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Dextrin–trypsin and ST-HPMA–trypsin conjugates: Enzyme activity, autolysis and thermal stability

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ABSTRACT

Using monomethoxy poly(ethylene glycol) (mPEG)–trypsin conjugates we recently showed that both PEG molecular weight (1100–5000 g/mol) and linker chemistry affect the rate of protein autolysis and thermal stability. These important factors are often overlooked but they can guide the early choice of optimal polymer/chemistry for synthesis of a lead polymer therapeutic suitable for later formulation development. As we are currently developing dextrin- and semi-telechelic poly[*N*-(2-hydroxypropyl)methacrylamide] (ST-HPMA)–protein conjugates as new therapeutics, the aim of this study was to examine the effect of polymer on activity, autolysis and its thermal stability using trypsin conjugates as a model and compare to the data obtained for mPEG conjugates. Trypsin conjugates were first synthesized using succinoylated dextrin (Mw ~ 8000 g/mol, dextrin I; or ~61,000 g/mol, dextrin II), and a ST-HPMA–COOH (Mw ~ 10,100 g/mol). The conjugates had a trypsin content of ~54, 17 and 3 wt% respectively with <5% free protein. When amidase activity (K_M, V_{max} and K_{cat}) was determined by using *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA) as substrate, trypsin K_M values were not altered by conjugation, but the V_{max} was ~6–7-fold lower, and the substrate turnover rate (K_{cat}) decreased by ~5–7-fold. The dextrin II–trypsin conjugate was more stable than the other conjugates and native trypsin at all temperatures between 30 and 70 °C, and also exhibited improved thermal stability in the autolysis assays at 40 °C.

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1. Introduction

Many peptide and protein drugs have benefited clinically from polymer conjugation; particularly using poly(ethylene glycol) (PEG). PEGylation can improve poor stability and low solubility, prolong circulation half-life, and reduce immunogenicity (reviewed in Harris and Chess, 2003; Pasut and Veronese, 2007; Veronese and Harris, 2008). Recently, a PEGylated anti-TNF antibody fragment, Cimizia® (Sandborn et al., 2005), came to market as a new treatment for Crohn's disease. It is also being developed as a treatment for arthritis. Many other polymers have been explored as candidates for protein conjugation (reviewed in Veronese and Morpurgo, 1999; Duncan, 2003; Pasut and Veronese, 2007), but so far PEG conjugates have found the widest clinical use. Our recent studies (Treetharnmathurot et al., 2008) used a series of PEG–trypsin conjugates that were synthesized using PEGs of different molecular weight (Mw 1100, 2000 and 5000 g/mol), and with different linking chemistries (succinic anhydride (SA), cyanuric chloride (CC) or tosyl chloride (TC)) to study the effect of chemical composition on bioactivity of the conjugated enzyme, its autolysis and its thermal stability. It was found that both PEG molecular weight and the linking chemistry used had subtle, but important, effects on these parameters.

As dextrin- (Duncan et al., 2008; Hardwicke et al., 2008; Ferguson and Duncan, 2006) and semi-telechelic poly[*N*-(2-hydroxypropyl)methacrylamide] (ST-HPMA)–protein conjugates (Ulbrich et al., 2000) are being developed as new therapeutics, the aim of this study was to examine the effect of polymer, and for dextrin molecular weight, on protein activity, autolysis and thermal stability. Trypsin conjugates were synthesized as model compounds (Schemes 1 and 2) using succinoylated dextrins of Mw = 8100 g/mol (dextrin I)) and Mw = 61,000 g/mol (dextrin II) or ST-HPMA–COOH of Mw = 10,100 g/mol. We have recently shown that biodegradable polymers, such as dextrin and hyaluronic acid, can be used to generate novel, bioresponsive protein conjugates for use in the context of a new concept called Polymer-masking-UnMasking-Protein Therapy (PUMPT) (Duncan et al., 2008; Gilbert and Duncan, 2006). In this approach, a biodegradable polymer

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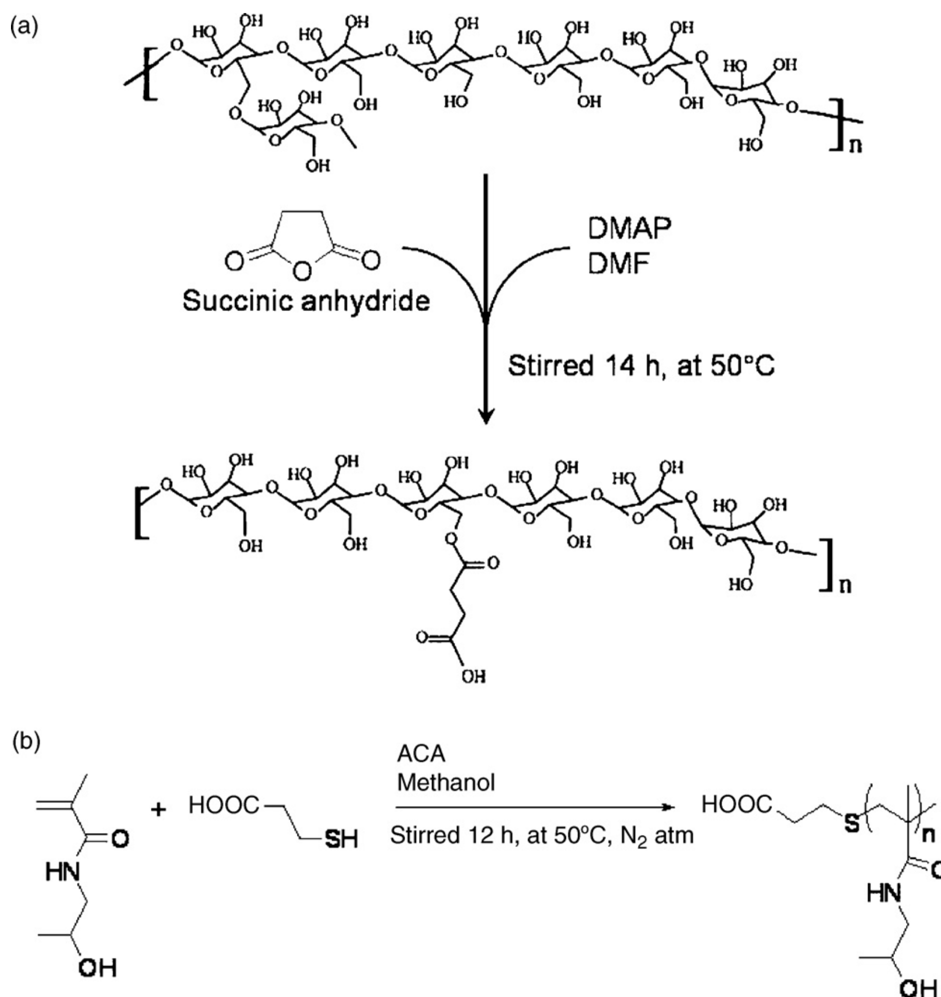


Scheme 1. Reaction scheme for synthesis of (a) succinoylated dextrin and (b) ST-HPMA–COOH.

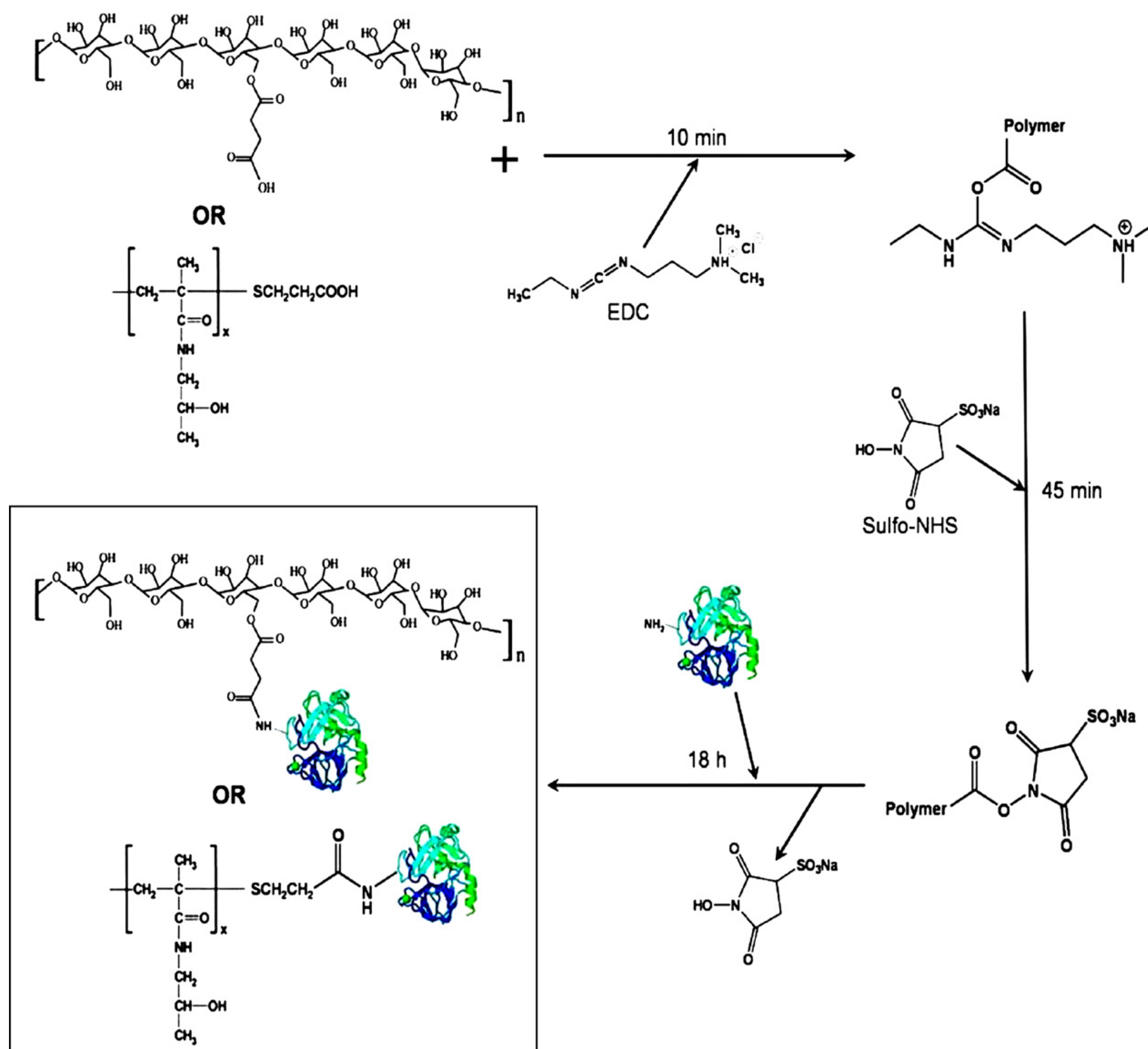
is used to transiently mask a protein thus stabilising/inactivating it during transit, and subsequently allowing triggered polymer degradation to 'unmask' the protein and thus restore its activity in a controlled manner. Dextrin, α-1,4 poly(glucose) with minimal branching in the α-1,6-position (<5%) is a useful tool as it is non-toxic and non-immunogenic, and has been FDA approval as the peritoneal dialysis solution (Icodextrin^{TM}) (Mistry and Gokal, 1994). It is also used as a controlled drug delivery formulation for 5-fluorouracil (Kerr et al., 1996). In the context of PUMPT, succinoylated dextrin has been used to prepare bioresponsive (α-amylase triggered degradation) conjugates of recombinant human epidermal growth factor (rhEGF), a polymer therapeutic designed to promote wound healing (Hardwicke et al., 2008), and phospholipase A_2 (PLA_2), an anticancer conjugate (Ferguson and Duncan, 2006). Both show pharmacologically important, bioactivity that is regenerated with time in the presence of α-amylase. Although a significant number of HPMA copolymer-anticancer drug conjugates progressed into clinical trial (reviewed in Duncan, 2006), these non-biodegradable polymers have also been used to create protein conjugates using either a multifunctional HPMA copolymer intermediate, or single point attachment using ST-HPMA (reviewed in Duncan, 2005; Oupicky et al., 1999; Ulbrich et al., 2000).

First, the polymer–trypsin conjugates were synthesized and characterized in respect of relative molecular weight (GPC), total (protein assay) and free protein content (SDS-PAGE electrophoresis and FPLC). Their retained enzyme activity (K_M, V_{max} and K_{cat}) was determined using *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA) as a substrate, and their thermal stability (30–70 °C) and autolysis rate at 40 °C compared to that of native trypsin and the previously studied mPEG–trypsin conjugates (Treetharnmathurot et al., 2008).

2. Materials and methods

2.1. Materials

Dextrin I from corn starch, trypsin (Type IX-S, from porcine pancreases, lyophilized powder), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS), bromothymol blue, deuterated dimethylsulfoxide (DMSO-d_6) and Nα-benzoyl-L-arginine *p*-nitroanilide hydrochloride were from Sigma (UK). Dextrin II was from the Centre for Polymer Therapeutics, Welsh School of Pharmacy, Cardiff University. *N*,*N*′-Dimethylformamide (DMF) and succinic anhydride were from Aldrich (UK), and *N*-hydroxysulfosuccinimide sodium salt and 4,4′-azobis(4-cyanopentanoic acid) (ACA) were from Fluka (UK). 4-Dimethylaminopyridine (DMAP) and sodium azide (NaN_3) were from Lancaster (UK), and 3-mercaptopropionic acid (MPA) was from Acros Organics (UK). All other chemical were of analytical grade. Conjugates were purified by dialysis using Spectra/Por Membranes (10,000 g/mol cut-off or 2000 g/mol cut-off) from BDH Merck (UK).



Scheme 2. Reaction scheme for synthesis of polymer–trypsin conjugates.

2.2. Analytical methods

FPLC. The polymer–trypsin conjugates were purified and characterized by FPLC run at room temperature (Amersham Pharmacia Biotech AKTA) using a Superdex™ 75-HR 10/30 300 GL column with phosphate buffer (pH 7.4; flow rate of 0.5 mL/min) as mobile phase, and a UV (280 nm) detector.

GPC. Gel permeation chromatography of both polymers and conjugates was undertaken using G3000PW_{XL} and G4000PW_{XL} columns in series and a TSK Progel PW_{XL} guard column (Viscotek, UK) with phosphate buffer (pH 7.4; flow rate 1.0 mL/min) as mobile phase, UV (280 nm) and differential 133 refractive index detectors (Gilson Inc., USA). After filtration through a single-use syringe filter (0.2 μm), 40 μL of the polymer solution (<10 mg/mL in the mobile phase) was injected onto the column, before elution for 30 min at 25 °C. Polysaccharide (pullulan) standards were used for calibration (Polymer Laboratories, UK) and all data were analyzed using the Caliber software (Polymer Laboratories, UK).

SDS-PAGE electrophoresis was performed using a denaturing gel consisting of a 4% stacking gel, tris (0.125 M, pH 6.8) and 12.5% separating gel Tris (0.375 M, pH 6.8) run at a constant voltage of 200 V for 1 h.

Circular dichroism (*CD*) *spectroscopy*: CD spectra were obtained using a Spectrometer Model 215 spectrometer (AVIV® Instrument Inc.) with quart cells of 1 mm path length (at 4 °C) and using samples dissolved in phosphate buffer (50 mM, pH 7.4). Spectra of dextrin II–trypsin conjugate (1 mg/ml trypsin-equiv.) and also dextrin II, succinoylated dextrin II, trypsin were obtained, the latter were used at concentrations equivalent to that present in the conjugate. Phosphate buffer was used as a blank and the results were expressed as mean molar residue ellipticity $[\theta]_{MR}$ (deg cm^2/dmol).

NMR: ^1H NMR spectra were acquired at 500 MHz using an UltraShield™ 500 spectrometer Bruker (UK), and analyzed by using TopSpin® 2.0 software. At least 40 mg of the polymer was placed in the NMR tube and dissolved in the appropriate deuterated solvent (0.6 mL).

IR: Dried and bulk samples (<5 mg) were scanned (128 scans) with wave numbers from 750 to 4000 cm^{-1} on a Nicolet Avatar E.S.P. 360 FT-IR Spectrometer from Thermo Scientific (UK). Data were plotted as transmittance (%) in function of the wave num-

ber (cm^{-1}) and analyzed using EZ OMNIC E.S.P 5.2 software from Thermo Scientific (UK).

2.3. Methods

2.3.1. Synthesis and characterization of ST-HPMA–COOH

ST-HPMA–COOH was synthesized as described by Wang et al. (2000) using methanol at 50 °C under a N_2 atm, ACA as the initiator ([I]/[M] = 4.30%) and MPA as the chain transfer agent (CTA) (Scheme 1). Briefly, HPMA (2.00 g, 14.00 mmol), ACA (168.20 mg, 0.60 mmol, [I]/[M] = 4.30%), MPA (21.60 μL, 2.48×10^{-1} mmol, [CTA]/[M] = 1.77%) were dissolved in methanol (20 mL) at room temperature in a round-bottom flask containing a magnetic stirrer. The mixture was then degassed for 0.5 h with N_2 before the flask was sealed and placed in an oil bath at 50 °C for 12 h. The reaction was stopped by cooling the mixture to room temperature and the homopolymer was then isolated and purified by precipitation from methanol into cold acetone (3×), and dried under vacuum at 40 °C. ST-HPMA–COOH was isolated with a 70% gravimetric yield, as a white solid and it was characterised by IR, ^{1}H NMR, ^{13}C NMR and GPC.

IR (in bulk, cm^{-1}): $\overline{\nu} = 3352$; 2972; 2900; 1660; 1530; 1388; 1330–1200; 1210–800.

^{1}H NMR (DMSO-d_6, ppm): δ = 0.8 (bs, 3H, C**H$_3$** polymer backbone); 0.9 (ss, 3H, C**H$_3$** pendant group); 1.7 (bs, 2H, C**H$_2$** polymer backbone); 2.9 (bs, 2H, C**H$_2$** pendant group); 3.7 (bs, 1H, C**H** pendant group); 4.7 (bs, 1H, O**H** pendant group); 7.2 (bs, 1H, N**H** pendant group).

^{13}C NMR (DMSO-d_6, ppm): δ = 15 (**C**H_3 polymer backbone); 17 (**C**H_2 polymer backbone); 22 (**C**H_3 pendant group); 45 (**C** polymer backbone); 48 (**C**H_2 pendant group); 65 (**C**H pendant group); 177 (**C**=O pendant group).

GPC (in PBS, flow rate 1 mL/min, columns calibrated with narrow pullulan standards): Mw = 10,100 g/mol, Mn = 7000 g/mol and Mw/Mn = 1.50.

2.3.2. Succinoylation of dextrin I (Scheme 1)

Following the method of Hreczuk-Hirst et al. (2001), dextrin I (1 g) was dissolved in DMF (10 mL) and succinic anhydride (91.3 mg) added followed by DMAP (40 mg). The reaction mixture was purged with N_2, sealed and left to stir for 14 h at 50 °C, under N_2. It was then poured into vigorously stirred diethyl ether (1 L) and stirred for 10 h. The ether was removed by filtration under vacuum and the remaining solid was dissolved in a minimum amount of distilled water, poured into a dialysis membrane (molecular weight cut-off 2000 g/mol) and dialyzed against distilled water for 48 h. The resulting solution was freeze-dried to yield succinoylated dextrin I. The acid groups were quantified by titration (using 1% bromophenol blue as indicator and NaOH as base), and the product was characterized by FT-IR, ^{1}H NMR and GPC. Succinoylated dextrin II was prepared in a similar way but using dextrin II (1 g) in DMF (10 mL) and succinic anhydride (182.5 mg) with DMAP (80 mg).

2.3.3. Synthesis of ST-HPMA–COOH– and dextrin–trypsin conjugates (Scheme 2)

2.3.3.1. Synthesis of dextrin I–trypsin conjugates. The succinoylated dextrin I (101.5 mg) was dissolved in distilled water (2 mL). EDC (15.9 mg) was added and the reaction mixture was stirred for 10 min. Then sulfo-NHS (16.8 mg) was added and the reaction mixture was stirred for a further 45 min. Trypsin (49.2 mg) was added to the mixture and the pH adjusted to pH ~8 by drop wise addition of NaOH, and the reaction mixture left to stir for 18 h at room temperature. The conjugate was purified by FPLC, and the collected fractions desalted using a dialysis membrane of molecular weight cut-off 2000 g/mol before freeze-drying. The conjugate was characterized by SDS-PAGE electrophoresis, GPC, and FPLC. The Bradford protein assay (Bradford, 1976) was used to estimate the total protein content with BSA standards.

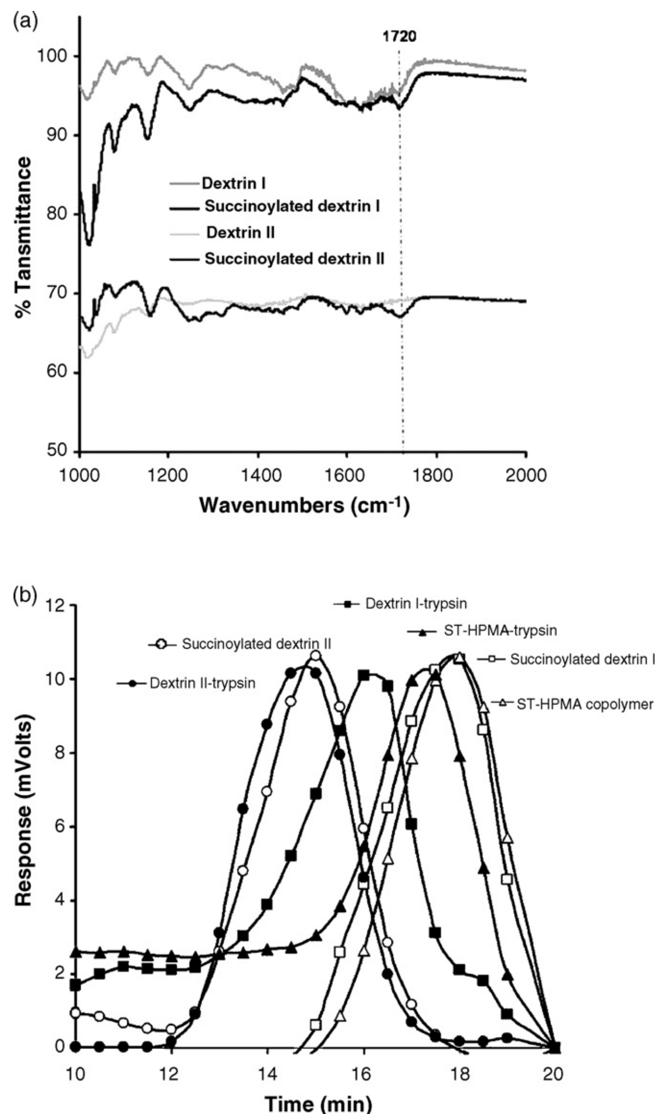


Fig. 1. Characteristics of succinoylated dextrin, and the trypsin conjugates. Panel (a) FT-IR of dextrin and succinoylated dextrin and panel (b) GPC of the polymeric intermediates and trypsin conjugates.

2.3.3.2. Synthesis of dextrin II–trypsin conjugates. In this case succinoylated dextrin II (154.8 mg) in distilled water (2 mL) was used, and the reaction conducted as described above but using EDC (21.7 mg), sulfo-NHS (22.4 mg) and trypsin (41.2 mg). All the other reaction conditions, purification and characterization were as described above.

2.3.3.3. Synthesis of ST-HPMA–trypsin conjugates. ST-HPMA–COOH (203.9 mg) was dissolved in distilled water (2 mL). EDC (21.6 mg) was added and the reaction mixture was stirred for 10 min then sulfo-NHS (24.8 mg) was added and the mixture stirred for a further 45 min. Trypsin (42.2 mg) was then added and further reaction conditions, purification and characterization were conducted as described above.

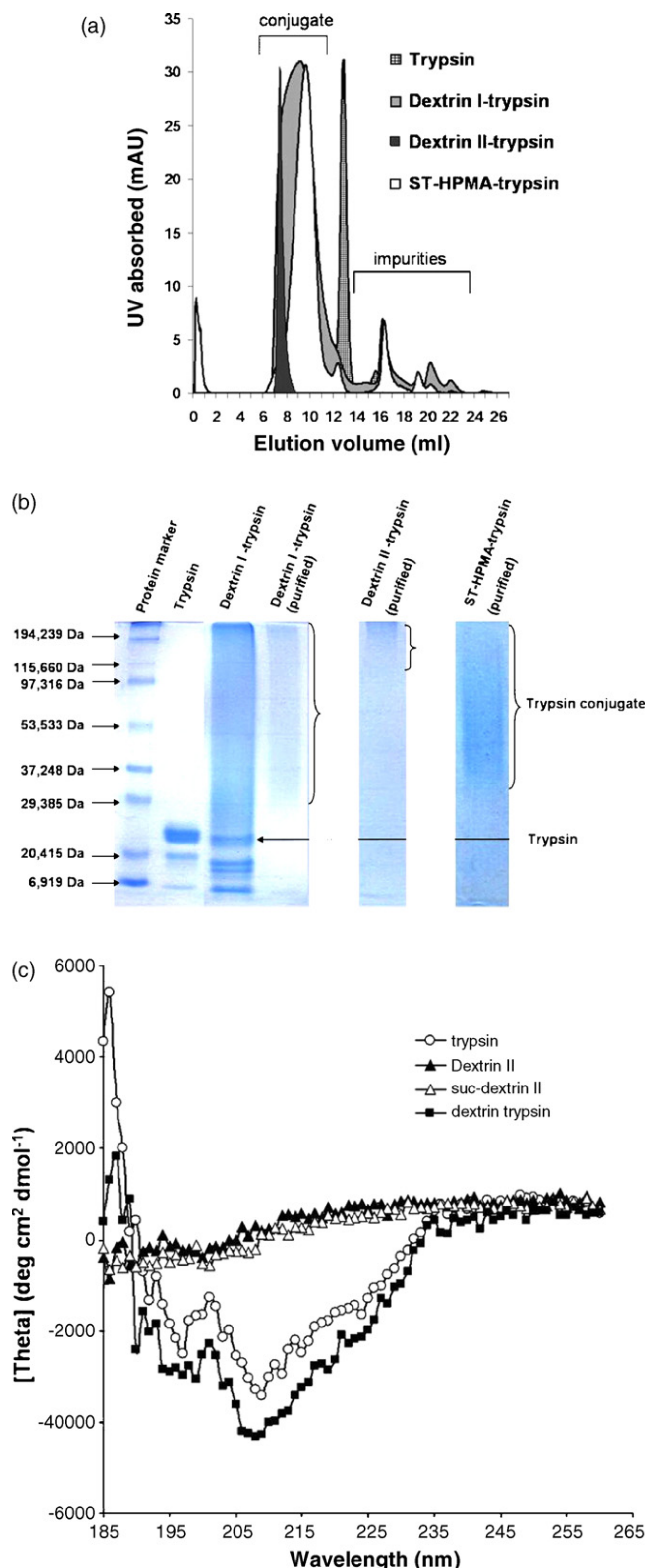


Fig. 2. Characterization of polymer–trypsin conjugates. Panel (a) FPLC of trypsin and conjugates, panel (b) SDS-PAGE of trypsin and conjugates, and panel (c) CD spectroscopy of dextrin, trypsin, succinoylated dextrin II and the dextrin II–trypsin conjugate.

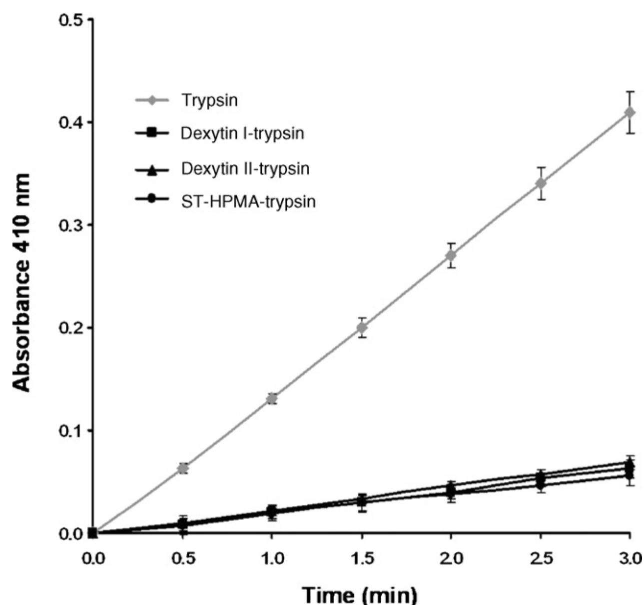


Fig. 3. Comparison of the enzymatic activity of native trypsin and the polymer–trypsin conjugates. In each case 2 mg/ml protein (trypsin) equivalent was used. The results represent mean ± S.D. ($n = 3$).

2.4. Determination of trypsin amidase activity

The activity of free and conjugated trypsin (8.3×10^{-5} mM) in Tris buffer (0.1 M containing 20 mM $CaCl_2$; pH 8.2) was measured with the substrate, BAPNA, using a method modified from Duncan et al. (2008). Briefly, BAPNA solution (stock solution 2 mg/mL in DMSO; 5–50 μL) was added to a quartz cuvette (1 mL) and made up to 980 μL with Tris buffer and equilibrated at 37 °C. To start the assay, free trypsin (stock solution 0.1 μg/μl in Tris buffer; 20 μL) or polymer–trypsin conjugates (20 μL; 2 μg/mL protein-equiv. in Tris buffer) was added to the cuvette and the solution was thoroughly mixed and measured the release of *p*-nitroaniline (PNA) at 410 nm (at 37 °C for 5 min) using a Cary1G UV-Vis spectrophotometer (Varian, Australia) (PNA $\varepsilon_{410} = 8800\,M^{-1}\,cm^{-1}$). The kinetic parameters K_M, V_{max} and K_{cat} were derived from the raw data of trypsin activity against a range of substrate concentrations (0.023–0.23 mM) using a Hanes-Wolfe plot (Cornish-Bowden, 2004).

2.5. Measurement of the thermal stability and autolysis of the trypsin conjugates

Thermal stability was assessed as follows: native trypsin and the polymer–trypsin conjugates (8.3×10^{-5} mM) were incubated in 0.1 M Tris–HCl (pH 8.2) containing 20 mM $CaCl_2$ for 10 min at temperatures between 30 and 70 °C (Zhang et al., 1999). The residual enzyme activity was then assayed using BAPNA (0.23 mM) for each sample as described above.

To determine autolysis, native trypsin and polymer–trypsin conjugates were incubated at 40 °C in 0.1 M Tris–HCl, pH 8.2 containing 20 mM $CaCl_2$ for 180 min. Aliquots (20 μL) of free trypsin or polymer–trypsin conjugates (2 μg/mL protein-equiv.) were removed at different times and assayed for retained amidase activity using BAPNA as described above.

3. Results

3.1. Synthesis and characterization of the trypsin conjugates

First the reactive polymeric intermediates were prepared. The ST-HPMA with a carboxylic acid end-group was obtained using

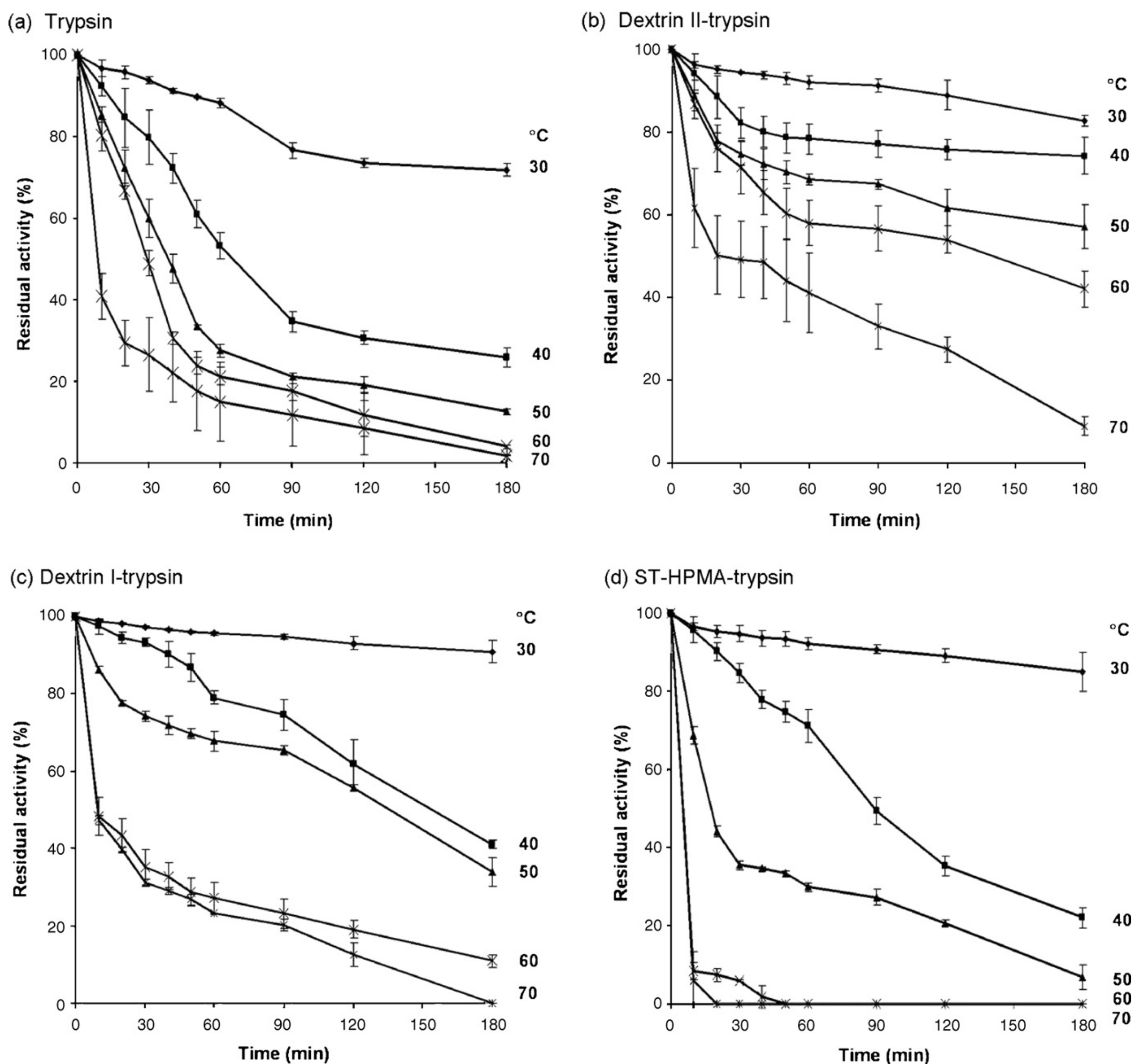


Fig. 4. Thermal stability the trypsin conjugates. The residual enzyme activity was measured with time during incubation (over 180 min) at temperatures between 30 and 70 °C. Panel (a) trypsin, (b) dextrin I–trypsin, (c) dextrin II–trypsin, and (d) ST-HPMA–trypsin. The results represent mean ± S.D. (n = 3).

MPA as the CTA (Dieudonne, 2008). The reaction yield was ~70%. FT-IR, ^{1}H NMR and ^{13}C NMR, and GPC confirmed absence of impurities, and GPC gave molecular weight characteristics of Mw = 10,100 g/mol, Mn = 7000 g/mol a polydispersity of 1.50. In the case of dextrin, FT-IR confirmed the presence of the characteristic ester peak at 1720 cm^{-1} in both succinoylated dextrin I and II intermediates (Fig. 1a). Titration revealed a succinoylation degree of 8.8 and 16.7 mol% respectively (Table 1). On GPC, the succinoylated dextrin eluted faster than dextrin (Fig. 1b). This is due to the increased molecular radius of the extended COOH-containing polymer chain compared to parent dextrin rather than a significantly increased polymer molecular weight after succinoylation.

During synthesis of the trypsin conjugates, a 1:1 molar ratio of polymer:protein was used. FPLC proved a useful tool to separate conjugate from free trypsin (Fig. 2a) and the resultant dextrin I–, dextrin II– and ST-HPMA–trypsin conjugates contained 54, 17

Table 1
Characteristics of the polymer conjugates.

Compound	Protein content (wt%)	Mw[a]	Mn[a]	Polydispersity (Mw/Mn)
Trypsin[b]	–	23,475[b]	–	–
Dextrin I	–	8,100	5,200	1.6
Succinoylated dextrin I	–	11,200	7,000	1.6
Dextrin I–trypsin	54	40,400	22,000	1.9
Dextrin II	–	61,000	36,000	1.6
Succinoylated dextrin II	–	73,800	40,000	1.9
Dextrin II–trypsin	17	87,400	51,000	1.7
ST-HPMA copolymer	–	10,100	7,000	1.5
ST-HPMA–trypsin	3.0	17,500	14,200	1.2

[a] Estimated using GPC and pullulan standards.
[b] Trypsin from porcine pancreas (223 amino acids) (http://www.expasy.ch/tools/protparam.html).

and only 3 wt% total protein respectively (Table 1). Whereas the dextrin II–trypsin conjugate contained no detectable free trypsin (on SDS-PAGE), the dextrin I– and ST-HPMA–trypsin conjugates contained 3.7% and 2.3% free trypsin respectively (Fig. 2b). The dextrin conjugates had a higher polydispersity of (Mw/Mn = 1.6–1.9) (Table 1) than the ST-HPMA–trypsin conjugate. The latter displayed a relatively low molecular weight (perhaps due to the low trypsin loading) and very low polydispersity (Mw/Mn = 1.2). In fact, the polydispersity of this conjugate was lower than seen for the parent polymer (Mw/Mn = 1.5) suggesting either preferential conjugation of lower molecular weight polymer chains or an unusual conformation of the conjugate (polymer wrapped around the protein). CD spectroscopy of dextrin II and succinoylated dextrin II showed a completely unstructured conformation, whereas the spectra obtained for trypsin and dextrin II–trypsin conjugates showed a negative minimum at 208 nm. The spectrum of the dextrin II–trypsin conjugate was very similar to that seen for trypsin (Fig. 2c), but it had a slightly decreased amplitude of the 208 nm. These results indicated that the dextrin conjugation did not disturb the trypsin structure significantly.

3.2. Enzyme activity, thermal stability and autolysis

All the conjugates displayed similar enzyme activity (13.8–18.6%) relative to the trypsin control (Fig. 3 and Table 2). Also their K_M values were similar to native trypsin. However the conjugates displayed V_{max} values that were ~6–7-fold lower, and the substrate turnover rate K_{cat} was decreased to ~5–7-fold (Table 2). The activity of both free trypsin and the trypsin conjugate progressively decreased with increasing incubation time at all temperatures (Fig. 4). The conjugates were inactivated when incubated at 70 °C for 180 min, with the exception of the dextrin II–trypsin which retained a small amount of activity at this temperature (Fig. 4b). Indeed the dextrin II–trypsin conjugate was more stable than all the other conjugates and native trypsin at all temperatures between 30 and 70 °C during an 180 min incubation. At 30 °C the dextrin I– and the ST-HPMA–trypsin conjugates were also more stable than native trypsin, and the dextrin I conjugate was also more stable at 40 and 50 °C (Fig. 4a, c and d). However, ST-HPMA–trypsin was much less stable than trypsin at 60 and 70 °C. Thermal stability was also compared for all temperatures following the shortest, 10 min, incubation period (Fig. 5a). This allowed data expression as T_{50} values (temperature at which 50% residual activity remains), and it can be seen that the values obtained were >70, 68, 60 and 53.5 °C for dextrin II–trypsin, trypsin, dextrin I–trypsin, and ST-HPMA–trypsin respectively.

All the polymer–trypsin conjugates synthesized here were less susceptible to autolysis at 40 °C than native trypsin (Fig. 5b), although only dextrin II–trypsin was more stable than the PEG–trypsin conjugates studied previously. The half-life ($t_{1/2}$) calculated from the time course for autolysis shown in Table 3 indicate an order of stability: dextrin II conjugate > dextrin I conjugate > ST-HPMA conjugate > native trypsin.

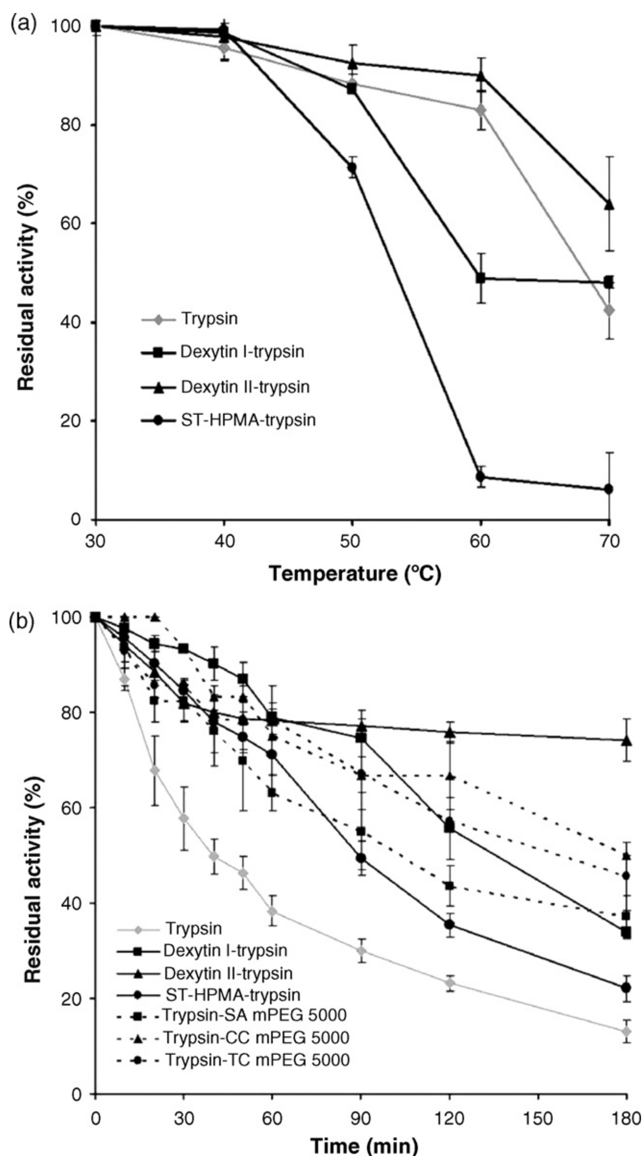


Fig. 5. Stability and autolysis of the trypsin conjugates. Panel (a) shows the relationship between trypsin or conjugate residual activity following incubation for 10 min at the temperatures shown. Panel (b) shows the autolysis of native and polymer–trypsin conjugates measured using BAPNA as a substrate. Full methods described in Section 2 and the results represent mean ± S.D. (n = 3).

4. Discussion

PEGylation is well established as a tool to improve the properties of biotech drugs including proteins, peptides and aptamers (reviewed in Ryan et al., 2008). The resulting conjugates have therapeutic and formulation advantages, and also with the potential to be cost-effective and even cost-saving (Eldar-Lissai et al., 2008; Gerkens et al., 2007). With more and more clinical applications realized, studies on PEG–protein conjugates continue to grow exponentially. They usually report the biological properties of novel conjugates, improved chemistry for conjugate synthesis (Kinstler et al., 2002; Brocchini et al., 2006), the effect of PEG molecular weight and branching on physico-chemical properties and biodistribution (Fee, 2007), and new techniques for purification and/or conjugate characterization (Na et al., 2008; Hardy et al., 2008). In spite of this success, PEG can also have disadvantages (reviewed in Gaberc-Porekar et al., 2008), not least the fact that this is a non-biodegradable polymer. This has brought increasing interest in alternative polymers such as poly(2-ethyl 2-oxazoline) (Mero et al., 2007), poly(vinylpyrolidone) (Kaneda et al., 2004) and in our case, dextrin (Duncan et al., 2008; Hardwicke et al., 2008; Ferguson and Duncan, 2006). However, for both PEG- and the other polymer-conjugates there have been disappointingly few studies on thermal stability and other formulation issues (e.g. Tattini et al., 2005; López-Cruz et al., 2006).

During conjugation, semi-telechelic polymers such as PEG and ST-HPMA form a single covalent attachment bringing advantages of minimal protein cross-linking and improved product homogeneity (Lu et al., 1998). Here a molar ratio of polymer:trypsin of 1:1 and

Table 2
Enzyme activity of the conjugates.

Compound	Activity[a] (%)	K_M[b] (mM)	V_{max}[b] (mM min^{-1})	K_{cat}[b] (s^{-1})
Trypsin	100	0.122 ± 0.031	0.0228 ± 0.0041	4.56 ± 0.83
Dextrin I–trypsin	15.1 ± 0.2	0.131 ± 0.005	0.0037 ± 0.0001	0.69 ± 0.01
Dextrin II–trypsin	18.6 ± 3.8	0.105 ± 0.062	0.0037 ± 0.0007	0.85 ± 0.17
ST-HPMA–trypsin	13.8 ± 1.6	0.136 ± 0.021	0.0033 ± 0.0004	0.63 ± 0.07

[a] Data shown relate to the trypsin control; mean ± S.D. (n = 3)
[b] Estimated using the Hanes-Wolfe plot.

gentle reaction conditions were chosen in the hope of generating, on average, conjugates containing one trypsin per polymer chain. It was anticipated that the dextrin conjugates would have more than one polymer attachment site per trypsin molecule. This is desirable in the context of PUMPT where the aim is to envelope the protein prior to conjugate activation. All the conjugates synthesized here were heterogeneous (not surprising due to the polydispersity of the polymeric intermediates), and contained little or no free trypsin. Although the GPC characterization is not quantitative, the estimated molecular weight, and also the trypsin content, of the dextrin conjugates (Table 1) suggested a ~1:1 or 1:2 polymer chain to protein content. By GPC, the ST-HPMA–trypsin conjugate apparently had lower molecular weight than native trypsin, but this was due to elution close to the bed volume of the column (Table 1). FPLC chromatogram clearly showed that the ST-HPMA–trypsin conjugate had a molecular weight that was higher than native trypsin (Fig. 2a).

Although, some previous studies report that PEG–trypsin and β-cyclodextrin–trypsin conjugates had an increase in enzyme activity compared to native trypsin (Abuchowski and Davis, 1979; Gaertner and Puigserver, 1992; Zhang et al., 1999; Fernandez et al., 2003), the three conjugates obtained in this study showed similar, and decreased trypsin activity (<20% the parent trypsin) (Fig. 3 and Table 2). This was consistent with the reduced trypsin activity reported by others following polymer conjugation (Zhang et al., 1999; Villalonga et al., 2000; Treetharnmathurot et al., 2008) which is usually attributed to modification of functional groups close to the active site resulting in steric hindrance, and/or refolding of the protein chain upon polymer attachment (Oupicky et al., 1999). Our previous studies with dextrin have confirmed that the protein masking resulting from dextrin conjugation can be regenerated (20–100%) on incubation with α-amylase, e.g. for dextrin–trypsin, dextrin–melanocyte stimulating hormone (MSH), dextrin-recombinant human epidermal growth factor, and dextrin-phospholipase A_2 conjugates respectively (Duncan et al., 2008; Hardwicke et al., 2008; Ferguson and Duncan, 2006).

The porcine pancreatic trypsin rate constants K_M, V_{max} and K_{cat} estimated reported here that were calculated using the Hanes-Wolfe plot (Table 2) differed from values reported by Johnson et al. (2002). For example, the K_{cat} of 4.56 s^{-1} (Table 2) is slightly higher than that the value of 2.89 s^{-1} estimated in their study using the least squares fit of the Michaelis–Menten equation to the reciprocal data using LUCENZIII, a computer software programme. It is well known that the analytical methodology and experimental conditions used, such as temperature and pH can affect K_M values and enzyme purity and enzyme concentration can cause variability in V_{max} values (Cornish-Bowden, 2004; Samoshina and Samoshin, 2005). Although Johnson et al. (2002) also used L-BAPNA as substrate, they performed their experiments at 35 °C (not 37 °C as used here), and using a different range of enzyme and substrate concentrations and also different incubation buffers. In these studies, although the K_M values obtained for free and conjugated trypsin were similar (suggesting the substrate had a similar trypsin affinity after conjugation), both the V_{max} and K_{cat} values estimated for the conjugates were reduced (~5–7-fold) after conjugation (Table 2).

Table 3
Half-life ($t_{1/2}$) min of polymer–trypsin conjugates.[a]

Compound	Half-life (min)[b]	Ratio of $t_{1/2}$ conjugate/trypsin
Trypsin	65 ± 1	
Dextrin I–trypsin	135 ± 2	2.08
Dextrin II–trypsin	>180	>2.77
ST-HPMA–trypsin	89 ± 1	1.37
Trypsin succinoylated mPEG[c]	102 ± 1	1.57
Trypsin cyanurate mPEG[c]	180 ± 1	2.77
Trypsin tosylate mPEG[c]	157 ± 3	2.41

[a] Experiments were conducted at 40 °C and the hydrolysis of BAPNA was used to measure residual trypsin activity.
[b] Data represent $t_{1/2}$ min (mean ± S.D.) (n = 3).
[c] Figures taken from Treetharnmathurot et al. (2008).

All the conjugates showed both increased thermal stability (at most temperatures) and better stability to autolysis than native trypsin (Figs. 4 and 5 and Table 3). The dextrin II–trypsin conjugate clearly demonstrated best stability in both assays. Its higher T_{50}, compared to native trypsin, was consistent with the studies of Murphy and O'Fagain (1996) who showed that covalent modification of trypsin with acetic acid *N*-hydroxy-succinimide ethyl ester increased the T_{50} by 5 °C, and Villalonga et al. (2000) who reported a T_{50} increase of 7 °C after carboxymethylcellulose conjugation (using the same experimental assay conditions). The higher molecular weight dextrin might be expected to provide enhanced protection from autolytic attack due to enhanced steric hindrance of the larger bulkier polymer chain. However, it is also known that the glycosidic –OH groups can create hydrogen-bonding protects and stabilizes proteins (Mislovicova et al., 2006). Interestingly, the ST-HPMA–trypsin conjugates displayed lower thermal stability at high temperatures, and had a lower $t_{1/2}$ for autolysis than the dextrin I–trypsin conjugate even though these polymers were of similar molecular weight. This may also reflect the better protective properties of the poly(glucose) polymer.

In conclusion, under specific conditions both dextrin and ST-HPMA were able to increase stability of conjugated trypsin. The dextrin II–trypsin conjugate was also most stable, and more stable than all the previously studied PEG–trypsin conjugates (Treetharnmathurot et al., 2008). This simple study underlines the potential of higher molecular weight dextrin II for protein conjugation, not only in the context of protein masking with subsequent regeneration of activity (PUMPT), but also as new biodegradable, polymer–protein conjugates with improved stability properties.

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