\mu$ l), and APS (8  $\mu$ l).
9. Pour the stacking gel onto the separating gel until the gel solution reaches the top of the glass plate. Insert the comb and allow the gel to polymerize.
10. Prepare the running buffer by diluting 30 ml of 10 $\times$  concentrated running buffer stock solution with 270 ml of distilled water.
11. Transfer the polymerized gel sandwich stack to the electrophoresis unit and secure it in place.
12. Connect the coolant ports of the electrophoresis unit to a circulating (cold) water bath.
13. To aid in loading the samples into the gel, wet the transparent well-locating decal and apply it to the front of the glass plate so that the appropriate edge outlines the sample wells.
14. Fill the upper buffer chamber with running buffer and then remove the comb.
15. Load the samples into the wells of the gel (load 10  $\mu$ l of the samples and 5  $\mu$ l of the protein molecular weight markers).
16. Fill the lower buffer chamber with running buffer and install the safety lid onto the electrophoresis unit.
17. Connect the electrophoresis unit to a power supply and run the gel at 40 mA (constant current mode) for ~6 min through the stacking gel, and then run the gel at 30 mA for ~40 min through the separating gel. (When running two gels, run at 80 mA for 6 min through the stacking gel and 60 mA for 40 min through the separating gel.)

18. After the bromophenol blue tracking dye has reached the end of the gel (or has passed completely through the gel), turn off the power and disconnect the electrophoresis unit from the power supply. Also, stop the circulating water bath.
19. To take the gel out of the sandwich stack, remove the spacers first and then carefully pry the aluminum backing plate with a spatula to strip the gel from the glass plate.
20. Briefly rinse the gel with distilled water.
21. To stain the protein bands, gently agitate the gel in staining solution for ~1 h.
22. Rinse the gel briefly with destaining solution, and then agitate the gel in destaining solution for 30 min. Replace with fresh destaining solution and agitate the gel for an additional 30 min (at this point, the gel background should not contain any blue color).
23. Analyze the stained gel using a gel imaging system.

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## 4. Notes

1. The recombinant biocatalyst (inactive pro-TG) can be produced in soluble form using *Escherichia coli* and IPTG as an inductor as described in detail in ref. 9. A very important point to note is the temperature shift prior to induction (from 37°C to 24°C) to prevent the formation of inclusion bodies. This step reduces the transcription and translation velocity, delivering the pro-enzyme in limited amounts and enabling the protein to fold properly. Alternatively, the autoinduction medium (9) can be used at an incubation temperature of 28°C.
2. Inactive pro-TG enzyme can be purified using metal affinity chromatography and is activated prior to use by removal of the pro-sequence using a suitable protease, such as dispase (8). The endogenous protease from *Streptomyces mobaraensis* can also be used instead; however, this enzyme is not commercially available and must be prepared in the laboratory.
3. In principle, commercially available transglutaminase can be used instead of the recombinant enzyme. However, commercial preparations of transglutaminase can in some cases be relatively crude and may contain proteases (which may not be visible on a Coomassie-stained SDS-PAGE gel). Such proteases can cause unwanted degradation of the protein substrate.
4. MTG is known to display a high degree of selectivity for glutamine (Gln) as the acyl donor, while the acyl acceptor can either be a lysine (Lys) residue or simply a primary amine

group attached to an alkyl chain having at least four carbon atoms. Accordingly, we used HMDA for the modification of HES, as well as MDC as a model acyl acceptor, where both have primary amine groups attached to alkyl chains with six and five carbon atoms, respectively.

5. Due to the fact that DCC can lead to the oxidation of alcohols in DMSO, the coupling of Z-QG to HES 70 is carried out in two steps: First, the activation of Z-QG using DCC and NHS is performed. Following this, the coupling of HES 70 to the activated peptide is achieved.
6. DMC is derived from casein by methylation (i.e., blocking) of all the lysine amino groups. Consequently, DMC can act as an acyl donor (i.e., presenting only glutamine residues) and is a useful compound for performing test conjugation reactions with acyl acceptors.
7. HSA functions as a positive control, where it is used to show that the purified rMTG has retained its bioactivity. In the native form, HSA does not act as a substrate for MTG and must first be denatured by reduction using DTT.
8. Since both HES 70-amine and DMC are polyvalent compounds, their crosslinking with rMTG can lead to the formation of very large aggregates, which can be seen as bands at the top of the stacking gel, as shown in Fig. 1.

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