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Thiolated polymers: Stability of thiol moieties under different storage conditions

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Abstract: The purpose of this study was to evaluate the stability of thiolated polymers - so-called thiomers. A polycarbophil-cysteine conjugate and a chitosan-thioglycolic acid conjugate were chosen as representative anionic and cationic thiomer. The thiol group bearing compounds L-cysteine and thioglycolic acid were introduced to polycarbophil and chitosan, respectively with a coupling reaction mediated by a carbodiimide. The resulting thiolated polymers were freeze-dried and the amount of thiol groups on the thiomer was determined spectrophotometrically. Each kind of polymer was directly used or compressed into 1 mg matrix-tablets. Polymers were stored for a period of six months at four different storage conditions, namely at –20°C (56% relative humidity; RH), 4°C (53% RH), at 20°C (70% RH), and at 22°C (25% RH). Samples were taken after 6 months to determine the formation of disulfide bonds and the remaining thiol groups on the polymer. When the polycarbophil-cysteine and chitosan-thioglycolic acid conjugate were stored as powder a decrease of free thiol groups was observed only after storage at 20°C and 70% RH. Both polymers were found to be stable under all storage conditions when compressed into matrix tablets. The results provide the base for the use of thiomers as auxiliary agents in commercial products.

Key-words: polycarbophil, chitosan, thiomers, stability

1. Introduction

Recently a new type of mucoadhesive polymeric excipients has been introduced in pharmaceutical literature. Thiolated polymers are polymers modified by the covalent attachment of thiol bearing moieties such as L-cysteine, cysteamine and thioglycolic acid. A number of well established hydrophilic polymers such as polycarbophil,

carboxymethylcellulose, sodium alginate, poly(acrylic acid) and the cationic polymer chitosan were already successfully modified[1-5].

In contrast to traditionally used mucoadhesive polymers, which adhere to the mucus by non-covalent bonds such as hydrogen bonds and ionic interactions[6], thiomers are presumed to be capable of forming covalent bonds leading to ***improved mucoadhesive properties***. The underlying mechanism is based on thiol/disulfide exchange reactions and on an oxidation process between the reactive thiol groups of the mucoadhesive polymer and cysteine-rich subdomains of the mucin glycoproteins[7]. For example, polycarbophil-cysteine conjugates and chitosan-thioglycolic acid conjugates display on freshly excised intestinal mucosa more than two- and four-times higher mucoadhesiveness, respectively compared to the corresponding unmodified polymers[1, 5]. Additionally, thiolated polymers show ***improved cohesive properties***. A simple pH-dependent oxidation process results in an inter- and/or intrachain disulfide bond formation within the polymeric network. This crosslinking takes place during the swelling process in aqueous media leading to a substantial stabilisation of e.g. matrix tablets comprising a thiomer[8]. Furthermore, thiolated polymers display ***enzyme inhibitory capabilities***, which render them useful in non-invasive peptide delivery. This is believed to be due to polymer conjugates complexing the divalent metal-ions, such as zinc ions, from the enzyme structure. Metalloproteases such as aminopeptidase N often responsible for the degradation of therapeutic peptides on mucosal membranes, can be inhibited by thiomers[9]. Recently, a ***permeation enhancing effect*** was demonstrated for thiolated polymers such as polycarbophil-cysteine conjugates and poly(acrylic acid)-cysteine conjugates[10, 11]. The proposed reaction mechanism for the permeation enhancement involves the opening of tight junctions. The cysteine moieties of the polymers reduce oxidised glutathione and therefore increase the concentration of reduced glutathione on the absorption membrane[11]. Reduced glutathione is able to inhibit the enzyme protein-tyrosine-phosphatase[12], which results in the phosphorylation of the membrane protein occludin. This leads to the opening of tight junctions[13].

The efficacy of thiomers has meanwhile also been verified by various *in vivo* studies. A significant reduction in the glucose level of diabetic mice, for instance, was achieved by the oral administration of insulin microtablets based on a polycarbophil-cysteine conjugate[14]. Furthermore, Guggi et al. could demonstrate a significant decrease in the

plasma calcium level after orally administered calcitonin being incorporated in thiolated chitosan[15].

As free thiol moieties are essential for all the features mentioned above, it was the aim of the present study to evaluate the long-term stability of these reactive residues being immobilised on the polymeric backbone. On this behalf two different types of thiomers, namely a polycarbophil-cysteine conjugate and a chitosan-thioglycolic acid conjugate were synthesised. These conjugates were lyophilised and stored at different temperatures as received or compressed to 1 mg matrix-tablets. Thiol-moieties were determined monthly spectrophotometrically with Ellman's reagent. Results obtained should provide helpful basic information concerning storage conditions of thiomers.

2. Materials and methods

2.1. Synthesis of the polycarbophil-cysteine conjugate

The polycarbophil-cysteine conjugate was synthesised as described previously[10]. Two grams of the sodium salt of polycarbophil (Noveon® AA1, MM ≥ 700 kDa) were hydrated in 500 ml of demineralised water. After activation of the carboxylic-acid moieties of the polymer with 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC; Sigma, St. Louis, MO) for 45 min, 400 mg of L-cysteine (Sigma, St. Louis, MO) were added. The reaction mixture was incubated for 3 h at room temperature at a constant pH of 5.0. Purification was performed via dialysis. To remove unbound cysteine the reaction mixtures were dialysed against 0.2 mM HCl and 2 µM EDTA to quench any further reaction, two times against the same medium but containing 1% NaCl, then 3 times exhaustively again against 0.2 mM HCl. After dialysis the pH of the aqueous polymer solutions was adjusted to 4.5 and the polymers were lyophilised (-30°C, 0.1 mbar; Christ Beta 1-8; Germany). Samples prepared in exactly the same way but omitting EDAC during the reaction served as control.

2.2. Synthesis of chitosan- thioglycolic acid conjugate

Thioglycolic acid (TGA; Sigma, St. Louis, MO) was attached covalently to chitosan (molecular mass: ~150 kDa; degree of deacetylation: 83 - 85%; Fluka Chemie, Buchs, CH) as previously described[5]. Immobilisation of TGA was achieved by the formation of amide

bonds between the primary amino groups of the polymer and the carboxylic acid group of thioglycolic acid. First, 1 g of chitosan were hydrated in 8 ml of 1 M HCl and dissolved by the addition of demineralised water in order to obtain a 1% solution of chitosan hydrochloride. Thereafter, EDAC was added in a final concentration of 125 mM in order to activate the carboxylic acid moieties of the afterwards added TGA. After the carbodiimide was completely dissolved in the chitosan hydrochloride solution, TGA was added in a ratio 1:1 (w/w; polymer to thioglycolic acid). The pH-value for the coupling reaction was adjusted to pH 5 using 1 M NaOH. The reaction mixture was incubated for three hours at room temperature under stirring. Samples prepared in exactly the same way but omitting EDAC during the coupling reaction served as controls for the analytical studies.
In order to eliminate unbound TGA and to isolate the polymer-conjugate, the reaction mixtures were dialysed against 5 mM HCl, two times against 5 mM HCl containing 1% NaCl, against 5 mM HCl and finally against 0.4 mM HCl. After adjusting the pH to 4.5 the aqueous polymer solutions were lyophilised.

2.3. Preparation of the polymer-matrix tablets

Half of the amount of each lyophilised thiomer was compressed (Hanseaten, Type EI, Hamburg, Germany) into 1 mg tablets (diameter: 2 mm; depth: 0.5 mm). The compression force was kept constant during the preparation of all tablets.

2.4. Storage conditions for the thiomers

The thiolated polymers were tested with regard to the stability of immobilised thiol groups against oxidation. Therefore, samples were stored either in an exsiccator at room temperature (22°C) and a relative humidity (RH) of 25%, in a refrigerator at 4°C / 53% RH, in a freezer at –20°C / 56% RH or in a climatic exposure test cabinet at 20°C / 70% RH. Samples were withdrawn monthly for a time period of 6 months and the amount of remaining thiol groups was determined.

2.5. Determination of the thiol group content

The amount of thiol groups immobilised on the polymer was determined spectrophotometrically with Ellman's reagent quantifying free thiol groups.

First, 0.5 mg each of the conjugate tablets and the uncompressed thiomers were hydrated in 0.25 ml of demineralised water. 0.25 ml of 0.5 M phosphate buffer pH 8.0 and 0.50 ml Ellman's reagent (3 mg 5,5'-dithiobis(2-nitrobenzoic acid)/10 ml of 0.5 M phosphate buffer pH 8.0) were added. The samples were incubated for 2 hours at room temperature. Thereafter, 0.30 ml of each sample were transferred into a microplate and the absorbency was measured at a wavelength of 450/620 nm with a microplate reader (Anthos reader 2001; Salzburg, Austria). L-Cysteine and thioglycolic acid standards were used to calculate the amount of thiol groups remaining on the thiomers.

To determine the degree of disulfide bond formation the reaction with Ellman's reagent was performed after reducing disulfides with sodium borohydride. To 0.5 mg of the polymers 0.35 ml water were added. After a hydration time of 30 min 0.75 ml 0.05 M tris buffer pH 6.8 and 1.00 ml 4% (w/v) sodium borohydride solution were added. The samples were incubated for 1 h at 38°C. Then remaining sodium borohydride was inactivated by addition of 0.20 ml 5 M HCl. The pH of the reaction mixture was adjusted to 8.0 with 1 ml of 1 M phosphate buffer pH 8.0. After addition of 0.10 ml Ellman's reagent (40 mg 5,5'-dithiobis(2-nitrobenzoic acid) in 10 ml of 1.0 M phosphate buffer pH 8.0) the samples were incubated for 2 h at room temperature. Measurement of the absorbency and quantification of thiol groups were performed as described above.

2.6. Statistical data analyses

Statistical data analyses were performed using the Student *t* test with $p < 0.05$ as the minimum level of significance.

3. Results and Discussion

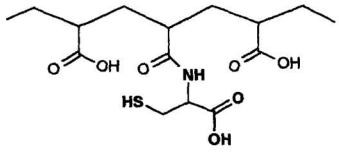
3.1. Synthesis and characterisation of the polymers

The lyophilised polymers were white and odourless powders with fibrous structure. All polymers were easily swellable in water. The efficacy of the purification step via dialysis after conjugate synthesis with EDAC was evidenced by the controls exhibiting only a negligible amount of free thiol groups.

The amount of attached L-cysteine was 48.73 ± 0.41 µM per g polycarbophil. The presumptive substructure of the resulting conjugate is shown in Figure 1. Since disulfide

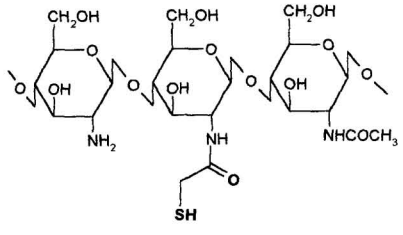
bonds were already formed during the coupling reaction, about 10% of these cysteine moieties were oxidised. Therefore, the polycarbophil-cysteine conjugate displayed 43.21 ± 2.77 µM free thiol groups per g polymer.

Figure 1. Presumptive substructure of a polycarbophil-cysteine conjugate



For the chitosan-thioglycolic acid conjugate (Fig. 2) the amount of covalently attached thioglycolic acid was determined to be 1473.28 ± 116.47 µM per g chitosan. During the coupling reaction 66 % of the thiol groups were oxidised and the resulting amount of free thiol groups was 499.45 µM ± 57.77 thiol groups per g polymer. The preliminary formation of disulfide bonds during the coupling reaction is advantageous because of the resulting crosslinking of the polymer. Consequently the cohesive properties of thiomer matrix tablets are increased and their stability and water uptake are positively influenced[8].

Figure 2. Presumptive substructure of a chitosan-thioglycolic acid conjugate



3.2. Stability of the thiolated polymers

Since polycarbophil and chitosan are commonly used as excipients in tablets their long term stability has already been confirmed[16, 17]. For this study these polymers were modified by the introduction of thiol groups.

The rate at which the oxidation of thiol groups takes place depends on the temperature and the pK_a value of the sulfhydryl groups and consequently on the pH within the conjugate. The pK_a value of the thiol group of thioglycolic acid covalently attached to chitosan was calculated by the Advanced Chemistry Development/Chem Sketch program to be 8.52. Therefore, the formation of disulfide bonds can take place at relatively lower pH-values compared to the polycarbophil–cysteine conjugate with sulfhydryl groups displaying a higher pK_a value of 10.2. To increase the stability of thiol groups the pH-value of the thiomers was adjusted to 4.5 before lyophilization. The results of the stability studies are shown in Table 1 and 2.

Table 1. Amount of free thiol groups in µM/g polymer of the polycarbophil-cysteine conjugate after 6 months under different storage conditions. The initial amount of thiol groups was 43.2 ± 2.8 µM/g polymer. [a] significant decrease of thiol groups ($p < 0.05$)

storage conditions temperature	humidity	powder	tablets
- 20°C	56%	43.0 ± 2.5	41.3 ± 0.8
+ 4°C	53%	42.2 ± 3.3	43.8 ± 1.7
+ 20°C	70%	36.4 ± 2.5[a]	38.8 ± 1.7
+ 22°C	25%	40.5 ± 1.7	46.3 ± 1.7

Table 2. Amount of free thiol groups in µM/g polymer of the chitosan-thioglycolic acid conjugate after 6 months under different storage conditions. The initial amount of thiol groups was 499.5 ± 57.8 µM/g polymer. [a] significant decrease of thiol groups ($p < 0.05$)

storage conditions temperature	humidity	powder	tablets
- 20°C	56%	451.2 ± 63.7	445.7 ± 24.2
+ 4°C	53%	457.7 ± 39.5	492.9 ± 54.9
+ 20°C	70%	375.4 ± 8.8[a]	438.0 ± 19.8
+ 22°C	25%	490.7 ± 4.4	462.1 ± 51.6

The total amount of L-cysteine and thioglycolic acid immobilised on the polymers did not change significantly for this time period under all storage conditions. The free thiol groups of both thiolated polymers were found to be stable against oxidation for at least 6 months when stored at –20°C and at 4°C. These findings were expected since the polymers are usually stored as lyophilised powder in the refrigerator and no significant changes in their properties were ever observed. Also at room temperature no significant decrease of free thiol groups was observed when the polymers were protected from humidity. In case of storage in the climatic exposure cabinet (20°C, relative humidity 70%) already after 2 months the amount of free thiol groups was significantly decreased by oxidation in both thiomers. Nevertheless, this degradation was only observed for polymers stored as lyophilised powders, where a decrease of 15% and 25% of the initial amount of free thiol groups was observed after 6 months for the polycarbophil-cysteine and chitosan-thioglycolic acid conjugate, respectively. This is probably due to a much smaller amount of polymer being actually exposed to the environment in tablets. Therefore, no significant decrease of free thiol groups was observed for the compressed polymers.

The results of this study confirm that polymer conjugates between studies can be safely stored in the refrigerator at 4°C. More detailed studies will have to be performed in the future for the use of these thiolated polymers as excipients in tablets. Nevertheless, our preliminary studies indicate that the achievement of long term stability of thiolated polymers will be feasible.

4. Conclusion

The immobilisation of thiol-bearing compounds on polymeric excipients such as polycarbophil and chitosan leads to a significant improvement of the mucoadhesive, cohesive, enzyme inhibitory and permeation enhancing properties of these polymers. Within this study the stability of free thiol groups - which are essential for the features mentioned above - was investigated. The stability of a polycarbophil-cysteine and a chitosan-thioglycolic acid conjugate under typical storage conditions over a time period of 6 months was confirmed.

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References

1. Bernkop-Schnürch A., Schwarz V., Steininger S. (1999), Pharm. Res. 16:876- 881.
2. Bernkop-Schnürch A., Kast C. E., Richter M. F. (2001), J. Control. Rel. 71:277-285.
3. Clausen A. E., Bernkop-Schnürch A. (2001), Eur. J. Pharm. Biopharm. 51:25-32.
4. Marschütz M. K., Bernkop-Schnürch A. (2002), Eur. J. Pharm. Sci. 15:387-394.
5. Kast C. E., Bernkop-Schnürch A. (2001), Biomaterials. 22:2345-2352.
6. Peppas N. A., Mikos A. G. (1990), Kinetics of mucus-polymer interactions, in Bioadhesion-Possibilities Future Trends, R. Gurny and H.E. Junginger, Editors. Wiss. Verl.-Ges., Stuttgart. p. 65-85.
7. Gum J. R. J., Hicks J. W., Toribara N. W., Rothe E. M., Lagace R. E., Kim Y. S. (1992), J. Biol. Chem. 267:21375-21383.
8. Clausen A. E., Bernkop-Schnürch A. (2001), Pharm. Ind. 63:312-317.
9. Walker G., Zarti H., Bernkop-Schnürch A. (2001), J. Pharm. Sci. 90:1907-1914.
10. Clausen A. E., Bernkop-Schnürch A. (2000), J. Pharm. Sci. 89:1253-1261.
11. Clausen A. E., Kast C. E., Bernkop-Schnürch A. (2002), Pharm. Res. 19:602-608.
12. Barrett W. C., DeGnore J. P., Konig S., Fales H. M., Keng Y. F., Zhang Z. Y., Yim M. B., Chock P. B. (1999), Biochemistry. 38:6699-6705.
13. Collares-Buzato C. B., Jepson M. A., Simmons N. L., Hirst B. H. (1998), Eur. J. Cell Biol. 76:85-92.
14. Marschütz M. K., Caliceti P., Bernkop-Schnürch A. (2000), Pharm. Res. 17:1468-1474.
15. Guggi D., Kast C. E., Bernkop-Schnürch A. (2002), 11th International Pharmaceutical Technology Symposium.
16. Illum L. (1998), Pharm. Res. 15:1326-1331.
17. Manji P., Durrani M., G-Jensen A., Whitaker R., Andrews A. (1995), Proc. Int. Symp. Controlled Release Bioact. Mater. 22:362-363.

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