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Effect of PEG molecular weight and linking chemistry on the biological activity and thermal stability of PEGylated trypsin

Benjaporn Treetharnmathurot [a], Chitchamai Ovartlarnporn [b], Juraithip Wungsintaweekul [c], Ruth Duncan [d], Ruedeekorn Wiwattanapatapee [a,*]

[a] *Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hatyai, Songkhla 90112, Thailand*
[b] *Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hatyai, Songkhla 90112, Thailand*
[c] *Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hatyai, Songkhla 90112, Thailand*
[d] *Centre for Polymer Therapeutics, Welsh School of Pharmacy, King Edward VII Avenue, Cardiff University, Cardiff CF10 3XF, UK*

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Abstract

PEGylated proteins are routinely used as therapeutics, but systematic studies of the effect of PEG molecular weight and linking chemistry on the biological activity and particularly the thermal stability of the conjugated protein are rarely made. Here, activated monomethoxypolyethylene glycol (mPEG)s (Mw 1100, 2000 and 5000 g/mol) were prepared using succinic anhydride (SA), cyanuric chloride (CC) or tosyl chloride (TC) and used to synthesise a library of trypsin conjugates. The enzyme activity (K_M, V_{max} and K_{cat}) of native trypsin and the mPEG-modified trypsin conjugates was compared using *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA) as a substrate, and their thermal stability determined using both BAPNA and *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrates to measure amidase and esterase activity respectively. The effect of conjugate chemistry on trypsin autolysis was also examined at 40 °C. PEG-trypsin conjugates containing the higher molecular weight of mPEG (5000 g/mol) were more stable than free trypsin, and the conjugate containing CC-mPEG 5000 g/mol had the best thermal stability.

Keywords: Monomethoxypolyethylene glycol (mPEG); PEG conjugates; Thermal stability; Trypsin

1. Introduction

The biotechnology revolution has produced many novel peptides and proteins that have become important new drugs. More than 80 are now marketed in the United States, and 350 are undergoing clinical trials (reviewed in Harris and Chess, 2003). However, protein drugs also possess several shortcomings that limit their usefulness. These include premature degradation due to susceptibility to destruction by proteolytic enzymes, short circulating half-life, low solubility, rapid kidney clearance of lower molecular weight proteins and potential immunogenicity. Conjugation of proteins and peptides to natural or synthetic polymers has shown the ability to solve many of these problems (reviewed in Harris and Chess, 2003; Pasut and Veronese, 2007).

Although a variety of polymers have been used for protein conjugation, polyethylene glycol (PEG) has been most popular due to its excellent water-solubility, and it is known to be safe being FDA approved for use in injectable, topical, rectal and nasal formulations. PEG is synthesised by anionic ring polymerisation of ethylene oxide using methanol or water as initiator to give a linear polyether that can be modified to ensure a single terminal reactive functional group e.g. monomethoxy PEG (mPEG). This enables protein modification without risk of cross-linking (reviewed in Pasut and Veronese, 2007; Duncan, 2003; Greenwald et al., 2003; Harris and Chess, 2003). Moreover, PEG polymerisation can be controlled to produce a wide range of discreet molecular weights (<5000–50,000 g/mol) with low polydispersity (Mw/Mn from <1.01 to 1.1 for higher PEGs of Mw ∼50,000 g/mol). The high hydration and flexibility of the PEG chain enables a reduction in antigenicity of proteins to which it is bound, and careful choice of PEG molecular weight enables fine tuning of plasma pharmacokinetics

* Corresponding author. Tel.: +66 74 288915; fax: +66 74 428148.
E-mail address: ruedeekorn.w@psu.ac.th (R. Wiwattanapatapee).

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and route of elimination (by renal and hepatic pathways) of specific peptides and proteins to suit their pharmacodynamic requirements.

Despite the large number of studies on PEG-protein modification, including the emergence of novel linking chemistry (Brocchini et al., 2006), there has been little attempt to systematically study the effect of PEG molecular weight and linking chemistry on protein properties such as thermal stability. Therefore, the aims of this study were to compare the enzyme activity (kinetic parameters), the thermal stability and autolysis of a library of PEG-modified trypsin conjugates synthesised using PEGs of different molecular weights (Mw 1100, 2000 and 5000 g/mol) and different linking chemistries. Trypsin (from bovine pancreas) was chosen as a convenient model protein. It is a pancreatic serine protease, composed of a single polypeptide chain of 223 amino acid residues, and it displays a narrow substrate specificity hydrolysing L-lysyl and L-argininyl bonds of polypeptides (amidase activity; Walsh, 1970 and esterase activity; Bergmeyer et al., 1974). As the covalent attachment of *p*-nitrophenyl chloroformate (NPC) activated mPEG to bovine pancreatic trypsin increased thermal stability (Gaertner and Puigserver, 1992; Zhang et al., 1999) the PEG-trypsin conjugates were synthesised using succinic anhydride (SA), cyanuric chloride (CC) or tosyl chloride (TC) to activate PEG (Scheme 1). The enzyme activities (K_M, V_{max} and K_{cat}) were determined by using *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA) and *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrates for amidase and esterase activity, respectively.

2. Materials and methods

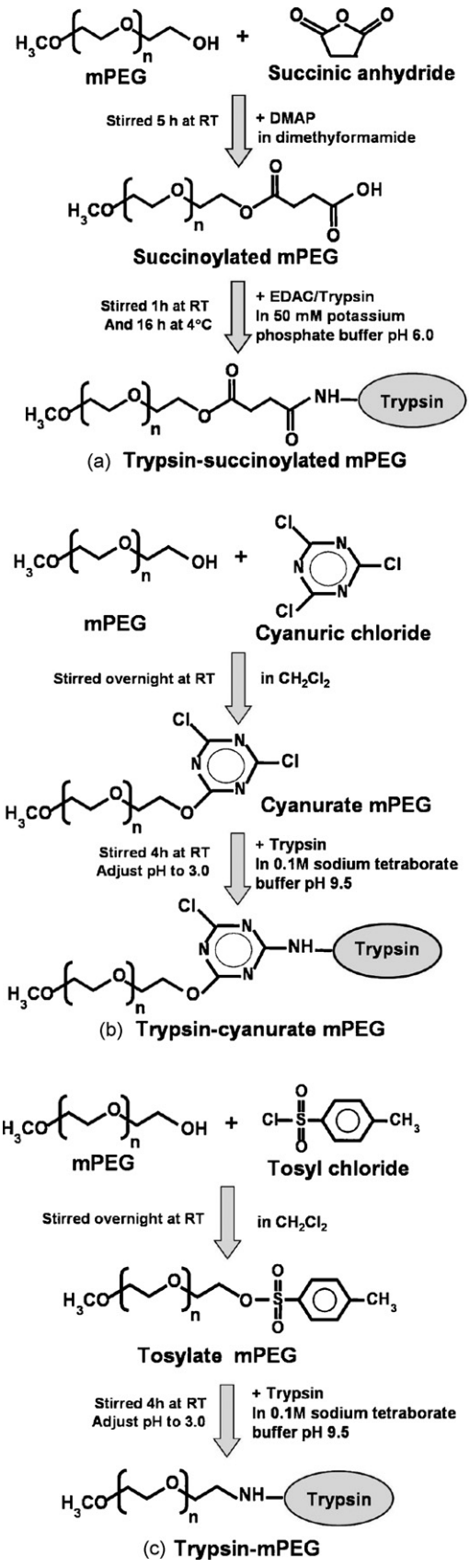
2.1. Materials

mPEGs of molecular weight 1100, 2000 and 5000 g/mol, succinic anhydride and 4-dimethylaminopyridine (DMAP) were from Fluka (Switzerland). Bovine pancreatic trypsin (EC 3.4.21.4), cyanuric chloride, tosyl chloride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 2,4,6-trinitrobenzene sulfonic acid (TNBS), BAPNA, BAEE, and the Coomassie blue for protein assay were from Sigma (Germany). All other reagents and chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of activated mPEG

2.2.1.1. Succinoylated mPEG (SA-mPEG) (Scheme 1a). mPEG (1100, 2000 or 5000 g/mol; 1 mmol) was dried by azeotropic distillation using toluene and then dissolved in anhydrous dimethylformamide (DMF, 10 ml). Succinic anhydride (4 mmol) was added, followed by DMAP (4 mmol). The mixture was stirred overnight at room temperature, the resulting polymer was then precipitated using ether, recrystallized twice from CH_2Cl_2/ether (1:40) and characterised by TLC (BuOH/AcOH/H_2O, 4:1:1) (Zalipsky et al., 1983), FTIR and ^1H NMR. The acid groups were quantified by titration against stan-



Scheme 1. Reaction scheme for synthesis of (a) trypsin-succinoylated mPEG conjugates; (b) trypsin-cyanurate mPEG conjugates; (c) trypsin-mPEG conjugates.

dardized NaOH solution using bromothymol blue as an indicator (Hreczuk-Hirst et al., 2001).

2.2.1.2. Cyanurate mPEG (CC-mPEG) (Scheme 1b). Cyanuric chloride (3 mmol) was dissolved in CH_2Cl_2 containing anhydrous sodium sulphate. mPEG (1100, 2000 or 5000 g/mol; 1 mmol) was added and the mixture was stirred overnight at room temperature. The solution was then filtered, and ether was added slowly with stirring. The finely divided precipitate was collected on a filter and re-dissolved in CH_2Cl_2. This precipitation and filtration process was repeated several times until the ether was free of residual cyanuric chloride as determined by TLC ($CHCl_3$/MeOH, 7:1) following the method of Schiavon et al. (2004). The product was characterised by FTIR and ^{13}C NMR.

2.2.1.3. Tosylate mPEG (TC-mPEG) (Scheme 1c). Tosyl chloride (3 mmol) was dissolved in CH_2Cl_2 containing anhydrous sodium sulphate. mPEG (1100, 2000 or 5000 g/mol; 1 mmol) and triethylamine (1 mmol) was added and the mixture was stirred overnight at room temperature. The solution was filtered, and ether was added slowly with stirring. The resulting white powder was collected on a filter and re-dissolved in CH_2Cl_2, washed several times until the ether was free of residual tosyl chloride as determined by TLC ($CHCl_3$/MeOH, 9:1). The product was characterised by FTIR and ^1H NMR.

2.2.2. PEGylation of trypsin

Trypsin was modified with the activated mPEGs synthesised above as follows.

2.2.2.1. Trypsin-succinoylated mPEG (Trypsin-SA-mPEG) (Scheme 1a). EDAC was added to the reaction mixtures containing trypsin dissolved in 10 ml of 50 mM potassium phosphate buffer, pH 6.0, and succinoylated mPEGs of molecular weight 1000, 2000 and 5000 g/mol using a molar ratio of trypsin: succinoylated mPEGs (1:20, 1:40 and 1:60). The solution was stirred for 1 h at room temperature and then for 16 h at 4 °C. It was then dialysed at 4 °C for 48 h against water using a dialysis membrane of molecular weight cut-off 12,400 and the product was finally lyophilized (Fernandez et al., 2003).

2.2.2.2. Trypsin-cyanurate mPEG (Trypsin-CC-mPEG) (Scheme 1b). Cyanurate mPEG was reacted with trypsin solubilised in 0.1 M sodium tetraborate buffer (pH 9.5, 2 mg/ml). As above a molar ratio of trypsin: cyanurate mPEGs of 1:20, 1:40 and 1:60 was used. The resulting mixture was stirred at room temperature for 4 h, and then brought to pH 3.0, and dialysed at 4 °C as above and the product was lyophilised (Abuchowski and Davis, 1979).

2.2.2.3. Trypsin-tosylated mPEG (Tryp-TC-mPEG or Trypsin-mPEG) (Scheme 1c). Tosylated mPEG was reacted with trypsin solubilised in 0.1 M sodium tetraborate buffer (pH 9.5, 2 mg/ml) using the same molar ratios as given above. The resulting mixture was stirred at room temperature for 4 h, the solution then brought to pH 3.0, and dialysed at 4 °C as above and the product was lyophilised (Gaertner and Puigserver, 1992).

2.2.3. Characterisation of the trypsin conjugates

In all cases, the Bradford protein assay was used to determine the total protein content of the conjugates. The extent of the protein modification was also estimated by determining the number of remaining free amino groups using the TNBS assay as described by Fields (1971). Briefly, sample (20 μl) was added to 0.1 M sodium tetraborate buffer in 0.1 M NaOH (0.5 ml) and the volume was made up to 1 ml. Then, TNBS solution (20 μl) was added and the solution was rapidly mixed. After 5 min the reaction was stopped by adding 0.1 M sodium phosphate buffer containing 1.5 M sodium sulfite (2.0 ml), and the absorbance at 420 nm was determined. A blank was prepared similarly without sample.

2.2.4. Determination of trypsin (native and conjugate) activity using BAPNA

Amidase activity of native and the PEGylated-trypsin conjugates was measured using BAPNA as a substrate (Murphy and O'Fagain, 1996). Briefly, 4.2 mM BAPNA (18.2 mg dissolved in 1 ml DMSO and added to 9 ml of 0.1 M Tris–HCl, pH 8.2 containing 20 mM $CaCl_2$) (400 μl) and 0.1 M Tris–HCl, pH 8.2 containing 20 mM $CaCl_2$ (580 μl) were added to a 1 ml cuvette. Then, 20 μl of free trypsin or PEG-trypsin conjugate (10 μg protein-equiv. in 0.1 M Tris–HCl, pH 8.2 containing 20 mM $CaCl_2$) was added and the solution was thoroughly mixed. The mixture was then incubated in the water bath at 30 °C for 10 min. The absorbance was read using a Shimadzu UV 1601 Spectrometer (Kyoto, Japan) at 410 nm, and one unit of activity defined as the amount of enzyme that hydrolyses 1 μmol BAPNA/min. The trypsin activity was calculated using a range of substrate concentrations (0.4–2.0 mM) and a molar extinction coefficient 8800 M^{-1} cm^{-1} for free *p*-nitroaniline (PNA). The reaction volume was always 1.0 ml. To calculate the kinetic parameters, a Lineweaver–Burk plot (Erlanger et al., 1961) was constructed as follows:

$$\text{Unit of activity (unit/mg)} = (\Delta \text{Abs}_{410\text{nm}} \times 1000 \times 1.0)/(8800 \times \text{mg protein})$$

2.2.5. Determination of trypsin (native and conjugate) activity using BAEE

Esterase activity of native and PEGylated-trypsin conjugates was determined at 25 °C using BAEE as substrate. Briefly, 20 μl of free trypsin or PEG-trypsin conjugate (10 μg protein-equiv.) was added to a cuvette containing 3 ml of 0.25 mM BAEE in 67 mM sodium phosphate buffer, pH 7.6, then immediately mixed thoroughly by inversion. The increase in absorbance at 253 nm was measured for approximately 10 min and the trypsin activity was calculated for a range of substrate concentrations 0.025–0.25 mM. One unit of esterase activity was defined as the amount of enzyme that hydrolyses 1.0 mmol of BAEE/min at

25 °C (Bergmeyer et al., 1974) as follows:

$$\text{Unit of activity (unit/mg)} = [(\Delta \text{Abs}_{253\text{nm}}\ \text{test} - \Delta \text{Abs}_{253\text{nm}}\ \text{blank}) \times 1000 \times 3.02]/\text{mg protein}$$

2.2.6. Evaluation of the thermal stability of PEG-trypsin conjugates

To test the thermal stability, native trypsin and the PEGylated conjugates were incubated in aqueous buffer (1.67 mM of 0.1 M Tris–HCl, pH 8.2 containing 20 mM $CaCl_2$ for amidase activity and 0.25 mM of 67 mM sodium phosphate buffer, pH 7.6 for esterase activity) for 10 min at temperatures between 30 and 70 °C. Then the residual trypsin for amidase or esterase activity was assayed using BAPNA or BAEE as described above. The activities of samples were compared to that at 30 °C (Zhang et al., 1999; Fernandez et al., 2002, 2003).

2.3. Evaluation of autolysis of PEG-trypsin conjugates

Native trypsin and the PEGylated-trypsin conjugates were incubated at 40 °C in aqueous buffer (0.1 M Tris–HCl, pH 8.2 containing 20 mM $CaCl_2$ for amidase activity and 67 mM sodium phosphate buffer, pH 7.6 for esterase activity) for 180 min. Aliquots of free trypsin or PEG-trypsin conjugate (10 μg protein-equiv.) (20 μl) were taken at different times and assayed for amidase or esterase activity using BAPNA or BAEE as described above.

3. Results and discussion

3.1. Synthesis and characterisation of the activated mPEG intermediates and PEG-trypsin conjugates

The succinoylation of mPEGs of different molecular weight was monitored by TLC. No free succinic anhydride was detected in the product, and the content of acid groups was 87–98% by titration. FTIR of SA-mPEG showed disappearance of the characteristic of –OH signals of mPEG at 3461 cm^{-1}, with the characteristic ester (1731 cm^{-1}) and carboxyl group (–COOH) of succinoyl moiety (1647 cm^{-1}). ^{1}H NMR confirmed the succinoylated mPEG characteristic peaks through the aliphatic protons of succinoyl moiety at 2.5–2.7 ppm (4H) and multiplet band of PEG protons at 3.5–4.2 ppm. The FTIR spectra of CC-mPEG showed disappearance characteristic of –OH moiety of mPEG at 3470 cm^{-1}, with a C=N characteristic peak at 1700 cm^{-1} and the skeleton vibration of the 1,3,5-triazine ring at 803 and 1500 cm^{-1}. ^{13}C NMR showed 1,3,5-triazine carbon ring at 172.1–174.2 ppm and carbon skeleton of PEG at 69.7–72.7 ppm. Similarly for the TC-mPEG the FTIR spectra showed disappearance characteristic of –OH moiety of mPEG at 3413 cm^{-1} with, in this case, the characteristic SO_2 (1700 cm^{-1}) and the aromatic (at 1398 and 1176 cm^{-1}) peaks. ^{1}H NMR confirmed aromatic protons at 7.4–7.8 ppm and multiplet band of PEG protons at 3.2–3.7 ppm (results not shown).

Table 1
Estimated number of amino groups substituted in trypsin

Conjugate	Molar ratios of trypsin and PEG	mPEG 1100[a]	mPEG 2000[a]	mPEG 5000[a]
Tryp-SA-mPEG	1:20	4.4 ± 0.7	4.6 ± 0.1	5.4 ± 0.1
Tryp-SA-mPEG	1:40	5.3 ± 1.2	3.2 ± 0.2	4.8 ± 0.1
Tryp-SA-mPEG	1:60	5.2 ± 0.8	2.6 ± 0.2	5.2 ± 0.1
Tryp-CC-mPEG	1:20	8.5 ± 0.4	6.9 ± 0.1	7.6 ± 0.2
Tryp-CC-mPEG	1:40	8.6 ± 0.1	5.2 ± 0.5	6.8 ± 0.1
Tryp-CC-mPEG	1:60	8.5 ± 0.3	4.0 ± 0.3	4.8 ± 0.8
Tryp-TC-mPEG	1:20	9.1 ± 0.1	9.7 ± 0.1	8.5 ± 0.4
Tryp-TC-mPEG	1:40	8.9 ± 0.1	9.5 ± 0.1	7.0 ± 0.2
Tryp-TC-mPEG	1:60	9.0 ± 0.1	8.9 ± 0.1	7.0 ± 0.8

It should be noted that native trypsin has ~11 amino groups in the TNBS assay.
[a] Data show mean ± S.D. ($n = 3$).

These activated mPEG intermediates were then used to prepare the library of trypsin conjugates. All the molecular weight PEGs produced comparable levels of amino groups substitution of trypsin (Table 1). When experiments were carried out to ascertain the optimum ratio between trypsin:activated mPEG (reactions at molar ratios of 1:20, 1:40 and 1:60), a reaction ratio of 1:20 gave an adequate degree of substitution; increased amounts of the activated mPEG concentration did not show a higher degree of substitution. The TNBS assay indicated that native trypsin had 11 accessible amino groups compared to the primary structure trypsin from bovine pancreas which contains 14 lysine amino groups (Walsh, 1970). After modification, it appeared that 4–9 mPEG molecules were covalently bound depending on the reaction conditions and specific mPEG used. For all PEG molecular weights, the degree of modification was achieved using TC-mPEG > CC-mPEG > SA-mPEG (Table 1). This is probably due to the fact that tosyl chloride is a better leaving group for the conjugation condition used.

3.2. Enzyme activity of trypsin and PEG-trypsin conjugates measured using BAPNA

The kinetic parameters obtained for both native trypsin and the PEGylated-trypsin conjugates (measured using BAPNA as substrate) are summarised in Table 2. All conjugates had lower activity than native trypsin. The conjugates synthesised using higher molecular weight PEGs displayed much lower K_M values indicating a higher affinity for the binding site of trypsin. Increasing molecular weight of mPEGs resulted in a decrease in the K_M and V_{max} values seen. These results were in contrast to the observation of Gaertner and Puigserver (1992) as they found that all conjugates had a higher amidase activity (in terms of percentage) than trypsin. In addition, the influence of the linkers on the enzyme efficiency of the conjugates as reported in Table 2 for K_{cat}/K_M values should be noted. Modification of trypsin with cyanurate mPEG increased this catalytic parameter more than seen for the other conjugates. The trypsin conjugate prepared using succinoylated mPEG displayed a higher rate of hydrolysis (mM/min) than other conjugates at 30 °C (Fig. 1). This was likely due to the fact that it contained fewer mPEG chains than the other conjugates resulting in lower steric hindrance of the trypsin active site (Veronese, 2001).

Table 2
Enzyme activity

Conjugate	Residual activity (%)	K_M (mM)	V_{max} (min)	K_{cat} (s^{-1})	K_{cat}/K_M (s^{-1} mM^{-1})
Trypsin	100	0.88 ± 0.09	0.177 ± 0.002	6.1 ± 0.8	6.9 ± 0.2
Tryp-SA-mPEG 1100	96.2 ± 2.0	1.15 ± 0.16	1.047 ± 0.389	6.6 ± 0.6	6.4 ± 0.6
Tryp-SA-mPEG 2000	76.8 ± 9.5	0.93 ± 0.09	0.457 ± 0.061	5.3 ± 0.8	5.1 ± 0.8
Tryp-SA-mPEG 5000	65.2 ± 8.3	0.79 ± 0.03	0.206 ± 0.026	4.0 ± 0.5	4.8 ± 0.9
Tryp-CC-mPEG 1100	17.5 ± 0.9	0.83 ± 0.09	0.104 ± 0.025	1.1 ± 0.2	1.4 ± 0.4
Tryp-CC-mPEG 2000	49.0 ± 9.8	0.45 ± 0.13	0.089 ± 0.017	2.7 ± 0.6	6.0 ± 1.0
Tryp-CC-mPEG 5000	92.9 ± 8.7	0.34 ± 0.07	0.040 ± 0.004	4.7 ± 0.5	14.4 ± 2.3
Tryp-TC mPEG 1100	19.9 ± 2.4	0.71 ± 0.11	0.160 ± 0.014	1.2 ± 0.1	1.7 ± 0.3
Tryp-TC mPEG 2000	68.5 ± 0.3	0.66 ± 0.02	0.063 ± 0.001	4.1 ± 0.1	6.1 ± 0.2
Tryp-TC mPEG 5000	67.6 ± 0.6	0.48 ± 0.06	0.036 ± 0.010	3.4 ± 0.5	7.1 ± 0.2

Kinetic parameters of native and PEG-trypsin conjugates. In all cases the conjugates were prepared using a molar ratio of 1:20 (mean ± S.D.; $n = 3$).

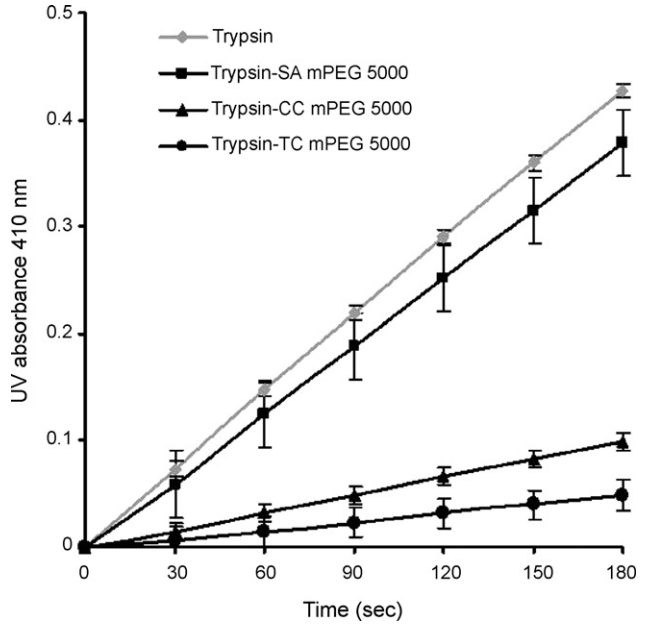


Fig. 1. Comparison of the activity of native and PEG-modified trypsin conjugates. The PEG used had a molecular weight of 5000 g/mol and in each case 10 μg/ml protein (trypsin) equivalent was used (mean ± S.D.; $n = 3$).

3.3. Thermal stability and autolysis of trypsin and PEG-trypsin conjugates

All mPEG-modified trypsins showed increased thermal stability compared to native trypsin (Figs. 2 and 3). This was true at all temperatures studied (except 70 °C) and when both BAPNA (Fig. 2) and BAEE (Fig. 3) were used as substrates. The highest activities were found when using the PEG with molecular weight of 5000 g/mol in all cases. This may be due to greater protection given by the higher molecular weight polymer chain; the hydrophilic PEG chain can swell and wrap around trypsin. Moreover, the PEG chain can form a highly hydrogen-bonded structure around the trypsin molecule (Gaertner and Puigserver, 1992; Zhang et al., 1999).

The time course for autolysis for native and modified trypsin at 40 °C was determined using both BAPNA and BAEE as substrates (Figs. 4 and 5 respectively). The half-life ($t_{1/2}$) of the PEG-trypsin conjugates was increased up to 4 fold compared to native trypsin (Table 3), and trypsin-modified with mPEG 5000 displayed the highest percentage retention of residual activity using both substrates and for all types of linker used. The values of residual activity measured using BAPNA as substrate were: native trypsin (13%), trypsin-SA-mPEG 5000 (37%), trypsin-CC-mPEG 5000 (50%) and trypsin-TC mPEG 5000 (39%) respectively. Stability was thus in the order CC > TC > SA, and moreover the same result was observed when using BAEE as substrate (residual activity 2, 8, 75 and 38% respectively).

All the mPEG-trypsin conjugates were less prone to autolysis compare to the native trypsin and they showed similar autolysis patterns. As the molecular weight of mPEG decreased the conjugates were less stable. This increased stability might be explained by specific modification of lysine residues (remembering the substrate specificity of trypsin) as

Table 3
Half-life of native and PEG-trypsin conjugates

Conjugate	Substrate[a]							
	BAPNA				BAEE			
	Trypsin	mPEG 1100	mPEG 2000	mPEG 5000	Trypsin	mPEG 1100	mPEG 2000	mPEG 5000
Trypsin	41 ± 1	–	–	–	8 ± 1	–	–	–
Tryp-SA-mPEG	–	45 ± 1	56 ± 1	102 ± 1	–	8 ± 1	9 ± 1	13 ± 1
Tryp-CC-mPEG	–	73 ± 1	119 ± 2	180 ± 2	–	15 ± 1	20 ± 1	>180
Tryp-TC-mPEG	–	95 ± 2	127 ± 3	157 ± 3	–	12 ± 1	15 ± 1	60 ± 1

Experiments were conducted at 40 °C and the hydrolysis of BAPNA and BAEE was used to measure residual trypsin activity.
[a] Data represent $t_{1/2}$ min (mean ± S.D.) ($n = 3$).

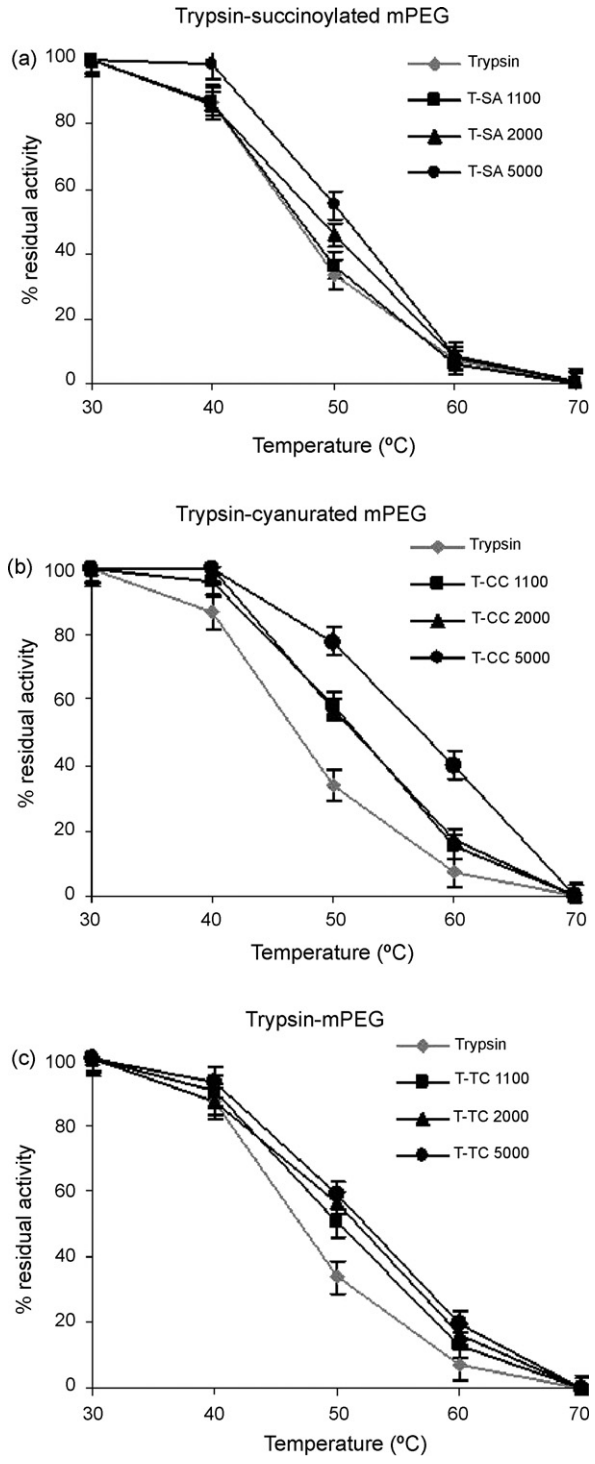


Fig. 2. Effect of temperature on the activity of native and PEG-modified trypsin conjugates measured using BAPNA as a substrate (mean ± S.D.; $n = 3$).

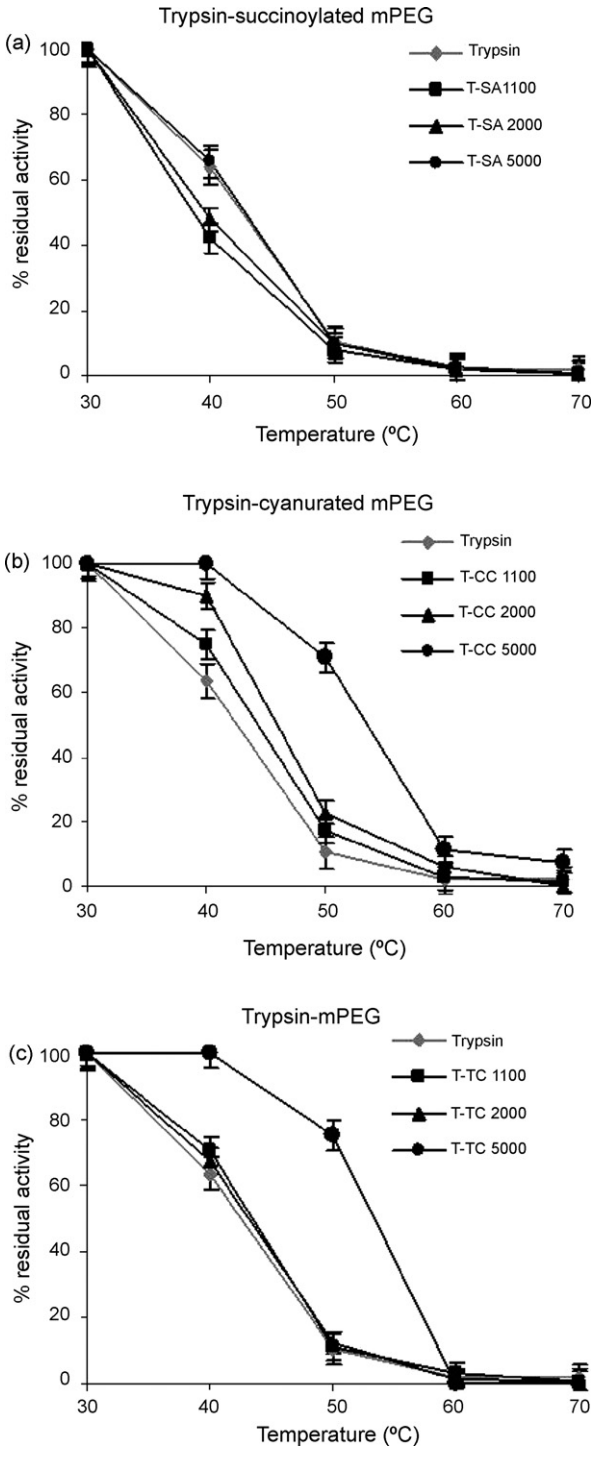


Fig. 3. Effect of temperature on the activity of native and PEG-modified trypsin conjugates measured using BAEE as a substrate (mean ± S.D.; $n = 3$).

this would inherently be expected to protect against autolysis, and/or the fact that the mPEG chains attached on the enzyme surface would anyhow cause non-specific steric hindrance (Murphy and O'Fagain, 1996). However, the observation that the higher molecular weight PEGs produced greater trypsin stability suggest a major role for the latter mechanism. The increasing of thermal stability of modified trypsins could also result from the formation of a highly hydrogen-bonded structure, with polymer wrapped around the protein (Gaertner and Puigserver, 1992; Zhang et al., 1999).

In the context of therapeutic protein formulation development, the active ingredient must be stable during formulation manufacture and for the shelf-life of the product. As thermal stability can be a good predictor of activity it is important to note that trypsin-modified with mPEG 5000 showed better stability

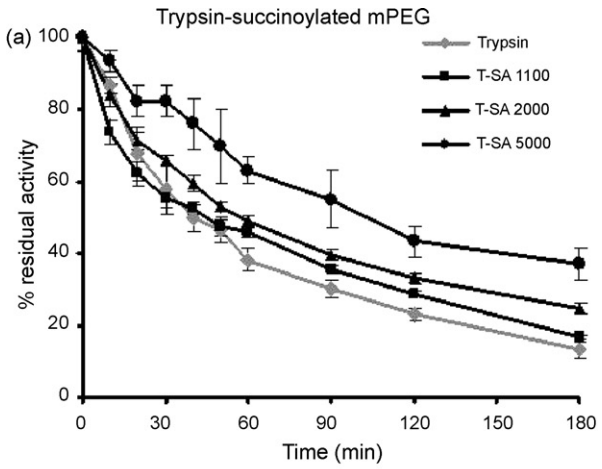


Fig. 4. Autolysis of native and PEG-modified trypsin conjugates measured using BAPNA as a substrate (mean ± S.D.; $n = 3$).

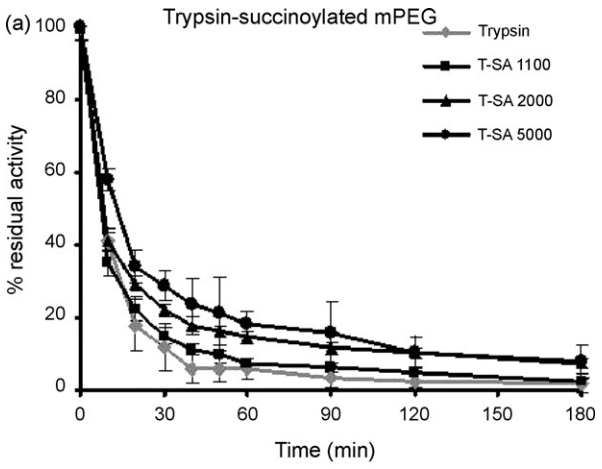


Fig. 5. Autolysis of native and PEG-modified trypsin conjugates measured using BAEE as a substrate (mean ± S.D.; $n = 3$).

compared to the native trypsin when tested at high temperature (Fig. 2). This study therefore underlines that, not only is the choice of PEG molecular weight and linking chemistry important in terms of retained biological activity of the protein, it may have an important impact on issues relating to formulation development.

4. Conclusions

In this study, trypsin was successfully conjugated with mPEG of molecular weight 1100, 2000 and 5000 g/mol. Although chemical modification of mPEG to trypsin could decrease activity of trypsin, but the modified trypsin conjugates containing the higher molecular mass mPEG showed higher affinity to bind-

ing site of enzyme with substrate, higher thermal stability, more stable against autolysis and had an increased $t_{1/2}$ compared to the native enzyme. The trypsin-conjugated mPEG 5000 having cyanurate linker demonstrated the best thermal stability.

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References

Abuchowski, A., Davis, F.F., 1979. Preparation and properties of polyethylene glycol-trypsin adducts. Biochim. Biophys. Acta 578, 41–46.

Bergmeyer, H.U., Gowehn, K., Grassi, M., 1974. Fundamental. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, vol. I. Verlag Chemie Weinheim. Academic Press, New York, pp. 517–576.

Brocchini, S., Balan, S., Godwin, A., Choi, J.W., Zloh, M., Shaunak, S., 2006. PEGylation of native disulfide bonds in proteins. Nat. Protoc. 1, 2241–2252.

Duncan, R., 2003. The dawning era of polymer therapeutics. Nat. Rev. Drug Discov. 2, 347–360.

Erlanger, B.F., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278.

Fernandez, M., Fragoso, A., Cao, R., Banos, M., Villalonga, R., 2002. Chemical conjugation of trypsin with monoamine derivatives of cyclodextrins Catalytic and stability properties. Enz. Microb. Tech. 31, 543–548.

Fernandez, M., Fragaso, A., Cao, R., Villalonga, R., 2003. Improved functional properties of trypsin modified by monosubstituted amino-b-cyclodextrins. J. Mol. Catal. B: Enzymatic 21, 133–141.

Fields, R., 1971. The measurement of amino groups in proteins and peptides. Biochem. J. 124, 581–690.

Gaertner, H.F., Puigserver, A.J., 1992. Increased activity and stability of poly(ethylene glycol)-modified trypsin. Enz. Microb. Tech. 14, 150–153.

Greenwald, R.B., Choe, Y.H., McGuire, J., Conover, C.D., 2003. Effective drug delivery by PEGylated drug conjugates. Adv. Drug Deliv. Rev. 55, 217–250.

Harris, J.M., Chess, R.B., 2003. Effect of pegylation on pharmaceuticals. Nat. Rev. Drug Discov. 2, 214–221.

Hreczuk-Hirst, D.H., German, L., Duncan, R., 2001. Dextrins as carrier for drug targeting: reproducible succinoylation as means to introduce pendant groups. J. Bioact. Compat. Polym. 16, 353–365.

Murphy, A., O'Fagain, C., 1996. Stability characteristics of chemically-modified soluble trypsin. J. Biotechnol. 49, 163–171.

Pasut, G., Veronese, F.M., 2007. Polymer-drug conjugation, recent achievements and general strategies. Prog. Polym. Sci. 32, 933–961.

Schiavon, O., Pasut, G., Moro, S., Orsolini, P., Guiotto, A., Veronese, F.M., 2004. PEG-Ara-C conjugates for controlled release. Euro. J. Med. Chem. 39, 123–133.

Veronese, F.M., 2001. Peptide and protein PEGylation: a review of problems and solution. Biomaterials 22, 405–417.

Walsh, K.A., 1970. Trypsinogens and trypsins of various species. In: Fleisher, S., Packer, L. (Eds.), Methods in Enzymology, vol. 19. Academic Press, New York, pp. 41–63.

Zalipsky, S., Gilon, C., Zilkha, A., 1983. Attachment of drugs to polyethylene glycols. Euro. Polym. J. 19, 1177–1183.

Zhang, Z., He, Z., Guan, G., 1999. Thermal stability and thermodynamic analysis of native and methoxypolyethylene glycol modified trypsin. Biotech. Tech. 13, 781–786.