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Synthesis and characterization of thiomers of polyaspartamide type

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Abstract

Synthesis of poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]–thioglycolic acid (PHEA–TGA) conjugate as a new polyaspartamide thiomer is described. The parent polymer PHEA is chemically modified by introducing sulphydryl-bearing compound thioglycolic acid.

By varying the reaction conditions several batches of PHEA–TGA conjugates were prepared and analyzed. Tensile studies revealed that total work of adhesion of PHEA–TGA increased more than twice compared to the unmodified polymer. Microparticles prepared from the thiolated polymer preserved its bioadhesive properties.
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Keywords: Thiomer; Polyaspartamide; Thioglycolic acid; Mucoadhesion; Microparticles

1. Introduction

Thiolated polymers (thiomers) represent a promising new generation of mucoadhesive polymers. They are supposed to interact with cysteine-rich subdomains of mucus glycoproteins thereby forming disulphide bonds between the mucoadhesive polymer and the mucus layer (Gum et al., 1992). Thiomers could provide prolonged residence time of drug delivery systems on various mucosal tissues compared to well established polymers, improved cohesive properties, show enzyme inhibitory capabilities and a permeation enhancing effect (Bernkop-Schnürch and Thaler, 2000; Bernkop-

Abbreviations: AS, atomic spectrometry; DMF, *N*,*N*′-dimethylformamide; DSC, differential scanning calorimetry; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent); DTT, dithiothreitol; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; PHEA, poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]; SEM, scanning electron microscopy; SNF, simulated nasal fluid; TG, thermogravimetry; TGA, thioglycolic acid; TWA, total work of adhesion

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Schnürch et al., 2001a, 2003). These features render thiolated polymers as useful excipients for various drug delivery systems.

Numerous thiomers have been synthesized and evaluated based both on anionic (polycarbophil, carboxymethylcellulose, alginate) and cationic polymers (chitosan). In order to introduce thiol moieties, sulphydryl-bearing compounds, such as cysteine, cysteamine or thioglycolic acid (TGA) have been used (see, e.g., Bernkop-Schnürch and Steininger, 2000; Kast and Bernkop-Schnürch, 2001; Bernkop-Schnürch et al., 2001b).

In this paper we report preparation and characterization of poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]–thioglycolic acid conjugate (PHEA–TGA), a new type of thiolated polyaspartamide. PHEA is chosen for modification since it is a hydrosoluble, nontoxic and nonantigenic polymer useful in preparation of various polymer–drug conjugates (see, e.g., Antoni et al., 1979; Giammona et al., 1998; Martinac et al., 2002; Van der Merwe et al., 2002).

2. Materials and methods

2.1. Instruments and materials

IR spectra were recorded on a GX FT-IR spectrometer (Perkin Elmer, UK). ^{1}H and ^{13}C NMR spectra were taken on a Varian Gemmini spectrometer (Varian, USA). Differential scanning calorimetry (DSC) was carried out with a type DSC Pyris 1 instrument (Perkin Elmer, UK). Thermogravimetric analyzer TGA 7 (Perkin Elmer, USA) was used for thermogravimetry (TG). Atomic spectrometry (AS) was carried on inductively coupled plasma atomic emission spectrometer (Vista Pro, Varian, USA). The weight average molecular weight was determined by size exclusion chromatography (SEC) with UV detector (Series II, Hewlet Packard, USA). Microparticles were prepared using spray dryer (Büchi 190, Flawil, Switzerland). Their characterization was done by Olympus BH-2 microscope, equipped with a computer-controlled image analysis system (Optomax V, Cambridge, UK) and JSM-5800 scanning electron microscope (Joel, Japan). The centrifugation was performed at spin 3500 × *g* on Labofuge 400 (Heraeus, Germany) using filter device Centricon® Plus 20 (molecular weight cut-off 5000, Amicon Bioseparations, Millipore, USA). Dialysis was made with cellulose dialysis tubings with a molecular weight cut-off 8,000–12,000 (Sigma, USA). For thin layer chromatography, silica gel sheets Kieselgel 60 F_{254} (Merck, Germany) were used. Solvent system was dichloromethane/methanol 1:1. For spot detection iodine vapour was used. Gel filtration molecular weight standards were purchased from Bio Rad Laboratories CA (USA). Thioglycolic acid and Ellman's reagent were purchased from Sigma-Aldrich (Germany), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and dithiothreitol from Sigma (USA), L-aspartic acid and ethanolamine from Kemika (Croatia). The amine was distilled prior to use. All solvents were of analytical grade purity and dry.

2.2. Synthesis

2.2.1. Synthesis of poly[α,β-(N-2-hydroxyethyl-DL-aspartamide)] (PHEA, **1**)

PHEA was synthesized following the procedure published previously (Neri et al., 1973; Zorc et al., 1993).

2.2.2. Synthesis of poly[α,β-(N-2-hydroxyethyl-DL-aspartamide)]–thioglycolic acid conjugates (PHEA–TGA, **2a–f**). General procedure

PHEA and the corresponding amount of TGA were dissolved in demineralized water in order to obtain 0.8% solution of PHEA (Table 1). The solution was cooled in ice bath and 1-ethyl-3-(3-dimetylaminopropyl)carbodiimide (EDAC) was added. The reaction mixture was stirred for 24 or 48 h at room temperature, protected from light. The analogous reaction without carbodiimide served as a control. The reaction mixture was dialyzed protected from light, lyophilized and stored at 4 °C until use. Yields: 68–96%.

2a: PHEA/TGA mass ratio 5:1; EDAC concentration 58 mM; reaction time 24 h; dialysis 3 days against 5 mM HCl in the presence of small amount of $Na_2S_2O_4$, 1 day against 1 mM HCl, room temperature. Yield: 96%.

2b: PHEA/TGA mass ratio 2:1; EDAC concentration 58 mM; reaction time 24 h; dialysis 3 days against 5 mM HCl in the presence of small amount of $Na_2S_2O_4$, 1 day against 1 mM HCl, room temperature. Yield: 92%.

Table 1
Reaction conditions for PHEA–TGA conjugates (**2a–f**) preparation and sulphur determination in the products

PHEA–TGA **2a–f**	pH value		PHEA/TGA mass ratio	EDAC concentration (mM)	Reaction time (h)	Total sulphur (μmol g^{-1} ± S.D.)	SH content (μmol g^{-1} ± S.D.)	S/SH molar ratio
	Start	End						
a	6	6	5:1	58	24	11.8 ± 0.12	6.08 ± 0.15	1.9
b	5	5	2:1	58	24	20.4 ± 0.04	5.75 ± 0.21	3.5
c	5	5	2:1	58	48	Not determined	4.7 ± 0.19	–
d	5	5	2:1	58	24	28.2 ± 0.09	9.96 ± 0.29	2.8
e	6	7–8	2:1	116	24	23.4 ± 0.04	3.6 ± 0.37	6.5
f	5	5	1:1	116	24	30.9 ± 0.08	10.53 ± 1.92	2.9
Control	2	2	2:1	–	24	3.4 ± 0.22	0.11 ± 0.11	–

2c: PHEA/TGA mass ratio 2:1; EDAC concentration 58 mM; reaction time 48 h; dialysis 3 days against 5 mM HCl in the presence of small amount of $Na_2S_2O_4$, 1 day against 1 mM HCl, room temperature. Yield: 95%.

2d: PHEA/TGA mass ratio 2:1; EDAC concentration 58 mM; reaction time 24 h; dialysis 3 days against 5 mM HCl in the presence of small amount of $Na_2S_2O_4$, 1 day against 5 mM HCl, 5–15 °C. Yield: 95%.

2e: PHEA/TGA mass ratio 2:1; EDAC concentration 116 mM; reaction time 24 h; dialysis 3 days against 5 mM HCl in the presence of small amount of $Na_2S_2O_4$, 1 day against 5 mM HCl, 5–15 °C. Yield: 70%.

2f: PHEA/TGA mass ratio 1:1; EDAC concentration 116 mM; reaction time 24 h; dialysis 3 days against 5 mM HCl in the presence of small amount of $Na_2S_2O_4$, 1 day against 5 mM HCl, 5–15 °C. Yield: 68%.

Control: PHEA/TGA mass ratio 2:1; reaction time 24 h; dialysis 3 days against 5 mM HCl in the presence of small amount of $Na_2S_2O_4$, 1 day against 5 mM HCl, 5–15 °C, protected from light.

2.3. Characterization

2.3.1. Determination of total sulphur, thiol and disulphide groups

The amount of free SH groups immobilized on the PHEA was determined after colorimetric reaction of thiols with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent). The conjugates or control (20 mg) were dissolved in 2 ml of demineralized water. Thereafter, 0.5 ml of Ellman's reagent was added (the reagent was dissolved in 0.07 M phosphate buffer containing 1 mM EDTA, pH 7.25, in final concentration of 1.5 μg ml^{-1}) (Fontana and Toniolo, 1974). After incubation for 3 h at room temperature, the absorbance at 408 nm was measured. The degree of thiolation was confirmed by the iodometric titration. The solution of 20 mg of the conjugate in 2 ml buffer solution, pH 3 ($NaHCO_3$/HCl) and 0.4 ml starch solution (1%, w/v) was titrated with 0.05 mM iodine solution until permanent light-blue colour.

The total sulphur content was determined by plasma atomic emission spectrometry (AS). The experimental conditions were: power of generator, 1.15 kW; argon flow rate for plasma, 15 l min^{-1}; side-flow, 1.5 l min^{-1}; feed rate of argon, 0.9 l min^{-1}; read time, 5 s. The absorbances were measured at wavelengths of 180.669 and 181.972 nm and the average concentration of sulphur was calculated.

Disulphide bonds quantification was done indirectly by comparing the content of thiol groups before and after treatment of product **2b** with dithiothreitol (DTT). The solution of 35 mg of conjugate in 4 ml of 0.07 M phosphate buffer (pH 7.25), 2 ml 0.1 M DTT and 4 ml demineralized water was incubated for 3 h at 60 °C. The solution was concentrated by centrifugation and reconstituted with demineralized water. The washing cycle was repeated until the excess of DTT was completely removed from the product (total consumption of demineralized water 110 ml). The efficacy of DTT removal was controlled by iodometric titration of filtrate.

The results for total sulphur, thiol and disulphide groups were expressed as micromoles per gram of polymers.

2.3.2. Stability of thiol groups in PHEA–TGA solution

In order to determine the stability of thiol groups in the conjugate solution, product **2b** was dissolved in 0.1 M acetate buffer pH 4.0, 0.1 M acetate buffer pH 5.0 or 0.1 M phosphate buffer pH 6.8 in a final

concentration of 2.2% (w/v). The solutions were incubated at 37 °C under permanent shaking. At predetermined time points, aliquots were withdrawn and the amount of thiol groups remained was determined spectrophotometrically with Ellman's reagent as described above.

2.3.3. Spectroscopic and thermogravimetric characterization

IR spectra of unmodified (**1**) and thiolated PHEA (**2**) were recorded using HATR method (transmittance, 1%; range 4000.0–370.0 cm^{-1}; number of scans, 16; resolution, 4.0 cm^{-1}; interval, 1.0 cm^{-1}). The absorption maxima for both compounds were practically the same. IR (HATR): ν_{max} 3279, 2927, 1636, 1522, 1226, and 1056 cm^{-1}.

^{1}H and ^{13}C NMR spectra were recorded at 500.13 MHz for the ^{1}H nucleus and at 125.67 MHz for the ^{13}C nucleus. Samples were measured in D_2O solutions in 5-mm NMR tubes. Chemical shifts are referred to TMS. ^{1}H NMR, δ (ppm): 4.77, 4.72, 3.65, 3.34, 2.82. ^{13}C NMR, δ (ppm): 175.42, 175.14, 174.73, 63.90, 63.46, 62.89, 62.83, 44.74, 44.67, 44.64, 44.47, 39.82.

Differential scanning calorimetry (DSC) was carried out at the following conditions: the step scan rate, 5 °C min^{-1} (holding time 1 min per step); temperature range 30–100 °C. Temperature calibration was performed by using indium and lead.

Thermogravimetric measurements conditions were: scan rate, 10 °C min^{-1} in temperature range 30–500 °C under nitrogen flow rate, 35 ml min^{-1}.

2.3.4. Molecular weight determination

The weight average molecular weight of unmodified polymer (**1**) and PHEA–TGA conjugates (**2**) was determined by size exclusion chromatography (UV detector, $\lambda = 200 \pm 10$ nm). The column set was composed of a precolumn and column BioSep-SEC-S 3000, 290 Å pore size (Phenomenex, USA). The experimental conditions were: mobile phase buffer solution pH 6.7 (50 mM KH_2PO_4 + 50 mM KCl); flow rate, 0.35 ml min^{-1}; injection volume, 5 μl. The column was calibrated by protein molecular weight standards: thyroglobuline, γ-globuline, ovalbumin, myoglobin and vitamin B-12. The column set, ionic strength and pH of the aqueous mobile phase were optimized prior the molecular weight determination.

2.3.5. Tensile studies

For tensile studies 30 mg of lyophilized unmodified polymer or PHEA–TGA conjugates was compressed into 5 mm diameter flat-faced test disc. A 2-cm long piece of freshly excised porcine nasal mucosa was mounted on the platform of the tension-compression stand. The disc and the mucosal surface were brought in contact in simulated nasal fluid (SNF: 8.77 g NaCl, 2.98 g KCl and 0.59 g $CaCl_2$ per 1000 ml of demineralized water), pH 6.4 at 22 °C (Cheng et al., 2002). The value of the force of detachment was measured as a function of displacement, by lowering the platform of the tension-compression stand at the constant rate (2 mm min^{-1}) until total separation of the components was achieved. The work of fracture, equivalent to the total work of adhesion (TWA) was calculated as the area under the obtained force/distance curve.

2.4. Preparation and characterization of microparticles

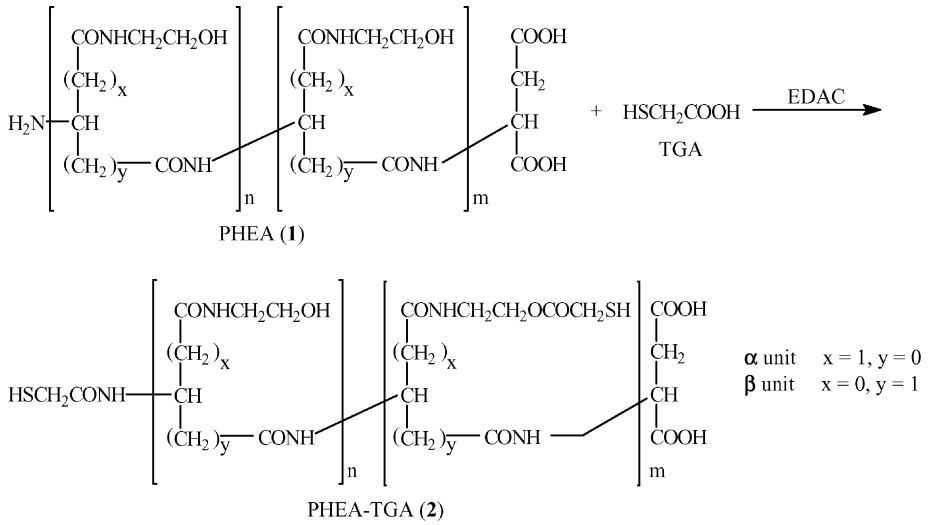
Microparticles were prepared by spray drying method. Unmodified (**1**) or thiolated PHEA (**2d**) were dissolved in ethanol/water mixture (3:2, v/v) in a final concentration of 2% (w/v). pH of the solution was adjusted to 4–5 by 0.1 M HCl or 0.1 M NaOH. The drying conditions were as follows: flow rate of 0.25 l h^{-1}, inlet air temperature of 120 °C and outlet air temperature of 90 °C.

The microscopical image analysis technique for determination of particle size and distribution was used. The shape and surface characteristics of the microparticles were observed by scanning electron microscopy. The microspheres were coated with gold and observed with scanning electron microscope (SEM).

The mucoadhesive properties of microparticles were investigated by the same method as for polymers.

3. Results and discussion

The parent polymer PHEA (**1**) was prepared by thermal polycondensation of L-aspartic acid in the presence of phosphoric acid and subsequent aminolysis of the resulting polysuccinimide with ethanolamine (Neri et al., 1973; Zorc et al., 1993). During reaction a partial racemization occurred and opening of the succinimide rings proceeded at two sights so the final polymer had



Scheme 1.

DL configuration and α,β structure (Neri et al., 1973; Kokufuta et al., 1978). Thioglycolic acid was thereby covalently attached to PHEA forming PHEA–TGA conjugate (**2**), a new thiomer of polyaspartamide type (Scheme 1). Formation of ester and amide bonds between hydroxyl and N-terminal amino groups in PHEA and carboxyl group of thioglycolic acid was achieved by carbodiimide method using EDAC as a coupling reagent.

Several batches of conjugates **2a–f** differing in total sulphur, thiol content and total sulphur/thiol ratio were prepared by varying the reaction and purification conditions: PHEA/TGA mass ratio, EDAC concentration, reaction time, pH and temperature during dialysis (Table 1).

The PHEA/TGA mass ratio during the reaction influenced the amount of sulphur and thiol groups in the final product **2** (Fig. 1). The highest coupling rate was achieved at the PHEA/TGA ratio 2:1. A lower amount of TGA (5:1) in the reaction mixture decreased the coupling rate, while the highest TGA ratio (1:1) did not significantly improved it.

The quantity of sulphur and thiol groups attached in conjugates **2** depended on EDAC concentration as well (Fig. 2). The product **2d** prepared with 58 mM EDAC concentration showed the highest SH content (9.96 μmol g^{-1}). Double EDAC concentration applied in synthesis of **2e** slightly decreased total sulphur content and significantly decreased SH content. This could

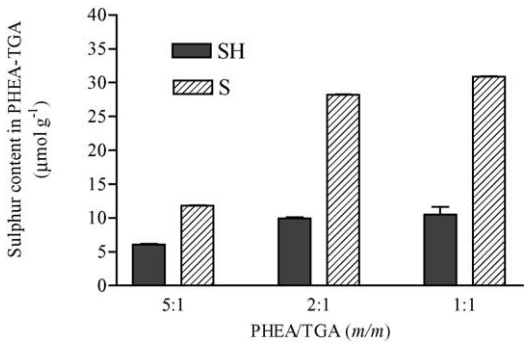


Fig. 1. The influence of PHEA/TGA mass ratio on the quantity of thiol groups and total sulphur in PHEA–TGA conjugates.

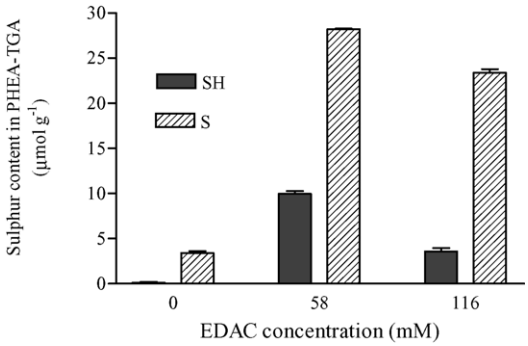


Fig. 2. The influence of EDAC concentration in coupling reaction on the quantity of thiol groups and total sulphur in PHEA–TGA conjugates.

be explained by oxidation of thiol groups caused by higher pH during the **2e** preparation. The control reaction performed analogously as synthesis of **2d** and **2e** but omitting EDAC gave the product with a negligible sulphur content. In this way, efficacy of coupling reaction was controlled: without a coupling agent binding of thioglycolic acid failed. In addition, dialysis efficacy was checked: the lack of sulphur in the product indicated that unbound thioglycolic acid and other small molecules were successfully removed.

The total sulphur/thiol ratio was mainly influenced by pH during the coupling reaction and dialysis. The highest ratio was obtained in product **2e**, which was prepared at pH 6 (Table 1). Prolonging the reaction time from 24 to 48 h did not increase the thiol content (Fig. 3).

The amount of free thiol groups bound to PHEA was determined by colorimetric method, after reaction of sulphydryl groups with Ellman's reagent. The results obtained by iodometric titration were in good correlation with the results of colorimetric determination.

The accuracy of analytical methods used was proved additionally by AS. The results for total sulphur determined by the method described above were in excellent agreement with the results obtained by AS. For example, total sulphur in product **2b** determined by the first method was 20.4 (SH content 5.75 ± 0.21 (mol g^{-1}, S-S content 8.5 ± 1.65 (mol g^{-1}) and by AS 22.8 μmol g^{-1}.

For all PHEA–TGA conjugates a higher content of total sulphur than thiol groups could be observed, indicating possibility of intra- and inter-molecular disulphide bond formation. Disulphide bonds were quantified indirectly by comparing the content of thiol groups before and after the treatment of conjugate **2b** with DTT which completely reduced disulphides. A large excess of DTT, neutral pH and elevated temperature were used to accelerate and complete the reaction (Zahler and Cleland, 1968; Morioka and Kobayashi, 1997). The results obtained proved that sulphur existed in the conjugates both in thiol and disulphide form.

It was reported that thiolated polymers readily oxidized depending on their pK_a and pH value of the thiomer solution. A higher pH led to a higher rate of thiolate anions, the active form for oxidation, resulting in inter- and intra-molecular bonds formation (Hornof et al., 2003). Stability of thiol groups in PHEA–TGA solutions at different pH values was followed and our results were in good agreement with the previous findings. A significant decrease in thiol group content of PHEA–TGA conjugate was observed at pH 6.8. During the 6-h period more than 90% of the thiol groups was oxidized. After 4 h of incubation a significant decrease in content of thiol groups could be observed even at pH 4. The results are presented in Fig. 4.

Spectroscopic data (IR and NMR) for parent polymer PHEA were in agreement with the values reported previously (Zovko et al., 2001; Kang et al., 2002). FTIR spectra of conjugates **2** were practically identical with the PHEA spectrum, while their ^{1}H NMR spectra showed slight differences in signals intensity in 8.5–7.0 ppm region, where thiol groups could be expected.

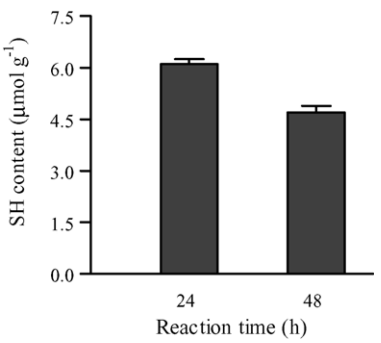


Fig. 3. The influence of reaction time on the quantity of thiol groups in PHEA–TGA conjugates.

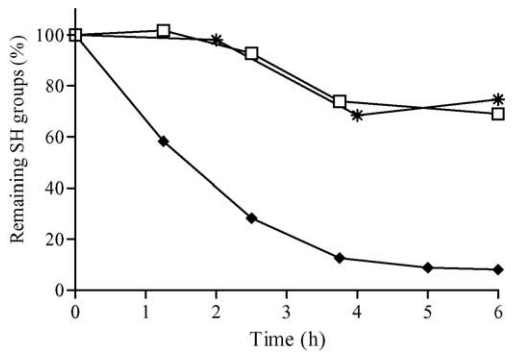


Fig. 4. Decrease in thiol group content in PHEA–TGA 2.2% solution in 0.1 M acetate buffer pH 4.0 (✱), 0.1 M acetate buffer pH 5.0 (□) and 0.1 M phosphate buffer pH 6.8 (♦). Indicated values are means of at least three experiments.

DSC measurements were carried out on **2d** product and different glass transitions were observed for the thiolated and unmodified polymer (at 55 °C for **2d** and 50 °C for PHEA) (Castelli et al., 2000). This can be explained by partial cross-linking of polymer chains via disulphide bonds. TG measurements displayed the water evaporation (4% approximately), starting at 35 °C and the degradation process starting at 190 °C for both polymers.

Weight average molecular weights determined by size exclusion chromatography were 67,039 and 68,873 Da for **1** and **2d**, respectively. As mentioned before, a total of 28.2 μmol of sulphur and 9.96 μmol of free thiol group per gram of **2d** conjugate were determined. According to this, approximately one polymer chain of PHEA linked two molecules of thioglycolic acid and at least one of them was cross-linked via a disulphide bridge so the weight average molecular weight of conjugate was higher than the weight of unmodified PHEA. The comparison of cumulative percent of molar mass for both unmodified and thiolated polymer is shown in Fig. 5.

Tensile studies demonstrated a clear correlation between the amount of polymer-attached TGA and mucoadhesive properties of the conjugates **2**. The observed TWA was higher for more thiolated conjugates. TWA of PHEA–TGA increased more than twice compared to the unmodified PHEA (Fig. 6).

In order to evaluate the influence of pH of the polymer on mucoadhesive properties, **2d** was dissolved in demineralized water in 2.2% (w/v) concentration and pH of the solution was left either unchanged (pH 2) or adjusted to pH 5 and pH 7, respectively. The solutions were lyophilized and the products thus obtained were analyzed for their mucoadhesive properties. According to the pH prior the lyophilization, the conjugates were assigned as **2d–2**, **2d–5** and **2d–7**. The results indicated that there was no significant difference in TWA between conjugates **2d–2** and **2d–5**, while TWA for conjugate **2d–7** decreased for 30% (Fig. 6). This could be

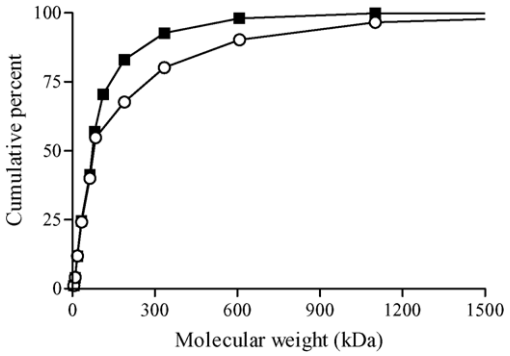


Fig. 5. Comparison of cumulative molar mass distribution for PHEA (■) and PHEA–TGA (**2d**) (○).

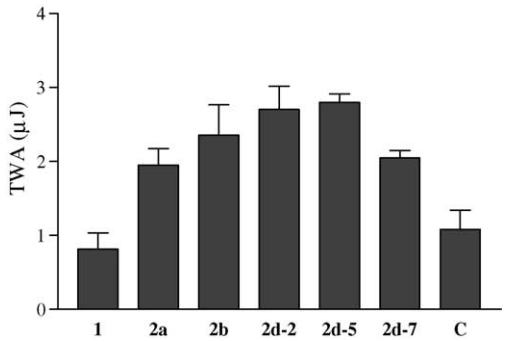


Fig. 6. Adhesive properties of PHEA, PHEA–TGA conjugates and control (**C**).

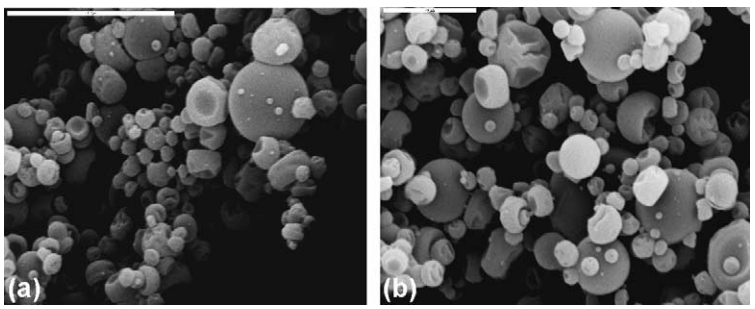


Fig. 7. SEM images of (a) PHEA and (b) PHEA–TGA microparticles.

explained by rapid disulphide formation at pH 7. Consequently, decreased number of thiol groups remained in reduced form and available for covalent binding to mucus glycoproteins.

Suitability of PHEA and PHEA–TGA (**2d**) for microparticles preparation was studied and compared. The microparticles were successfully prepared from both polymers using spray-drying method (Fig. 7). The microparticles were polydisperse, with smooth surface and similar in size (mean particle diameter was 3.41 ± 1.43 mm and 3.58 ± 1.66 mm, respectively). Tensile studies showed that mucoadhesive properties of both types of microparticles were comparable.

4. Conclusions

Thioglycolic acid was covalently attached to PHEA, a hydrosoluble polymer of polyaspartamide type. Thiolation of PHEA polymer significantly improved its bioadhesive properties. It is possible to prepare the microparticles of such modified polymer preserving its bioadhesive properties. The potential use of the PHEA–TGA conjugates in preparing of mucoadhesive delivery system will be the subject of future studies.

Acknowledgement

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