J. DRUG DEL. SCI. TECH., 15 (6) 411-417 2005

Synthesis and *in vitro* characterization of a novel poly(acrylic acid)-glutathione conjugate

K. Kafedjiiski[1], M. Werle[2], F. Föger[1], A. Bernkop-Schnürch[1]*

[1]Department of Pharmaceutical Technology, Institute of Pharmacy, Leopold-Franzens-University Innsbruck, Innrain 52, Josef Möller Haus, 6020 Innsbruck, Austria
[2]ThioMatrix GmbH, Research Center, Mitterweg 24, 6020 Innsbruck, Austria
*Correspondence: andreas.bernkop@uibk.ac.at

The purpose of the present study was to improve the multifunctional properties of poly(acrylic acid) by the covalent attachment of glutathione. The adhesive properties of poly(acrylic acid)-glutathione (PAA-GSH) conjugate were evaluated in vitro *on freshly excised porcine mucosa via tensile studies and the rotating cylinder method. The permeation enhancing effect of the conjugate in combination with glutathione was evaluated in Ussing chambers by using sodium fluoresceine as model compound. The resulting PAA-GSH conjugate displayed 353.7 ± 41.8 (n = 3) µmol immobilized free thiol groups and 309.8 ± 27.3 (n = 3) µmol disulfide bonds per gram polymer. In aqueous solutions, the modified polymer demonstrated improved cohesive properties. Due to the immobilization of glutathione, the swelling velocity of the polymer was 4-fold accelerated. Tensile studies showed that the mucoadhesive properties of poly(acrylic acid) were strongly improved by the covalent attachment of glutathione. The adhesion time of PAA-GSH was more than 14-fold higher in comparison to unmodified poly(acrylic acid). Furthermore, the conjugate exhibited a 1.77-fold higher permeation enhancing effect compared with the control. According to the results of the present study, PAA-GSH conjugate represents a very promising novel thiomer for the development of various mucoadhesive drug delivery systems.*

Key words: Poly(acrylic acid)-glutathione – Glutathione – Poly(acrylic acid) – Mucoadhesion – Permeation enhancement.

Mucoadhesive polymers have recently gained considerable attention as platforms for controlled drug delivery. Initially, the advantages of mucoadhesive drug delivery systems were considered to be based on their potential to prolong the residence time at the site of absorption, and to provide an intimate contact with the mucus membrane. Later it was found that certain mucoadhesive polymers, such as polyacrylic acids and chitosan exhibit multifunctional properties and can modulate the permeability of the epithelial tissues. In some cases, existing polymers were modified, while in others, new materials were developed [1]. Biological mucoadhesives, such as plant lectins, show specific interactions with cell surfaces and mucin and are regarded as 'second generation' bioadhesives [2]. The development of polymer systems that are able to interact with their environment in an "intelligent" manner has led to novel mucoadhesive materials [3]. Polymers with thiol groups were also investigated as a new generation of mucoadhesive polymers [4]. Because of the immobilization of thiol groups on generally established mucoadhesive polymers such as poly(acrylates), their mucoadhesive properties are strongly improved. The enhancement of mucoadhesion can be explained by the formation of disulfide bonds between the polymer and cysteine-rich subdomains of mucus glycoproteins [5]. Apart from their improved mucoadhesive properties, thiomers also exhibit permeation enhancing [6], strong cohesive and enzyme inhibitory properties [7].

Although results obtained so far with thiolated polymers are very promising, further improvements in their features seem feasible. The properties of thiomers are defined by the nature of polymer, the ligand and the degree of modification. Among anionic polymers generated so far, poly(acrylic acid)-cysteine conjugate displayed the most favorable features. The studies revealed that non-crosslinked PAA-cysteine of 450 kDa exhibited the highest mucoadhesive [8] and permeation enhancing properties [9]. The combination of thiomer with reduced glutathione (GSH) led to a significant improvement in the permeation enhancing effect of thiomers [10, 11]. The underlying mechanism of this system is based on the inhibition of the protein tyrosine phosphatase (PTP) being involved in the opening process of the tight junctions. The reduced form of free GSH reacts with PTP via thio/disulfide exchange reaction causing inactivation of PTP [12]. The inhibitory effect of glutathione is limited as it is rapidly oxidized on the mucosal surface. Recent studies supported the assumption that thiomers expressing reactive thiol groups might be able to reduce oxidized glutathione (GSSG) thereby increasing the amount of reduced glutathione on the absorption membrane. Therefore, the permeation enhancing effect of the thiomer/ GSH system depends on the reducing properties of thiomers. Attempts to further improve the permeation enhancing effect of the thiomer/ GSH system include the combination with permeation enhancers acting in different way [13] and optimization of the molecular mass of the thiomer [9]. Unfortunately, no further progress was made.

A new strategy, which is the subject of this study, is the direct immobilization of free GSH on poly(acrylic acid), which might lead to a new class of thiomers offering comparatively high permeation enhancing properties. The reasons for such expected features of the novel PAA-GSH conjugate are based on the unique ligand GSH [14, 15]. Reduced glutathione is the major non-protein thiol present in elevated amounts in living cells. GSH acts as a redox buffer to prevent oxidative damage due to its potent reducing and nucleophilic properties. It plays a role in other cellular reactions including reduction of ribonucleotides, regulation of protein and gene expression via thio/disulfide exchange reactions. Another consideration for immobilizing GSH on the polymer backbone is the impact of gastrointestinal conditions on thiomer/GSH permeation enhancing system. Observing *in vivo* environment, a subsequent dilution of free GSH cannot be excluded. Thus, the permeation enhancing effect of free glutathione in the thiomer/GSH system might be lost. In contrast, the thiomer has a high molecular weight to be absorbed and remains concentrated on the absorption membrane. As poly(acrylic acid) and glutathione are generally regarded as non-toxic, the new PAA-GSH conjugate is most likely to exhibit a toxicologically harmless profile.

In order to verify this hypothesis, it was the aim of this study to synthesize and characterize a novel PAA-GSH conjugate. It was achieved by the covalent attachment of glutathione to poly(acrylic acid) via the formation of amide bonds between the amino group

of the amino acid and a carboxylic acid group of the polymer. The influence of the immobilization of thiol moieties on the mucoadhesive properties of the conjugate was also investigated. In addition, essential thiomer features such as cohesive properties, swelling behavior and permeation enhancing effect were evaluated.

I. MATERIALS AND METHODS

1. Materials

Poly(acrylic acid) (exclusively linear, MM: 450 kDa; PAA450), glutathione reduced form (GSH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 5,5'-dithiobis(2-nitrobenzoic acid) and N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) were all purchased from Sigma (St. Louis, MO, USA). Sodium fluorescein (Na-Flu) was obtained from Fluka Chemie (Buchs, Switzerland). All chemicals were of analytical grade.

2. Synthesis of poly(acrylic acid)-glutathione conjugate

Reduced glutathione was attached covalently to poly(acrylic acid) via the formation of amide bonds between the amino group of glutathione and a carboxylic acid group of the polymer. First, 1 g of PAA 450 was hydrated in 80 ml demineralized water and the pH value of the obtained polymer solution was adjusted to 5.5 by the addition of 5 M NaOH. Then, the carbodiimide (EDAC) dissolved in 5 ml demineralized water was added in a final concentration of 50 to 300 mM, as shown in *Table I*, in order to activate the carboxylic acid moieties of poly(acrylic acid). After 15 min incubation time under stirring at room temperature, 2 g of reduced glutathione in 10 ml demineralized water was added and the pH was readjusted to 5.5. The reaction mixture was then allowed to proceed for 3 h at room temperature under stirring. The resulting PAA-GSH conjugate was isolated by dialyzing first against 1 mM HCl, twice against 1 mM HCl but additionally containing 1% NaCl and then twice against 1 mM HCl. Controls were prepared and isolated in the same way as the polymer conjugate but omitting the carbodiimide (EDAC) during the coupling reaction. After dialysis, the pH value of the polymers and corresponding controls were readjusted to 4 and frozen aqueous polymer solutions were lyophilized at -50°C and 0.01 mbar (Lyolab B; Inula, Austria). Samples were stored at 4°C until further use.

3. Determination of the thiol group and disulfide bond content

The amount of thiol groups immobilized on PAA-GSH conjugate was determined spectrophotometrically using Ellman's reagent as described previously [4].

Disulfide content was determined after reduction with $NaBH_4$ and addition of 5,5'-dithiobis(2-nitrobenzoic acid) as described by Habeeb [16].

4. Oxidation of thiol groups

PAA-GSH conjugate was hydrated in demineralized water in a final concentration of 0.5% (w/v) at pH 4. The pH of the solution was adjusted to 7 and 8, respectively. Samples were incubated at 37ºC under continuous shaking. At predetermined time points, aliquots of 200 μl were withdrawn and 50 μl of 1 M HCl was added in order to stop any further reactions. The amount of remaining thiol groups was determined via Ellman's reagent.

5. Tablet manufacture

Lyophilized PAA-GSH conjugate and controls were compressed into 30 mg, 5.0 mm diameter flat-faced tablets (single punch eccentric press-Korsch EK, Germany). The compaction pressure (force of 11 kN) was kept constant during the preparation of tablets. Tablets were checked for resistance to crushing (Schleuniger type apparatus) according to the European Pharmacopoeia. The tensile strength (σ_x) of the tablets was then calculated using the following equation [17]:

$$\sigma_x = 2P/\pi dt$$

where P is crushing strength (N), d is the diameter of the tablet (mm) and t is the thickness of the tablet (mm). The mean tensile strength of at least five tablets was calculated.

6. Evaluation of the swelling behavior

The water-absorbing capacity was determined by a gravimetric method. In brief, 30 mg each of lyophilized PAA-GSH conjugate and the corresponding control were compressed (single-punch eccentric press-Korsch EK, Germany) to 5.0 mm diameter flat-faced tablets. Tablets were fixed to a needle and immersed in a beaker containing 100 mM phosphate buffer pH 6.8 at 37°C. At predetermined time points the swollen tablets were taken out of the incubation medium, excess water was removed, and the amount of water uptake was determined gravimetrically [18].

7. Disintegration studies

The stability of the polymer tablets and the corresponding control tablets were analyzed in 100 mM phosphate buffer pH 6.8 at 37 ± 0.5°C with the disintegration test apparatus according to the European Pharmacopoeia. The oscillating frequency was adjusted to 0.5 s^{-1}.

8. *In vitro* evaluation of the adhesive properties

8.1. Tensile studies

Thirty milligrams of lyophilized PAA-GSH conjugate and controls was compressed to tablets. Each tablet was glued to a stainless steel flat disc (8 mm in diameter), which was attached to a laboratory stand with a nylon thread (15 cm). The porcine mucosa was fixed on a glass support using a cyanoacrylate adhesive, placed in a beaker containing 400 ml of 100 mM phosphate-buffer saline pH 6.8. The beaker was placed on a balance and raised by a mobile platform until the mucosa came into contact with the tablet. The contact was determined when the nylon thread holding the tablet became bent. After 30 min incubation at 25°C, the mucosa was pulled down from the tablet at a rate of 0.1 mm/s. Data points were collected by a personal computer (Windwedge software, TAL Technologies Inc., Philadelphia, PA) connected to the balance and then data were transferred to Excel 97 (Microsoft, USA). The total work of adhesion (TWA), representing the area under the force/distance curve and the maximum detachment force (MDF) were calculated [18].

8.2. *In vitro* mucoadhesion studies with the rotating cylinder method

Polymer and control tablets were attached to a freshly excised

Table I - Amount of thiol/disulfide groups immobilized on PAA-GSH conjugate.

No. of the conjugate	Polyacrylic acid (g/80 ml)	GSH (g)	EDAC (mM)	Thiol groups (µmol/g polymer ± SD, n = 3)	Disulfide groups (µmol/g polymer ± SD, n = 3)
PAA- GSH 1	1.0	2.0	50	61.9 ± 3.3	62.1 ± 6.4
PAA- GSH 2	1.0	2.0	100	126.5 ± 5.5	80.7 ± 19.9
PAA- GSH 3	1.0	2.0	150	175.2 ± 19.8	121.5 ± 9.6
PAA- GSH 4	1.0	2.0	200	353.7 ± 41.8	309.8 ± 27.3
PAA- GSH 5	1.0	2.0	300	324.4 ± 48.4	301.1 ± 27.8