

Spray-dried mucoadhesives for intravesical drug delivery using *N*-acetylcysteine- and glutathione-glycol chitosan conjugates

Nunzio Denora,^{1,‡,*} Angela Lopodota,^{1,‡} Mara Perrone,¹ Valentino Laquintana,¹ Rosa M. Iacobazzi,^{1,2} Antonella Milella,³ Elisabetta Fanizza,^{3,4} Nicoletta Depalo,⁴ Annalisa Cutrignelli,¹ Antonio Lopalco,⁵ and Massimo Franco.¹

¹*Department of Pharmacy - Drug Sciences, University of Bari "A. Moro", Orabona, St. 4, 70125 Bari, Italy.*

²*Istituto tumori IRCCS "Giovanni Paolo II", Flacco, St. 65, 70124 Bari, Italy.*

³*Department of Chemistry, University of Bari "A. Moro", Orabona, St. 4, 70125 Bari, Italy.*

⁴*CNR-Institute for Physical and Chemical Processes UOS, Bari, Italy.*

⁵*Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas 66047, USA.*

* Corresponding Author. Tel: +39 080 544 2767. E-mail: nunzio.denora@uniba.it (N.D.).

‡ These authors contributed equally to this study as first authors.

ABSTRACT

This work describes *N*-acetylcysteine (NAC)- and glutathione (GSH)-glycol chitosan (GC) polymer conjugates engineered as potential platform useful to formulate micro- (MP) and nano- (NP) particles via spray-drying techniques. These conjugates are mucoadhesive over the range of urine pH, 5.0 to 7.0, which makes them advantageous for intravesical drug delivery and treatment of local bladder diseases. NAC- and GSH-GC conjugates were generated with a synthetic approach optimizing reaction times and purification in order to minimize the oxidation of thiol groups. In this way, the resulting amount of free thiol groups immobilized per gram of NAC- and GSH-GC conjugates was 6.3 and 3.6 mmol, respectively. These polymers were completely characterized by molecular weight, surface sulfur content, solubility at different pH values, substitution and swelling degree. Mucoadhesion properties were evaluated in artificial urine by turbidimetric and zeta (ζ)-potential measurements demonstrating good mucoadhesion properties, in particular for NAC-GC at pH 5.0. Starting from the thiolated polymers, MP and NP were prepared using both the Büchi B-191 and Nano Büchi B-90 spray dryers, respectively. The resulting two formulations were evaluated for yield, size, oxidation of thiol groups and *ex-vivo* mucoadhesion. The new spray drying technique provided NP of suitable size ($< 1 \mu\text{m}$) for catheter administration, low degree of oxidation, and sufficient mucoadhesion property with 9 and 18% of GSH- and NAC-GC based NP retained on pig mucosa bladder after 3 h of exposure, respectively.

Statement of Significance

The aim of the present study was first to optimize the synthesis of NAC-GC and GSH-GC, and preserve the oxidation state of the thiol moieties by introducing several optimizations of the already reported synthetic procedures that increase the mucoadhesive properties and avoid pH-dependent aggregation.

Second, starting from these optimized thiomers, we studied the feasibility of manufacturing MP and NP by spray-drying techniques. The aim of this second step was to produce mucoadhesive drug delivery systems of adequate size for vesical administration by catheter, and comparable mucoadhesive properties with respect to the processed polymers, avoiding thiolic oxidation during the formulation. MP with acceptable size produced by spray-dryer Büchi B-191 were compared with NP made with the apparatus Nano Büchi B-90.

KEYWORDS: oxidation, glycol chitosan, mucoadhesion, spray drying, bladder drug delivery

1. Introduction

Mucoadhesion is most commonly defined as the ability of natural or synthetic polymers to adhere to the surface of mucosal tissues. Mucoadhesive drug delivery systems (MDDS) offer the advantages of extending the residence time at the site of interest, protecting the drug from enzymatic degradation and increasing its bioavailability, making less frequent dosing possible [1-3]. Hence, MDDS have become a useful approach to target and release the drug cargo to particular sites or tissues such as the bladder mucosa [3,4,5]. Although bladder diseases have a considerable impact on society, their treatment remains an important challenge for research. Intravesical instillation therapy using MDDS has become a promising approach for the treatment of bladder diseases, such as interstitial cystitis and bladder cancer [6,7]. Such approach has the advantage to selectively deliver high concentrations of drugs to the target site, limiting the undesirable side effects, compared to oral or intravenous therapies. However, local administrations into the bladder of drugs via solutions is often ineffective due to the low permeability of urothelium, the short residence time, and the pH of urine, typically 5.0–7.0, may limit solubility or permeability at some drugs. Moreover, the instilled drug solutions become diluted with urine and are washed out of the bladder during voiding, necessitating repeated infusions of the drug. Several natural polymers have been already used as MDDS. Among them, chitosans (CS) are an interesting class of mucoadhesive cationic polymers composed of D-glucosamine and N-acetyl-D-glucosamine units. However, there are some limitations in the use of these polymers as bladder MDDS, such as the tendency to form aggregates and loss of surface charge at pH values greater than 5.5 [8]. Hence, the use of a chemically modified CS characterized by an

ethylene glycol portion, and known as glycol chitosan (GC), is more suitable when a pH independent water solubility and a positive charge retention are desirable at physiological or urine pH values. Recently, it has been shown that chemical modification of the structure of some polymers, such as CS, GC, polyacrylic acid, cellulose derivatives, pectin, and alginates, can improve the mucoadhesive properties by the introduction of thiol groups, displaying potential utility for intravesical drug delivery [3,5,7]. However, besides this advantage, thiomers show reasonably low stability in aqueous solutions, as they are subject to thiol oxidation at pH 6 unless sealed under inert conditions [9]. In fact, it has been reported that N-acetylcysteine- and glutathione-GC polymer conjugates, with some mucoadhesive properties, are characterized by high amounts of oxidized thiols, namely disulfide groups. Most likely this can be ascribed both an oxidizing synthetic procedure and an excessive purification of raw materials detrimental for mucoadhesion properties [10]. Hence, the optimization of synthetic approach and purification of thiomers and their suitable formulations would be highly advantageous in developing MDDS. In a study conducted by Barthelmes et al., thioglycolic CS microparticles (MP) and nanoparticles (NP) were generated by spray-drying and ionic gelation [3]. In vitro and in vivo mucoadhesion tests revealed that thiolated MP and NP were more adherent to the mucosa than the unmodified materials. Furthermore, a direct comparison between thiolated MP and NP indicated a longer retention of NP in the rat bladder. Inspired from these findings, the aim of the present study was first the synthesis of N-acetylcysteine- and glutathione-GC conjugates (NAC-GC and GSH-GC), preserving the oxidation state of the thiol moieties, and second, the feasibility to formulate the above mentioned thiomers as MP and NP by using spray-drying techniques. Namely, GSH- and NAC-GC polymer conjugates were here synthesized by developing appropriate synthetic processes and purification methods which offer some significant advantages over those already reported in literature [10], such as high control on their molecular weight, retention of their water solubility at pH of at least 5.0, as well as, lower amount of surface disulfide groups, thus ensuring improved mucoadhesive properties of the two novel thiolated GC derivatives. Furthermore, an innovative approach was here designed and implemented to clearly discriminate the thiol groups from the disulfide ones by means XPS-ESCA analysis and mostly by exploiting a selective derivatization of the two polymer conjugates carrying the thiol groups with a molecule close in structure to the Ellman's reagent [10,11].

Finally, for the first time these new polymers were formulated as MP and NP by spray drying technique. In particular, NP were prepared by Buchi Spray Dryer B-90 that recently was advised as a valid tool in the pharmaceutical field [12], thus providing opening and promising instructions to process glycol chitosan and their derivate polymers in this new apparatus, never

studied up to now. The MP and NP based on the two novel thiolated GC-polymer derivatives and characterized by good bio-adhesively on bladder mucosa represent promising carriers, which can be potentially able to improve the therapeutic efficiency of specific drug by increasing its bioavailability, solubility, and retention time and thus finally resulting useful for local intravesical treatment of bladder diseases

2. Materials and Methods

Glycol chitosan (degree of polymerization ≥ 400 ; GC), glutathione (GSH), *N*-acetylcysteine (NAC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), 5,5'-dithiobis(2-nitrobenzoic acid) (Ellmann's reagent), 4,4'-bis(trifluoromethyl)-2,2'-dinitrodiphenyldisulphide (BTDDS), sodium-borohydride, *N*-hydroxysuccinimide (NHS), porcine stomach mucin (type II, bound sialic acid $\sim 1\%$), fluorescein diacetate (FDA) and the component for artificial urine were purchased from Sigma-Aldrich, Italy.

2.1. Synthesis of glutathione-glycol chitosan polymer conjugate (GSH-GC)

The synthesis of GSH-GC polymer conjugate was accomplished using a modified procedure previously reported [10]. In particular, we adapted this synthetic procedure from that developed for GSH-CS polymer conjugate [9]. Briefly, GSH (1.5 g, 4.8 mmol), NHS (0.6 g, 5.8 mmol), and EDAC (1.1 g, 5.8 mmol), were dissolved in 10 mL of a hydrochloric acid solution (pH 5.0). After 30 min, GC (0.5 g) dissolved in the same acid solution (10 mL), was added dropwise over the course of 5 min. To optimize the synthetic procedure, three different batches were planned differing on the reaction time of 1, 3 and 6 h. After 1, 3 and 6 h of incubation under continuous stirring at room temperature, the corresponding polymer conjugates were purified using an Amicon Centrifugal Filtration Device (100 kDa MWCO regenerated cellulose membrane). The loaded device was centrifuged at 4,000 rpm for 20 min, concentrating the GSH-GC polymer conjugate solution to approximately 1 mL. This concentrated material was diluted in an additional 10 mL of fresh Milli-Q water and centrifuged again under identical conditions. Each sample was washed three times. The final concentrated GSH-GC dispersions were frozen at $-20\text{ }^{\circ}\text{C}$ and lyophilized (Christ Alpha 1-4 LSC) for 24 hours under reduced pressure (0.016 mbar). The recovered solid polymers were sealed under nitrogen and stored in desiccator at $4\text{ }^{\circ}\text{C}$ until further use.

2.2. Synthesis of *N*-acetylcysteine-glycol chitosan polymer conjugate (NAC-GC)

For the synthesis of NAC-GC polymer conjugate, we used the same procedure adopted for GSH-GC thiomers above described. In particular, NAC (0.8 g, 4.8 mmol), NHS (0.6 g, 5.8 mmol), and EDAC (1.1 g, 5.8 mmol) were dissolved in hydrochloric acid solution (pH 5.0), and after 30 min a solution of GC (0.5 g) in 10 mL of hydrochloric acid solution (pH 5.0), was added dropwise to the first solution over the course of 5 min. Similarly, to the preparation of GSH-GC, three batches were prepared at reaction times of 1, 3, and 6 h. The resultant reaction mixtures were stirred at room temperature. NAC-GC polymer conjugates were further purified as described above for the GSH-GC thiomers.

2.3. Characterization of GSH- and NAC-GC polymer conjugates

The newly synthesized thiomers GSH- and NAC-GC polymer conjugates were fully characterized by FT-IR, ¹H-NMR, and Size Exclusion Chromatography (SEC). FT-IR spectra were carried out on a PerkinElmer 1600 FT-IR spectrometer, using 2% of sample in KBr pellets. ¹H-NMR spectra were recorded on Varian Mercury 300 MHz instrument, using D₂O as solvent (0.1 g/mL). ¹H chemical shifts were referenced by using the residual protic peak of the solvent as internal reference (4.80 ppm for HOD). The average molecular weights and molecular weight distributions of the starting polymer and its conjugates were determined by SEC using a Waters Associates (Milford, Ma) Model 1515 HPLC isocratic pump, a Waters 2414 differential RID detector and an UltraHydrogel TM 500 column (7.8 x 300 mm, 5 μm). Since it is well known in the literature that acidic mobile phases are optimum for the SEC characterization of CS and its chemical derivatives [13-15], we did not use a previously reported SEC method [10], that used 0.05 M phosphate buffered solution (PBS) at pH 7.2, in order to avoid precipitation of the polymers. As a mobile phase, we used acetate buffer solution, pH 4.5, (0.15 M acetic acid, 0.1 M sodium acetate), modified with 0.05 M NaNO₃ to overcome undesirable ionic interactions, at 30 °C with a flow rate of 0.6 mL/min. Test solutions were prepared in acetate buffer, pH 4.5, at a concentration of 3 mg/mL and 20-μL samples were injected onto the column. Dextran standards (M_p = 10,000-1,000,000 Da, Sigma-Aldrich) were used for calibration. Chromatographic data were processed with Waters Associates (Milford, Ma, US) Breeze software. The derivatization degree (DI) of the thiolated polymers at different reaction times was determined through the analysis of the molecular weights using the following equation:

$$DI (\%) = \frac{Mn (thiomer) - Mn (GC)}{[MW (thiol moiety) - 18]400} \times 100$$

where Mn (thiomer) is the number average molecular weight of the thiolated polymers GSH- or NAC-GC, Mn (GC) is the number average molecular weight of the GC, MW (thiol moiety) is the molecular weight of NAC or GSH, and 400 is the degree of polymerization.

2.4. Quantitative analysis of thiol and disulfide groups

The degree of substitution of glycol chitosan with NAC and GSH was determined photometrically with Ellman's reagent according to a procedure previously described [16,17], to quantify free thiol groups (partial Ellman's assay). Aliquots of 1.0 mg of the conjugates (GC-NAC, GC-GSH), of the polymer (GC) and of control (NAC, GSH), was hydrated in 250 μ L of water and 250 μ L of 0.05 M phosphate buffer pH 8.0, before adding 500 μ L of Ellman's reagent (3.0 mg of DTNB dissolved in 10 mL of 0.05 M phosphate buffer pH 8.0). The samples were incubated for 3 h at room temperature. After mixing and centrifugation for 5 min at 13,000 rpm, 200 μ L of the clear supernatant was transferred into a microplate and the absorbance was determined at a wavelength of 450 nm with a microplate reader (Perkin Elmer 2030 multilabel reader Victor TM X3). In order to calculate the amount of polymer free thiol groups, NAC and GSH standards were used. The quantity of polymer disulfide bonds (total Ellman's assay), was quantified as follows: all the samples (1.0 mg), as above listed, were hydrated in 250 μ L of water and 250 μ L of 0.05 M phosphate buffer, pH 8.0, and then diluted with 500 μ L of a freshly sodium-borohydride solution (4.0% m/v) in order to reduce the existing disulfide bonds. The mixture was incubated for 1 h in a water bath at 37 ± 0.5 °C. Afterwards, the residual sodium-borohydride, was deactivated with 100 μ L of 5 M HCl, and then the mixture was diluted with 900 μ L of 0.05 M phosphate buffer pH 8.0. Finally 2 mL of DTNB (Ellman's reagent, 0.03% m/v in 0.05 M phosphate buffer pH 8.0) were added. Following 15 min incubation at room temperature, aliquots of 200 μ L were transferred into a microplate and the absorbance was measured at 450 nm as described above. The amount of disulfide bonds was calculated subtracting the quantity of free thiol groups from total thiol moieties present on the polymer.

2.5. X-ray Photoelectron Spectroscopy (XPS) analysis

XPS analyses were carried out with a Thermo Fisher Scientific Theta Probe spectrometer equipped with a monochromatic Al K α X-ray source (1486.6 eV) with a spot size of 300 μ m, corresponding to a power of 70 W and at a take-off angle of 53° with respect to the sample normal. Survey spectra (0 - 1300 eV) were acquired at a pass energy of 200 eV with an energy step size of 1 eV. High-resolution spectra were recorded at a pass energy of 100 eV with a step size of 0.05 eV. In the set conditions, the overall energy resolution was 0.9 eV. Samples were charge-compensated by means of a low energy-electron flood gun (1 eV). Charge correction of the spectra was performed by taking the alkyl type carbon (C-C, C-H) component of the C1s spectrum as internal reference (Binding Energy, BE=285.0 eV). XPS analysis was repeated on three different spots of each sample. High-resolution spectra of detected elements were acquired for quantitative and detailed BE chemical shift analysis. Atomic percentages were calculated using the Scofield sensitivity factors set in the Thermo Avantage V4.87 software (Thermo Fisher Scientific) and a non-linear Shirley background subtraction algorithm. The high-resolution spectra were fitted with mixed Gaussian-Lorentzian peaks after a Shirley background subtraction. The determined standard deviation in the peak position was \pm 0.2 eV.

Since thiol groups cannot be distinguished from the disulfide ones with an ordinary XPS analysis, due to the very low difference in chemical shifts of these two functionalities [11], derivatization reactions of the thiomers were carried out to obtain more quantitative information on the thiols groups. More specifically, a molecule close in structure to the Ellman's reagent was selected, 4,4'-bis(trifluoromethyl)-2,2'-dinitrodiphenyldisulphide (BTDDS) and the derivatization protocol was optimized as reported below. Based on this reaction, the thiol groups on the polymers can be quantified based on the 1:1 ratio with respect to both, the nitro group and the trifluoromethyl ones of the reagent.

Derivatization reactions of thiomers were carried in order to quantify the free thiol groups (partial assay), and the total thiol moieties (reduced and oxidized thiols groups in the form of disulfide bonds; total assay) present on GSH- and NAC-GC polymer conjugates. First, for the partial assay, 1 mg of GSH- or NAC-GC polymer conjugates was hydrated in 500 μ L of fresh Milli-Q water followed by the addition in a dropwise manner of a DMSO solution of BTDDS (5.3 mg dissolved in 1 mL of DMSO). After addition of the DMSO solution, the mixture acquired a opalescent light yellow coloration and was stirred for 6 h at room temperature. Then, 3.5 mL of ethanol were added and the resulting clear solution was transferred into dialysis membrane tubing (Spectra/Por[®] 14 kDa MWCO, RC), and the resulting functionalized polymer conjugates were exhaustively dialyzed in two steps for 12 h each, against first deionized water/ethanol solution 1:1 v/v and finally just deionized water.

Lastly, the dispersion was lyophilized (Christ Alpha 1-4 LSC) under vacuum and stored in a desiccator until further use.

For the total assay, the samples (1 mg), as listed above, were hydrated in 200 μ L of fresh Milli-Q water and then diluted with 200 μ L of a freshly prepared sodium-borohydride solution (4% m/v) in order to reduce the existing disulfide bonds. The mixture was incubated for 1 h in a water bath at 37 ± 0.5 °C. Afterwards, the residual sodium-borohydride was deactivated with 100 μ L of 5-M HCl. The mixture was diluted with 500 μ L of DMSO followed by the addition in a dropwise manner of a DMSO solution of BTDDS (5.3 mg dissolved in 500 μ L of DMSO) and stirred for 6 h at room temperature. As described for the partial assay, the mixture was diluted with 3.5 mL of ethanol and then transferred into dialysis membrane tubing (Spectra/Por[®] 14 kDa MWCO, RC) and purified by dialysis as above described.

2.6. Solubility of GSH- and NAC-GC polymer conjugates in artificial urine

The solubility of thiomers was assessed in artificial urine (9.3 g/L urea, 1.17 g/L NaCl, 0.75 g/L KCl, 0.67 g/L creatinine pH.6.8 [18] adjusted to four different pH values (5.0, 6.0, 7.0, and 8.0) with 5-M HCl or 1-M NaOH. Combinations of artificial urine and thiomers (GSH-GC or NAC-GC) or pristine GC (at 1, 1.5 and 2% w/v) were mixed and stirred overnight at a controlled temperature of 25 ± 0.5 °C. The solubility of thiomers was determined by visual inspection and compared with that observed for pristine GC and with CS as reference. Moreover, the assessment of water solubility of CH, GC and thiolated polymers was also measured by turbidity. Samples (2% w/v) were dissolved in deionized water, and the transmittance was measured at 600 nm using a Perkin-Elmer Spectrometer Lambda Bio20 and a quartz cell (1 cm of optical path length).

2.7. In vitro evaluation of the mucoadhesive properties

The mucoadhesive characteristics of synthesized polymers and the corresponding parent were evaluated *in vitro* by turbidimetric and zeta (ζ)-potential measurements as previously reported with some modifications [19,20]. In detail, 100 mg of mucin were dispersed in 100 mL of two simulated artificial urine media (pH 5.0 or 7.0), under stirring at 37 ± 0.5 °C for 24 h. Then, 10 mg of each polymer were placed in tubes with screw caps and solubilized with 2.5 mL of artificial urine medium at pH 5.0 or pH 7.0 and combined with 2.5 mL of mucin dispersion at the same pH value. The samples were left in a thermostatic bath, and at scheduled times (0, 1, 2, 5, 8, and 24 h) the interaction between

mucin and the tested polymer was evaluated by UV (Perkin Elmer UV/Vis spectrometer lambda BIO 20) in transmittance mode at 650 nm. Carbopol 934 was included in the study as control. All experiments were carried out at 37 ± 0.5 °C in triplicate.

For ζ -potential measurements, mucin samples were prepared by dispersing mucin (2 mg/mL) in artificial urine at pH 5. To this suspension an equal volume of polymeric suspension (2 mg/mL) in same medium was added. Interaction with mucin was examined at scheduled time (0, 5, 30, 60, 90, 120, 150 and 180 min). Then, 200 μ L of each sample was diluted with 800 μ L of KCl solution (1 mM) and the ζ -potential of these samples was determined using a Zetasizer Nano ZS (Malvern, UK) instrument.

2.8. Evaluation of the swelling behavior

The swelling behavior of GSH-GC or NAC-GC was determined by a gravimetric method, GC was used as control. Exactly weighed aliquots of the studied polymers were compressed into 5.0 mm diameter flat-faced tablets with a pressure of 10 kN. The tablets were placed on a 5 mL sintered glass filter (\varnothing 10 mm; porosity: G3) and left to swell by immersing the filter plus support in a beaker containing 5 mL of the artificial urine (pH 5.0 or pH 7.0). After a fixed time (60 min), the excess of liquid was removed by percolation at atmospheric pressure. The filter was placed in a properly sized centrifuge test tube, then centrifuged at 6,000 rpm for 15 min and weighed. The weight swelling ratio (q) was calculated as: $q = W_f/W_i$, where W_f and W_i are the weights of polymers after (final) and before (initial) the experiment, respectively.

2.9. Preparation of micro- and nano-particles by Spray-drying process

Four different formulations were prepared by spray-drying technique. MP were obtained starting from NAC- or GSH-GC by a Mini spray dryer B-191 (Büchi Flawil, Switzerland). In detail, MP were prepared by solubilizing 200 mg of NAC- or GSH-GC in 20 mL of acetic acid aqueous solution (0.7% v/v) in order to obtain a polymeric concentration of 1% w/v. The solution was processed by Mini spray dryer B-191 adopting the following operative conditions: atomizing air flow rate 500-600 L/h, T inlet 145 °C, aspirator 90%, pump 10%, and nozzle 0.7-0.5 mm. For MP loaded with the fluorescent marker, we used the same procedure with minor modification. In particular, FDA was added to the polymeric solution at a final concentration of 0.03% w/v and the suspension was homogenized with an ultra-Turrax T25N (Janke and Kunkel, Germany) for 2 minutes at 16,000 rpm prior to processing. Then, this suspension was processed as reported above.

NP were obtained starting from the above cited polymers by a Nano spray dryer B-90 (Büchi Flawil Switzerland). The unique feature of this technology is the droplet generation through a piezoelectric driven actuator that operates at a specific ultrasonic frequency and thus creating a mist of droplets with extremely ultra-fine particle size. The dried particles are electrostatically charged and collected at the collecting electrode surface with minimal particle wastages and high formulation yields [21].

For NP formulations, a preliminary study was conducted to select some parameters, which influence mostly the process. In detail, it was investigated the solvent of polymeric solution feed (water or acetonitrile), the concentration of polymeric feed (0.25, 0.5, and 1% w/v), the spray mesh (4.0, 5.5, and 7.0 μm) the sample flow rate (5-60 mL/h), and the spray rate (100-35%). The spray rate can be only adjusted indirectly by the relative volume flow, which passes through the membrane.

The best formulation was obtained by a procedure as follows: 125 mg of thiomers were solubilized in 50 mL of a 1/1 (v/v) mixture of acetonitrile and acetic acid aqueous solution (0.7% v/v). These solutions were atomized in the Nano spray dryer B-90 to form NP. This apparatus was connected to a cooling unit, the Inert Loop B-295, for safe operation of solvents in a closed-mode configuration. Nitrogen was used as an inert gas, at 1.5 bar, to avoid forming an explosive gas mixture. CO₂ gas was used to build the electrical field for separation of the particles at 1.5 bar. The spray-drying experiments were performed by setting a pressurized N₂/CO₂ at a flow rate of 100 L/min, T inlet 40 °C using spray meshes with 7.0 μm at an ultrasonic frequency (60 kHz) feed rate 5 mL/h, and spray rate 35-45%. In these conditions outlet temperature ranged from 30-32 °C.

Similarly, NAC- or GSH-GC NP containing FDA were prepared by solubilizing FDA (0.03% w/v) in the feed solution.

2.10. Characterization of micro- and nano-particles

The prepared MP and NP were evaluated regarding yield, particle size distribution, morphology, entrapment efficiency and load of FDA. The following equation was used to calculate the percentage yield of the spray dried product:

$$\% \text{ Yield} = \text{weight of recovered MP (NP)} / \text{weight of processed polymers}$$

The particle size distribution was determined directly after the spray-drying process. The particle size was determined by direct observation using a light stereomicroscope (LEICA III) equipped with a

Panasonic camera (WV CP 230) interfaced with an image analysis program (Leica Qwin 2.4 software). The average diameter value with relative standard deviation was obtained through evaluation of arithmetic mode average of the individual values of at least 200 particles for each formulation.

A Zeiss Sigma field emission scanning electron microscope (FE-SEM) operating in the range of 0.5-20 kV and equipped with an in-lens secondary electron detector was used for particle characterization. Sample powders were set onto stainless-steel stub sample holders, by double-sided conductive carbon, and a low accelerating voltage of about 1.5 kV was applied, allowing to reduce the charging on the surface of the organic sample tape. Statistical analysis of the size (MP and NP average size and size distribution) of the samples were performed by using a freeware image analysis program.

FDA entrapment efficiency percentage (EE %), and payload were determined starting from 10 mg of FDA loaded MP or NP digested in 3 mL of 5 M NaOH for 3 hours at 37 ± 0.5 °C. This solution was appropriately diluted and assayed for fluorescence at an excitation wavelength of 450 nm and an emission wavelength of 520 nm using Perkin Elmer 2030 multilabel reader Victor TMX3. The quantity of FDA was calculated starting from a calibration curve of FDA (concentration range: 8×10^{-4} - 1.25×10^{-5} mg/mL, $R^2 = 0.996$) treated with the same procedure reported above [22], using the following equations:

$$\text{EE (\%)} = \text{actual loading FDA/theoretical loading FDA}$$

$$\text{Payload} = \mu\text{g of FDA recovered from carrier/mg of carrier}$$

Where actual loading is FDA recovered in the particles, and theoretical loading is the total amount of FDA used in the preparation.

2.11. *Ex vivo mucoadhesion study*

Porcine urinary bladders (10-12 months) obtained from a local slaughterhouse (Saponaro and Ciavarella Bari, Italy) were used for the evaluation of mucoadhesive properties of NP. A reported analytical method to determine the amount of remaining fluorescent marker (FDA) on the mucosa was used with some modifications [22]. The experiments were performed by using glass static vertical diffusion Franz cells and porcine bladder. Each bladder was washed with 0.05 M PBS pH 7.4, cut in pieces and frozen at -20 °C until its use. For the experiments, a piece of bladder was pre-equilibrated in PBS solution at 25 ± 0.5 °C for 1 h, and then was sandwiched securely between donor and receptor

compartments of the Franz cell with the mucosal side facing the donor compartment and equilibrated at 37 ± 0.5 °C, 100% of humidity for 10 min. The receptor compartment was filled with 10 mL of 0.05 M PBS pH 7.4, which was continuously thermostated at 37 ± 0.5 °C throughout the experiments. A suspension of NP (3 mg) in artificial urine, pH 5.0, (1 mL) was introduced in the donor half-cell and the top was covered with Parafilm to prevent evaporation. After 60 min of contact between NP and bladder mucosa, the piece of bladder (about 2 cm²) was recovered, and to simulate voiding urine, it was fixed on an inclined plane and washed out dropwise with artificial urine (10 mL). Then, the tissue was again mounted on the Franz cell with 1 ml of artificial urine in donor chamber and the wash out was repeated for other two times after 1 h of incubation. Latterly, the tissue was treated with 20 mL of 5 M NaOH for 60 min in order to hydrolyze FDA, loaded in the NP adherent at the mucosa, into sodium fluorescein. The percentage of FDA that remained was considered an index of mucoadhesion.

2.12. Statistical Data Analysis

Statistical significance was assigned to $p < 0.05$ (*) and calculated using a one-way analysis of variance (ANOVA) followed by the Bonferroni's Multiple Comparison Test (GraphPad Prism version 5 for Windows, GraphPad Software, San Diego, CA).

3. Results and Discussion

Two thiolated GC polymer conjugates were designed and developed in order to retain the water solubility of the pristine polymer at pH of at least 5.0 without losing the advantages of improved mucoadhesive properties. Namely, GSH- and NAC-GC polymer conjugates were synthesized by carefully controlling their molecular weight and surface sulfur content and extensively characterized in terms of solubility at different pH values, substitution and swelling degree. Furthermore, starting from the two novel thiolated GC derivatives, the corresponding MP and NP were prepared (Chart 1), thus formulating more promising carriers for intravesical drug delivery. Finally, particle yield, size and morphology, oxidation of surface thiol groups and *ex-vivo* mucoadhesion property were investigated and evaluated.

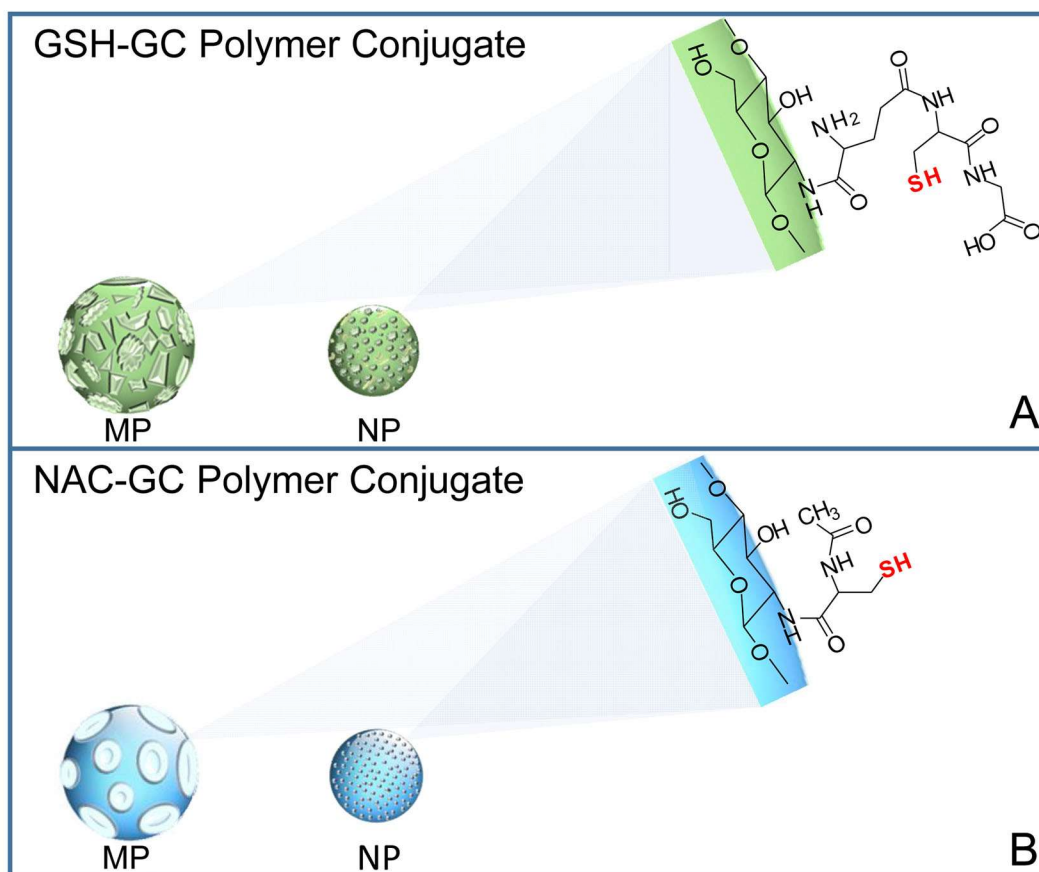


Chart. 1. Schematic illustrations of MP and NP prepared starting from GSH- (A) and NAC-GC (B) polymer conjugate.

3.1. Synthesis of GSH- and NAC-GC polymer conjugates

Synthetic pathways of GSH- and NAC-GC polymer conjugates, and their chemical substructure are reported in Fig. 1. In a similar fashion already reported in literature for the synthesis of GSH- and NAC-CS and GC polymer conjugates [9,10,16], GSH- and NAC-GC thiomers were obtained by the formation of amide bonds between primary amino groups linked to the C-2 of pyranose ring of GC backbone and glycine carboxylic acid groups or cysteine carboxylic acid groups of GSH and NAC, respectively. Thiol groups of polymers, and other thiols in the proteins, can be oxidized to disulfides in aqueous solutions by intra- and/or intermolecular reactions [23,24]. The pH affected the oxidation lability of thiol groups. In particular, it was demonstrated that the rate of cysteine oxidation to cystine was accelerated at higher pH values, when the sulfhydryl group of the amino acid was deprotonated [25]. Hence, reactions were carried out in an acidified solution at pH 5.0, instead of pH 6.0 as reported by Trapani et al. [10]. Furthermore, since the thiol groups oxidation strongly depends on the reaction time and the adopted purification procedure, suitable methods were explored as above mentioned.

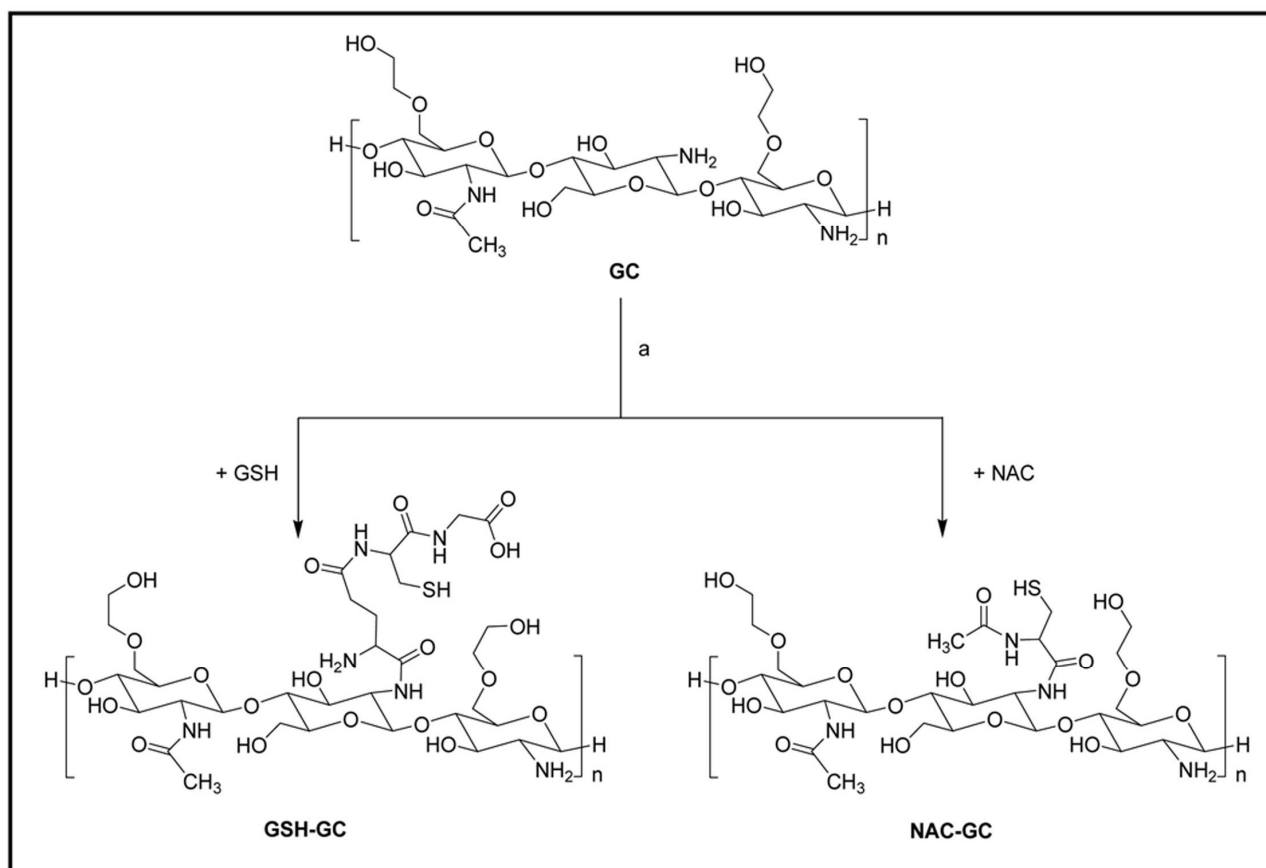


Fig. 1. Synthetic pathway of GSH- (left) and NAC-GC (right) polymer conjugate. a) NHS and EDAC in hydrochloric acid solution (pH 5.0) at r. t.

The obtained thiomers appeared odorless powders with a fibrous structure and easily hydratable in aqueous solution, forming a solution of initially low viscosity. The chemical structure of GSH- and NAC-GC polymer conjugates were confirmed via $^1\text{H-NMR}$ and FT-IR (Fig. S1 and Fig S2) and are in good agreement with that previously reported [10].

3.2. Determination of the thiol group content

In order to select the batch with the maximum amount of free thiol groups immobilized on the polymeric backbone during the conjugation, and with the lowest content of disulfide bonds, all samples were subjected to the Ellman's assay (total and partial). The total thiol moieties ($\Sigma\text{-SH}$) given by the sum of reduced (-SH) and oxidized (-S-S-) thiol groups were determined via Ellman's reagent, quantifying free thiol groups (partial assay) and disulfide bonds, after their reduction with NaBH_4 at $37\text{ }^\circ\text{C}$ for one hour. The amount of disulfide bonds was calculated subtracting the quantity of free thiol groups from total thiol moieties present on GSH- and NAC-GC polymer conjugates.

Ellman's assay results recorded for samples prepared at different reaction times are listed in Table 1, where the percentage of disulfide bonds on the total thiol group basis is indicated as $(-S-S-/\sum-SH)$. As shown in Table 1, GSH- and NAC-GC polymers synthesized with the optimized procedure monitoring temperature, pH and reaction times followed by fast purification, exhibited a content of free thiol groups that reached the maximum after 3 h of reaction. In particular, GSH- and NAC-GC are characterized by 3.6 and 6.3 mmol of immobilized free thiol groups, and 0.2 and 0.8 mmol of disulfide bonds per gram of polymer conjugate, respectively. These findings confirm a high degree of chemical modification of the starting GC polymer associated to low level of thiol oxidation. Interestingly, as presented in Table 1, GSH- and NAC-GC are characterized by a total amount of disulfide bonds that reached the highest percent (12.8 and 26.5%, respectively) after 6 h of reaction, and these values are much lower than those previously reported (50.8 and 50.4%, respectively) [10]. This significant decrease in oxidation of the immobilized thiol groups on the GSH- and NAC-GC polymer conjugates is probably due to the more acidic reaction conditions at pH 5.0, optimized reaction time (*i. e.* 3 h) and the rapid purification method. Moreover, from the results listed in Table 1, it could be also observed that the ease of oxidation of dithiols ($-S-S-$) increased as the number of thiols ($\sum-SH$) in the polymer increased. This suggested that thiol oxidation was also related to spatial factor. As the reaction requires two residues, when the thiol groups of two GSH or NAC residues were brought near each other, an oxidation reaction could generate a disulfide bond. By preserving the thiol groups of the polymers from oxidation, the disulfide formation between thiomers and mucin should be significantly promoted improving the mucoadhesion properties of the synthesized thiomers.

Table 1. Amount of immobilized thiol groups in reduced and oxidized form for GSH- and NAC-GC at different reaction times.

Polymer	Reaction time (h)	-SH			$\sum-SH$	$-S-S-/\sum-SH$ (%)
		-S-S-		(mmol/g polymer)		
GSH-GC ^a	1	2.3 ± 0.1	0.2 ± 0.1	2.5	8.0	
	3	3.6 ± 0.1	0.2 ± 0.1	3.8	5.2	
	6	3.4 ± 0.2	0.5 ± 0.1	3.9	12.8	
GSH-GC ^b	7	2.8 ± 0.1	2.9 ± 0.5	5.7	50.8	
NAC-GC ^a	1	4.9 ± 0.2	0.6 ± 0.2	5.5	10.9	
	3	6.3 ± 0.1	0.8 ± 0.1	7.1	11.2	

	6	6.1 ± 0.3	2.2 ± 0.4	8.3	26.5
NAC-GC^b	7	6.2 ± 0.2	6.3 ± 0.3	12.5	50.4

^a Data are mean values of three separate experiments. ^b Taken from Trapani et al. [10]. -SH: free thiol group; -S-S-: disulfide bond; Σ -SH: total amount of thiol moieties. Data represents mean \pm SD, $n=3$.

3.3. Molecular weight by size exclusion chromatography

The average molecular weights and molecular weight distributions of GC and their conjugates synthesized at different reaction times were determined by SEC using a suitable mobile phase and results are reported in Fig. S3 and Table 2. Molecular weight characterization of CS and its derivatives by SEC have been reported by several authors which used selective conditions to dissolve the sample and to avoid chemical interaction between the column matrix and the sample molecules as well. Precisely, acetate buffer at pH 4.2-4.5 have been previously used [13-15]. Instead, Trapani et al., used different conditions to elute the samples, exactly PBS at pH 7.2 [10]. To eliminate ionic interactions between resin surface and sample polymers GC, GSH- and NAC-GC, in the present study acetate buffer solution pH 4.5 with a neutral salt as sodium nitrate 0.05 M was used as mobile phase. In particular, as reported in Table 2 the value of weight average molecular weight (M_w) observed for the pristine GC was 273.8 kDa, and as expected, increasing values were recorded for GSH- and NAC-GC. Particularly, the measured molecule weights for GSH- and NAC-GC thiomers proportionally improved with increasing reaction times, but plateaued after 3 h. In fact after extending the reaction time to 6 h, only a slight increase of molecular weights was recorded. The polydispersity index (PDI) of all conjugates was in the range 3.3-3.8, suggesting a large dispersity as for the pristine GC (Table 2).

Table 2. Average molecular weights and polydispersity of GC and their conjugates with NAC and GSH prepared at different reaction times and SEC derivatization degree.

Polymer	Reaction time (h)	M_n^a	M_w^b			PDI ^e (M_w/M_n)	DI ^f (%)
			M_p^c	M_{z+1}^d	(kDa \pm SD)		
GC	-	75.5 \pm 0.5	273.8 \pm 1.6	265.3 \pm 8.4	649.7 \pm 8.5	3.3 \pm 0.3	-
NAC-GC	1	77.6 \pm 0.3	284.2 \pm 1.8	271.8 \pm 5.2	671.9 \pm 3.7	3.3 \pm 0.1	3.6

	3	89.0 ± 17.4	295.0 ± 13.1	283.1 ± 5.5	775.4 ± 130.2	3.3 ± 0.1	23.3
	6	89.9 ± 10.8	297.7 ± 17.2	289.0 ± 10.4	804.9 ± 63.4	3.3 ± 0.1	24.8
GSH-GC	1	82.7 ± 12.1	229.4 ± 47.5	251.1 ± 13.6	566.1 ± 175.2	3.4 ± 0.4	6.2
	3	123.2 ± 13.5	344.6 ± 32.4	274.7 ± 5.0	977.1 ± 126.7	3.8 ± 0.1	41.3
	6	123.4 ± 14.1	347.9 ± 28.3	277.2 ± 8.0	992.5 ± 130.2	3.8 ± 0.5	41.4

^a Number average (Mn) molecular weight; ^b Weight average (Mw) molecular weight; ^c Molar mass at the peak maximum (Mp); ^d Higher average (Mz+1) molecular weight; ^e Polydispersity index (PDI); ^f Derivatization degree (DI).

These results together with those observed with Ellman's assay suggested that 3 h of reaction time is a suitable period to obtain GSH- and NAC-GC polymers with an adequate conjugation. As a consequence, the remaining studies were conducted on batches of GSH- and NAC-GC polymer conjugates produced with a time reaction of 3 h.

3.4. X-ray Photoelectron Spectroscopy (XPS) analysis

XPS analyses were carried out to obtain information on the surface distribution of reduced (*i.e.* the -SH) and oxidized (*i.e.* the -S-S-) thiol groups immobilized on GSH- and NAC-GC thiomers. An ingenious approach was here contrived and explored to clearly discriminate the thiol groups from the disulfide ones. In particular, a selective derivatization of the two polymer conjugates carrying the thiol groups with a molecule (BTDDS) close in structure to the Ellman's reagent was achieved, as schematically illustrated in Fig. 2. XPS analysis was also performed on the molecule used for the derivatization reaction, in order to check its stoichiometry (spectra not reported). This analysis confirmed that the ratio between the trifluoromethyl (CF₃) groups (292.9 eV) percent to nitro (NO₂) ones is 1.0. Furthermore, the NO₂ to -S-S- ratio was found to be equal to 1.0, as well. Finally, the analysis revealed that part of the NO₂ groups are oxidized to nitrate, since a second component set at 406.7 eV is present next to the main one at 405.6 eV, in the N1s high-resolution spectrum (not reported) [11].

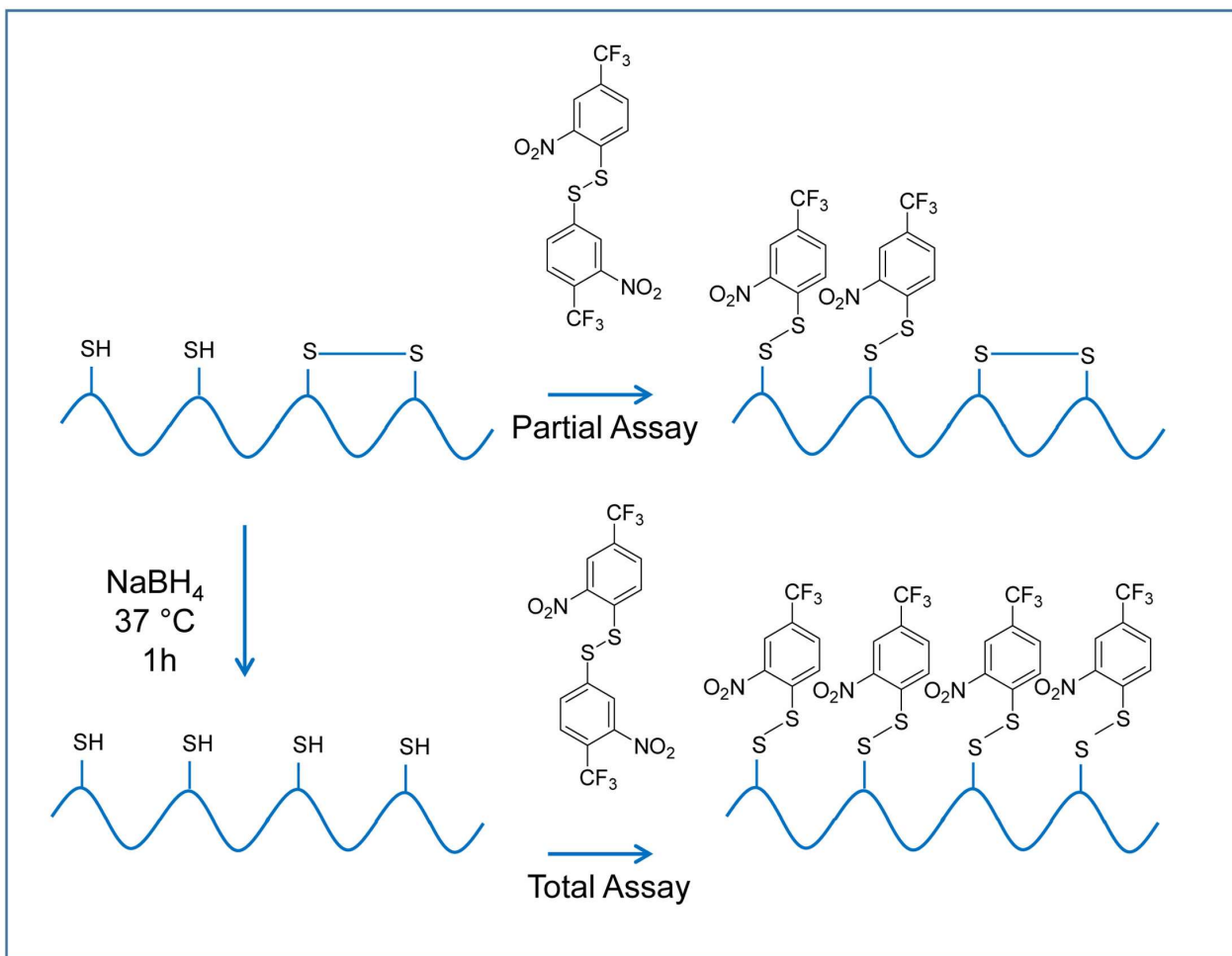


Fig. 2. Derivatization reactions of GSH- and NAC-GC polymer conjugates with BTDDS to quantify the free thiol groups (partial assay) and the total thiol moieties (total assay) present on the thiomers.

The S2p and N1s high-resolution spectra of the pristine GSH- and NAC-GC polymers, as well as, their corresponding spectra after derivatization in the total assay are presented in Fig. 3. The S2p signal of pristine NAC-GC polymer is composed of a doublet, due to the spin-orbit coupling, with the main S2p_{3/2} peak set at 163.1 eV, typical of -SH and -S-S- functionalities (Fig. 3 a). In the N1s spectrum can be clearly observed the three peaks corresponding to amine (399.2 eV), amide (400.5 eV) and protonated amine (401.6 eV) moieties, respectively (Fig. 3 b). Conversely, in the S2p spectrum of the same polymer after the derivatization reaction in the total assay, the doublet assigned to thiols or unreacted disulfide groups shifts at 163.9 eV (S2p_{3/2}), and a second one appears at 167.7 eV (S2p_{3/2}) which is typical of sulfur bonded to oxygen (Fig. 3 c). In the N1s spectrum, beside the low binding energies components corresponding to amine, amide and protonated amine groups, two additional peaks appear at 405.9 eV and 407.4 eV, due to NO₂ and NO₃ groups, respectively (Fig. 3

d). Once more, nitrate groups apparently come from the oxidative degradation of nitro groups. In the C1s spectrum, the main difference that can be observed upon derivatization in the total assay, is the appearance of the peak due to CF₃ groups (not shown).

The S2p signal of the pristine GSH-GC polymer shows already two doublets: a peak centred at 163.6 eV (S2p_{3/2}), ascribable to -SH and -S-S groups, and a peak of much lower intensity, at 168.2 eV (S2p_{3/2}), due to oxygen-containing sulfur species (Fig. 3 e). The corresponding N1s spectrum consists of three peaks: amine one at 399.5 eV, the amide one at 400.2 eV and the protonated amine at 401.8 eV (Fig. 3 f). Upon derivation in the total assay, the S2p spectrum shows an increase of the oxygen-containing sulfur species, while in the N1s spectrum, nitro groups appear at 406.0 eV, along with the component at 407.4 eV due to nitrates (Fig 3 g and h). Even in this case, the derivatization reaction leads to the appearance of the CF₃ peak in the C1s spectrum (not reported).

Quantitative information on the free thiol and disulfide bonds can be inferred from curve fitting of the S2p, N1s and C1s high-resolution spectra (Table 3). The amount of disulfide groups can be calculated by subtracting the amount of free thiol groups, given by the percent of NO₂ and CF₃ groups (Fig 3, Partial Assay), from the sum -SH and S-S % reported in Table 3. This outcome can be further confirmed by comparing the results of the partial and the total assay: the disulfide group percent, indeed, can be also obtained subtracting the thiol percent of the partial assay from the corresponding of the total one. From the partial assay carried out on NAC-GC, it can be concluded that the free thiol group percent is 2.4%, which is the percent of NO₂ and CF₃ groups (Fig. 2). Since the corresponding -SH or -S-S % is 2.5%, the disulfide group percent amounted to 0.1%, within the experimental error of the measurements. Likewise, the amount of disulfide moiety obtained by comparing the total assay with the partial one is equal to 0.1-0.2%, thus highlighting that the both analysis techniques indicated a negligible amount of disulfide bonds in the NAC-GC.

In the case of GSH-GC (partial assay), the amount of thiol groups as determined by the derivatization reaction is 2.5%, and the percent of disulfide groups amounted to 0.3%, thus resulting within the experimental error of the measurements ($\pm 0.3\%$). The same conclusions can be drawn for the GSH-GC sample (total assay), with a total amount of -SH groups of 2.8% and only 0.3% of -S-S- bonds (subtracting the -NO₂ (or CF₃) percent). The difference between the total thiols and the free ones of the partial assay yields a disulfide bond percent of 0.7%, which is quite within the experimental error.

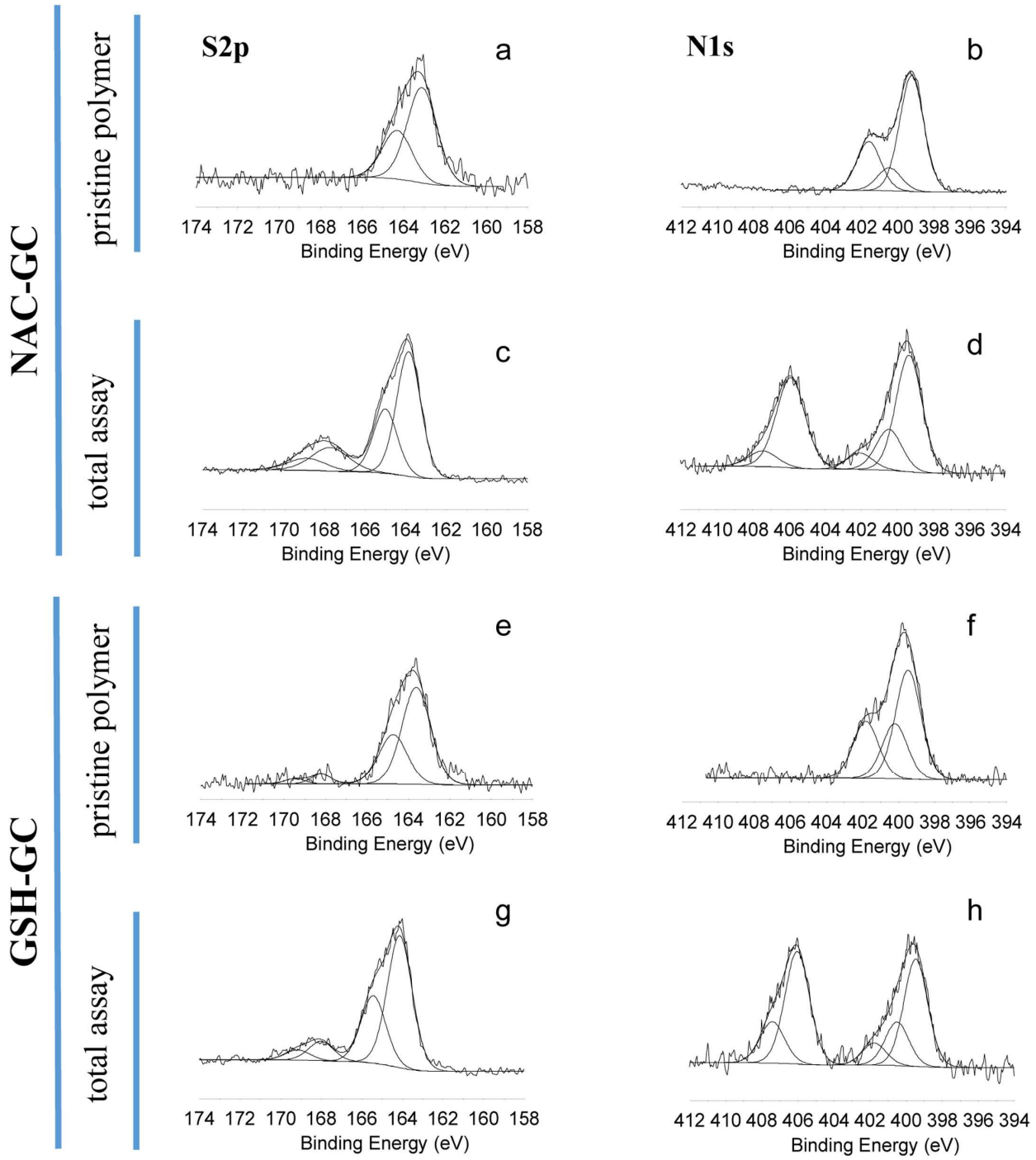


Fig. 3. XPS S2p and N1s high-resolution spectra of the pristine GSH- and NAC-GC polymer (a, b, e and f) and their corresponding spectra after derivatization reaction in the total assay (c, d, g and h)

Table 3. XPS quantitative results from curve fitting of S2p, N1s and C1s spectra.

Polymer	Assay	-SH and -S-S- -NO ₂ ^a -CF ₃		
		(%)		
NAC-GC	partial	2.5	2.4	2.4
	total	2.6	2.5	2.5
GSH-GC	partial	2.8	2.5	2.5
	total	3.2	2.8	2.8

^a This is actually the sum of the NO₂ and NO₃ percent, coming this latter from oxidation of nitro groups as stated in the text.

3.5. Solubility and swelling studies in artificial urine

The relative solubility of GSH- and NAC-GC polymer conjugates at 20 mg/mL in artificial urine in the pH range of 5.0-8.0 was checked and the results are shown in Table 4. GC was selected as starting polymer for the conjugations because it is more hydrophilic than CS. Indeed CS is considered a capable pharmaceutical excipient, with interesting properties such as mucoadhesion, sustained release and permeation-enhancing effects, but these characteristics are considerably influenced by its insufficient aqueous solubility and pH dependence [26]. To overcome this limitation, several CS derivatives have been proposed in literature [26-29]. In this study, starting from GC, two thiomeric derivatives were prepared to increase both solubility and mucoadhesive capacity in biological fluids such as urine. As shown in Table 4, the synthesized derivatives GSH- and NAC-GC are easily hydratable at the concentration assayed (2% w/v) in the range of studied pH (5.0-8.0). In particular, their solubility values were higher than parent GC (~ 1.5% w/v) and CS (< 1.5% w/v), selected as references. The water solubility of the CS, GC and of the thiolated polymers is influenced by the molecular weight, acetylation degree as well as by type of substituents linked to the polymer backbone, and were evaluated in terms of turbidity [30]. GC derivatives have shown to be completely soluble in a concentration range of 1-2% w/v. They swell and then gave clear aqueous solutions, as detected by the transmittance values (Fig. 4). Based on this improved fundamental property, it was concluded that GSH- and NAC-GC polymers are better excipients than GC and CS to develop bladder formulations, where wide variations in individual urine pH are possible.

Table 4. Solubility of CS, GC, GSH- and NAC-GC polymers (2% w/v) at different pH values in artificial urine.

pH	Polymer			
	CS #	GC*	NAC-GC	GSH-GC
5	-	-/+	+	+
6	-	-/+	+	+
7	-	-/+	+	+
8	-	-/+	+	+

+ complete dissolution; -/+ incomplete dissolution; - not soluble. # solubility < 1.5% w/v; * Solubility GC ~ 1.5% w/v.

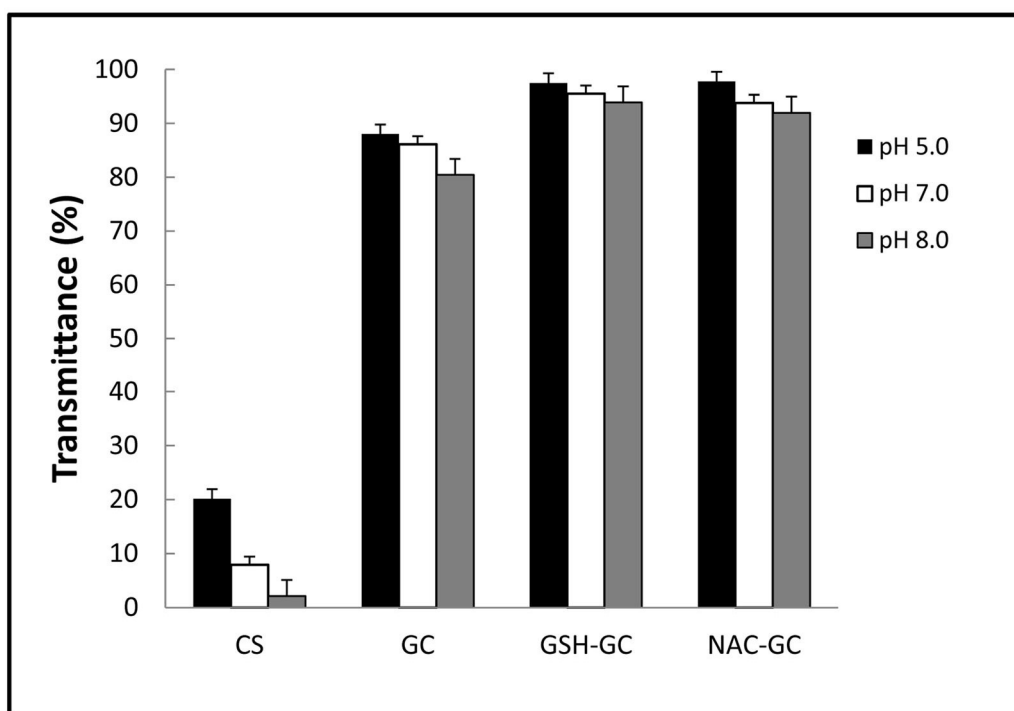


Fig. 4. Dependence of solubility of CH, GC, NAC-GC and GSH-GC in pure water on pH value ranging from 5 to 8. Data represents mean \pm SD, $n=3$.

Preliminary swelling studies were carried out in artificial urine at pH 5.0 and 7.0 by gravimetric methods, evaluating water-uptake capacity of the examined polymers. In particular, after an incubation period of 60 min at 37 °C, GC, NAC- and GSH-GC polymer conjugates displayed a high water uptake in both media, increasing their weight by about 2.0, 2.5 and 2.4 fold, respectively. Probably, the GC derivatives have a greater water uptake than GC because they are more soluble in

aqueous medium, as above demonstrated. These results are in good agreement with the observation made for mucoadhesive gels previously reported by Mortazavi et al. [31].

3.6. Mucoadhesion studies

In order to evaluate if the synthesized thiomers, GSH- and NAC-GC, can be a useful platform to prepare MP and NP to deliver drugs into the bladder by intravesical administration, we decided to study their mucoadhesion properties in artificial urine using both turbidimetric and ζ -potential measurements. For the turbidimetric study it is known that mixing polymer solutions with mucin results in a time dependent reduction in transmittance, due to polymer-mucin aggregates formation, that is observed up to a constant reading [30,31]. The results obtained with GSH- and NAC-GC are shown in Fig. 5 together with those of the parent GC and the well-known mucoadhesive polymer Carbopol 934. In detail, the mucoadhesion properties of the examined polymers in artificial urine at pH 7.0 have the following rank order: NAC-GC > GSH-GC > Carbopol 934 > GC. In artificial urine at pH 5.0 the rank order is: NAC-GC > Carbopol 934 ~ GSH-GC > GC.

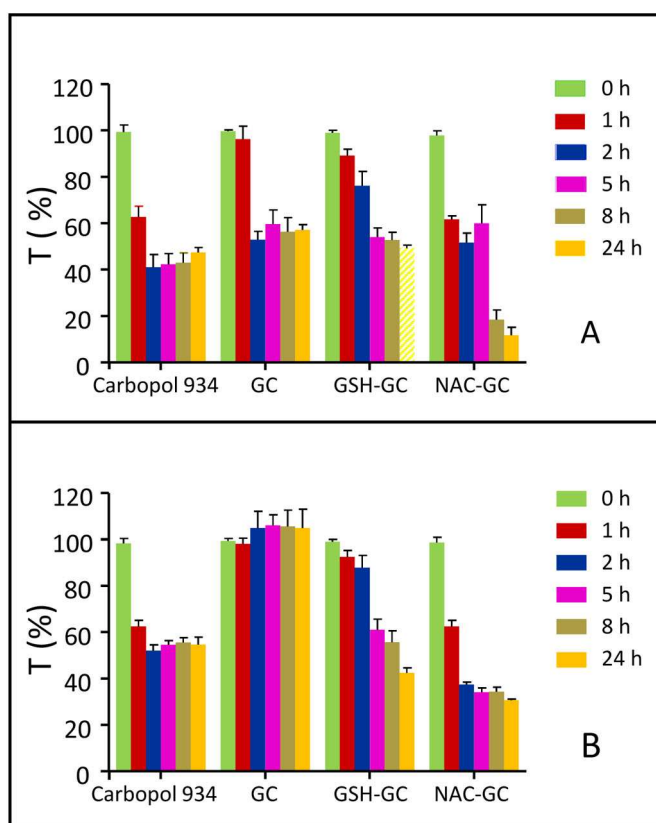


Fig. 5. Turbidimetric measurements of interaction between mucin and polymer in artificial urine at pH 7.0 (A), and pH 5.0 (B). Data represents mean \pm SD, $n=3$.

Based on this evidence it is clear that at pH 7.0 the GC thiomers have good mucoadhesive properties, even better than Carbopol 934. These results are in agreement with the general tendency of thiomers to form bridging structures, as disulfide bonds, between the thiolated polymer and the gel layer mucus of mucosa [33,34]. In fact, thiomers mimic the natural mechanism of secreted mucus glycoproteins, which are covalently anchored in the mucus layer by the formation of disulfide bonds. Interestingly, at pH 5.0 we observed a similar trend, which is probably strictly associated to the pH value. In fact, it is known in literature that the reactivity of thiol groups inside the polymeric network is mainly controlled by the pH of the thioimer, whereas the reactivity on the surface of the polymer is more controlled by the pH of the surrounding medium [33]. Consequently, at pH 5.0 probably most of the superficial thiol groups are not oxidized to disulfides, increasing the number of thiol groups that might interact with those of the mucus. In addition, at pH 5.0 other factors could be essential for the mucoadhesiveness. For instance, at this pH value the polymeric chain, bearing a more cationic character, can lead to a stronger ionic interactions with anionic substructures such as sialic acid and sulfonic acid of the mucus layer providing their mucoadhesivity [35]. NAC-GC has shown the maximum mucoadhesivity at both pH 5.0 and 7.0. This probably due to the high prevalence of thiolic groups in this polymer conjugate as determined by Elmann's assay and XPS analysis as well. Effects of local electrostatic fields could play an important role on the interaction of polymers with mucina. The presence of a carboxylic group on GSH-GC might decrease the interaction with mucina at pH 5 and 7. Moreover, at pH 5.0, NAC-GC has more pronounced mucoadhesion, probably due to the increased electrostatic interaction with mucin. To confirm this statement an interaction study between mucin and NAC-GC was conducted in artificial urine at pH 5.0 by the ζ -potential measurements. It is known that the surface properties of the mucin particles can be changed by the adhesion of the polymer if the polymer has mucoadhesive property. The occurrence of such change can be detected by measuring the ζ -potential [20]. In this study we compared NAC-GC polymer conjugate with pristine GC and two well-known mucoadhesive polymers: CS [20] and NAC-CS, synthesized as reported in literature [16]. Fig. 6 shows that the addition of polycation polymers to the mucin dispersion leads to an increase in ζ -potential, from negative values of mucin alone (-10 mV) to positive ones (+24 mV), reaching a plateau corresponding to the full coverage of mucin surface with the tested polymer. This variation could be explained with the ionic interaction between negatively charged sialic acid residues in mucin and positively charged amino groups in GC and NAC-GC [36].

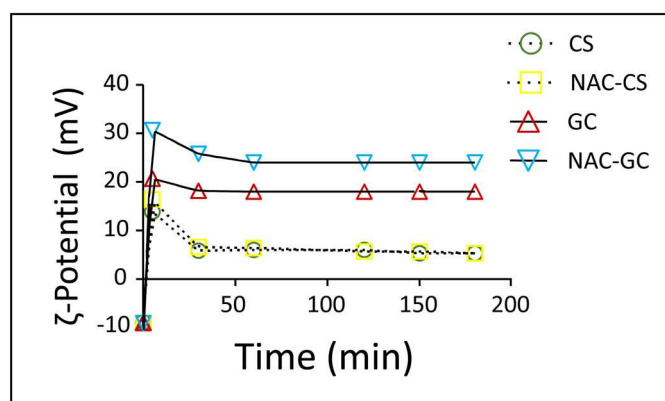


Fig. 6. Change in observed ζ -potential of mucin particles when mixed with polymers solution 0.1% w/v. Concentration of mucin suspension 1% w/v.

In particular, NAC-GC reached a positive value similar to that of GC and higher than CS. Moreover the gap in NAC-GC was larger than GC (from +32 to +24, and from +21 to +18 mV, respectively). Although the process of mucoadhesion cannot be exhaustively described by just one of theories proposed in literature due to its relative complexity, the highest positive charge values recorded for NAC-GC polymer compared with those obtained for the other investigated cationic polymers (CS, GC and NAC-CS) clearly suggest that, according the electronic theory, the mucoadhesive ability can be mainly ascribable to the ionic interactions between these polymers and biological substrates [2]. Namely, when both materials possessing opposite electrical charges come into contact, they transfer electrons leading to the building of a double electronic layer at the interface, where the attractive forces within this electronic double layer determines the mucoadhesive strength [2]. Furthermore, since the new synthesized polymers are more hydrophilic than parent GC and the well-known mucoadhesive CH, as suggested by the solubility and swelling studies, an intimate contact (wetting) can also occur between the mucoadhesive and mucous membrane, thanks to their good affinity, thus promoting the first step of mucoadhesive process in an easy and fast manner. This phenomenon is essential, since micro and nanoformulation which can be obtained starting from the here synthesized thiolated polymer conjugates, could be retained in an improved intimate contact with the absorption site.

Conversely, in the second step of the mucoadhesive process (consolidation stage), the most reliable mechanism being responsible for the enhanced or improved mucoadhesion property of thiolated chitosans is based on their *in situ* cross-linking property. During and after the interpenetration process, which could be verified for mucoadhesive polymers, disulfide bonds are formed within the thiolated chitosans itself leading to additional anchors via chaining up with the mucus gel layer [34, 35, 37-39]. Therefore, also in our case it is reasonably to guess that the formation of

disulfide bonds between NAC-GC and mucin may play an important role giving rise to better mucoadhesive properties than those of pristine GC. This effect was less evident at pH 7.0 where the variations of ζ -potential were less significant (data not shown). Therefore, this technique confirms the excellent mucoadhesive properties of NAC-GC especially in acidic environment (pH 5.0). This thiomers shows a better behavior than NAC-CS, which has been proposed as suitable mucoadhesive polymer in previous studies [16]. This is in contrast with the findings already reported on NAC-GC [10], which indicated NAC-CS more mucoadhesive than NAC-GC at a different pH. Probably, this incongruence might be explained by the substantial reduction of disulfide bonds in the new synthesized NAC-GC, which might promote a better mucoadhesion. On this basis, it is possible that the dehydration theory can be also relevant: when a material capable of rapid gelation in an aqueous environment is brought into contact with a second gel as mucous, water movement occurs between gels until equilibrium is achieved [2].

3.7. Preparation MP and NP

Starting from GSH- and NAC-GC polymer conjugates, we decided to investigate the feasibility to formulate them as suitable MDDS for intravesical administration. NP and MP engineered via spray-drying technique, have been proposed because this method is well applied in pharmaceutical industry due to its wide applicability, flexibility and easy scale up [38]. Recent advances in the spray-drying have led to new innovative technologies such as the Nano Spray Dryer B-90 (Fig. 7) developed by Büchi Labortechnik AG.

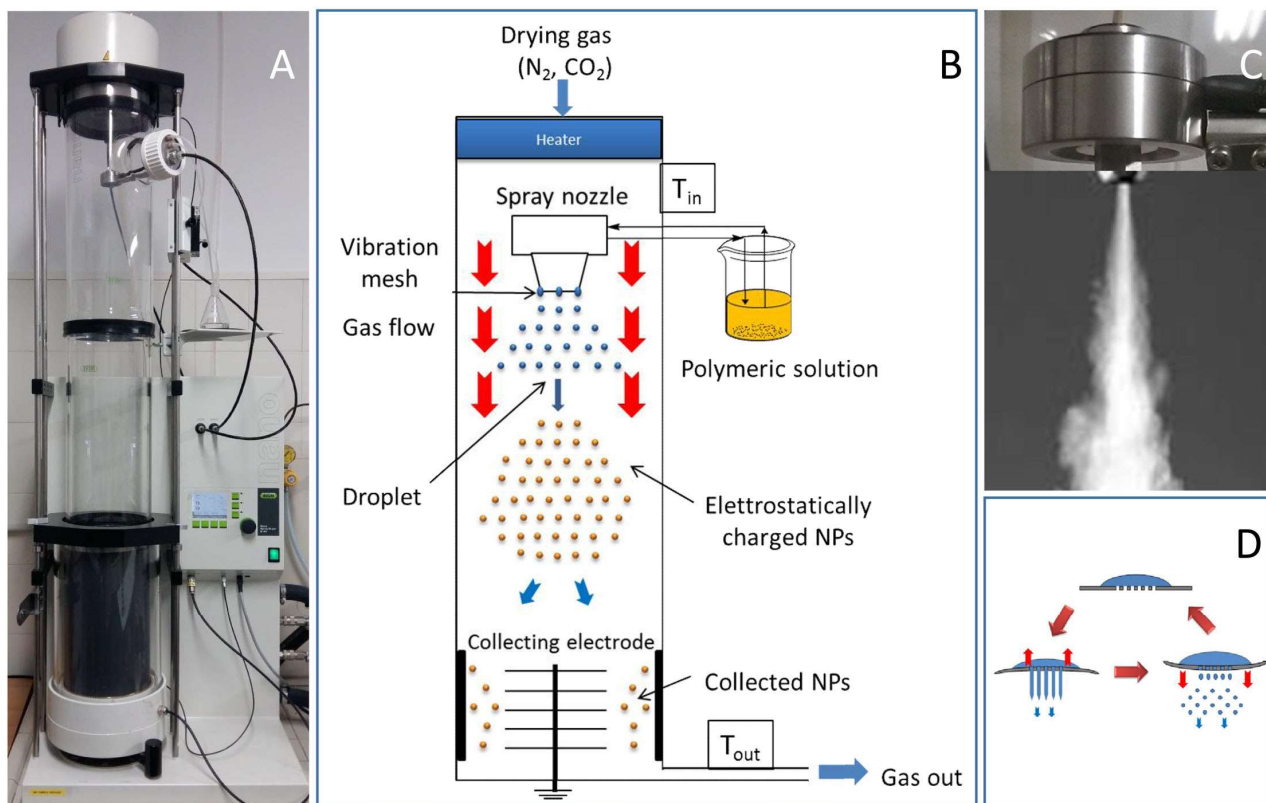


Fig. 7. Picture (A) and schematic illustration (B) of Nano Spray Dryer B-90. Picture of spray nozzle (C). Schematic illustration of vibration mesh size and droplet generation (D).

Recently, Li et al. [12] investigated the advantages and limitations of this new spray-drying process, analyzing the particle size, distribution, homogeneity, morphology and formulation yields of various polymeric materials. Moreover, different experimental conditions to produce drug encapsulated NP or drug crystals have been proposed.

In the present study both MP and NP were prepared using a Büchi conventional or new ultrasonic, spray dryer: namely Micro B-191 and Nano B-90, respectively. In the first approach a feed constituted of GSH- or NAC-GC in aqueous solution was nebulized. NP were prepared starting from a mixture of acetonitrile/water because aqueous solution at different percentages of tested polymers (1-0.25% w/v) were not suitable for an adequate atomization. The process variables were appropriately set up for both instruments. In literature these variables, developed for the classic apparatus such as the micro B-191, are reported for CS and for some CS derivatives [3,12, 38-41]. Hence, MP were produced adopting the previously reported methods with minor modifications.

To our knowledge, no work reports process condition for CS, GC or its derivatives formulations using the new spray dryer B-90. Although we are aware that a thorough study, involving an experimental design, is needed to optimize the instrumental and process parameters, in this first phase the

potentiality and the capacity of the device to produce GSH- and NAC-GC NP, were investigated. In this first step, some factors were identified as responsible to influence strongly the process including the failure. Indeed, an optimized process generates NP with appropriate characteristics of size, size distribution, that are closely related to the bioavailability of the formulation, and high yield of process essential to evaluate an economic advantage. Therefore, we identified basic conditions in order to atomize and dry NAC- and GSH-GC polymers. In this regard, some general considerations are important to be reported: *i*) polymeric aqueous solutions at concentrations of 1, 0.5 and 0.25% w/v are not proper to atomize the feed, even with cap of 7 μm meshes; *ii*) organic solutions such as acetonitrile can allow the atomization of feed at polymeric concentration of 0.25% w/v; *iii*) spray rate must be set to values lower than 50%; *iv*) rate feed must have a suitable speed, which can be selected among the options of the peristaltic pump. These conditions were chosen after several justified attempts aimed to achieve NP with nanometric size, narrow size distribution and high yield. Generally, in this process the formation of the NP is complex and is governed by an interplay of polymer concentration in the droplet, pore size of the vibrating membrane and frequency of membrane (fixed in this study at 60 kHz). The droplet size is mostly influenced by the pore size of the membrane. A smaller pore size will however increase spraying time. The choice of the solvent feeding is critical to the process. This parameter with the polymeric concentration feed have a strong influence on size and size distribution as reported by Drahein et al. [42]. As first attempt, water was selected as solvent of the feeding solution. Unfortunately, this choice proved wrong as the experiments failed and no final product was achieved. This condition was verified at different aqueous polymeric concentration (1, 0.5 and 0.25% w/v) with all the cap size membranes (4.0, 5.5, and 7.0 μm). Probably viscosity and density of these solutions blocked the vibrating membrane. This problem was overcome by using the organic solvent acetonitrile but a yield under 10% was obtained with a polymeric concentration feed of 0.25% w/v. This surprisingly low value may be due to a fast evaporation of solvent forming a polymeric film on collecting electrode that sensibly decreasing the amount of NP recovered. As an alternative, the mixture water/acetonitrile 1/1 v/v was studied because it permit the solubilisation of hydrophilic and lipophilic drugs with high efficiency of encapsulation. The polymeric concentration of feed was fixed at low value (0.25% w/v) in order to produce particles in nanosize range, but this procedure led to a low yield (maximum 25%). To increase this parameter, polymeric concentration at 0.5% w/v was considered, but in this case the spraying was irregular and not continuous. Indeed, generally a constant spraying was hard to realize and it was obtained with low flow feed rate (5 mL/h) and low spray rate 35%.

3.8. Characterization of MP and NP

The GSH- and NAC-GC polymer conjugate based formulations were characterized by determining parameters such as particle yield, size and morphology, as well as, content of thiol moieties and disulfide bonds. In addition, efficiency of encapsulation (EE%) and payload (μg FDA/mg particles) were calculated for formulations containing the fluorescent probe FDA. In detail, both GSH- and NAC-GC MP were produced with yields always higher 50%, while the corresponding NP were recovered with low yields in the range of 15-25% (Table 5).

The average diameters achieved, by stereomicroscopy measurements, for GSH- and NAC-GC MP were found approximately of 2 μm . The corresponding SEM micrographs, reported in Fig. 8 B and E highlight the formation of particles characterized by size heterogeneity with an average size of $2.2 \pm 0.3 \mu\text{m}$ and $2.1 \pm 0.5 \mu\text{m}$, respectively; in addition MP have spherical collapsed or dimpled shape.

Table 5. Yield, diameter (by light stereomicroscope) and FDA efficiency of encapsulation (EE%) of MP and NP obtained by spray drying.

Polymer	Formulation	Yield (%)	Size (μm)	EE ^a (%)	Payload ^b ($\mu\text{g}/\text{mg}$)
NAC-GC	MP	60.5 ± 5.0	1.90 ± 0.6	15.2 ± 2.2	7.6 ± 1.1
	NP	20.0 ± 1.5	0.90 ± 0.5	69.3 ± 5.0	385.2 ± 27
GSH-GC	MP	53.2 ± 3.0	2.27 ± 0.7	18.2 ± 3.1	10.3 ± 1.7
	NP	15.0 ± 3.0	0.80 ± 0.3	100 ± 3	714.2 ± 21
GC	MP	57.5 ± 8.0	1.80 ± 0.8	12.2 ± 3.0	6.8 ± 1.3
	NP	25.0 ± 2.1	0.80 ± 0.6	90.4 ± 6.0	400.3 ± 37

Data are mean values of three repeated experiments. ^a Efficiency of encapsulation = actual loading/theoretical loading *100; ^b μg of FDA/mg of particles. Data represents mean \pm SD, $n=3$.

This morphology is probably due to the water evaporation during the drying process. Indeed, the formed MP in the drying chamber have a consolidated polymeric wall, but aqueous vapor present in the particles creates holes on surface to escape collapsing the polymeric particles. This is also reported in previous works that prepared MP by spray-drying technique [38]. Furthermore, comparing the SEM micrograph of GSH-GC MP with that of NAC-GC MP, a different morphology of the dimples, whose formation occurs on MP surface during the drying process, can be observed, probably due to a different degree of hydrophilicity of the two pristine GSH- and NAC-GC polymer conjugates (Fig

8 A and D). Namely, NAC-GC MP are characterized by the presence on their surface of quite regular spherical dimples, while the surface of GSH-GC MP appears more crumpled, owing the occurrence of dimples with very irregular shape. The NP, as expected, have average diameters smaller than the MP, resulting by stereomicroscopy measurements of about 0.9 and 0.8 μm for NAC- and GSH-GC MP, respectively (Table 5). In particular, NP size was anyhow reduced by 50% ($< 1 \mu\text{m}$), resulting in particles more appropriate for administration by a catheter. According these results, SEM investigation performed on NAC- and GSH-GC NP micrographs revealed the production of well formed NP, which appear smaller than MP, spherical in shape and with average size of $800 \pm 300 \text{ nm}$ and $700 \pm 150 \text{ nm}$, respectively. The NAC-GC NP has a broader size distribution than those obtained starting from GSH-GC polymer conjugate, since the formation of a very low amount of bigger dimpled shaped particle can be still observed along with smaller and well formed NAC-GC NP characterized by quite smooth surface (Fig. 8 C and F).

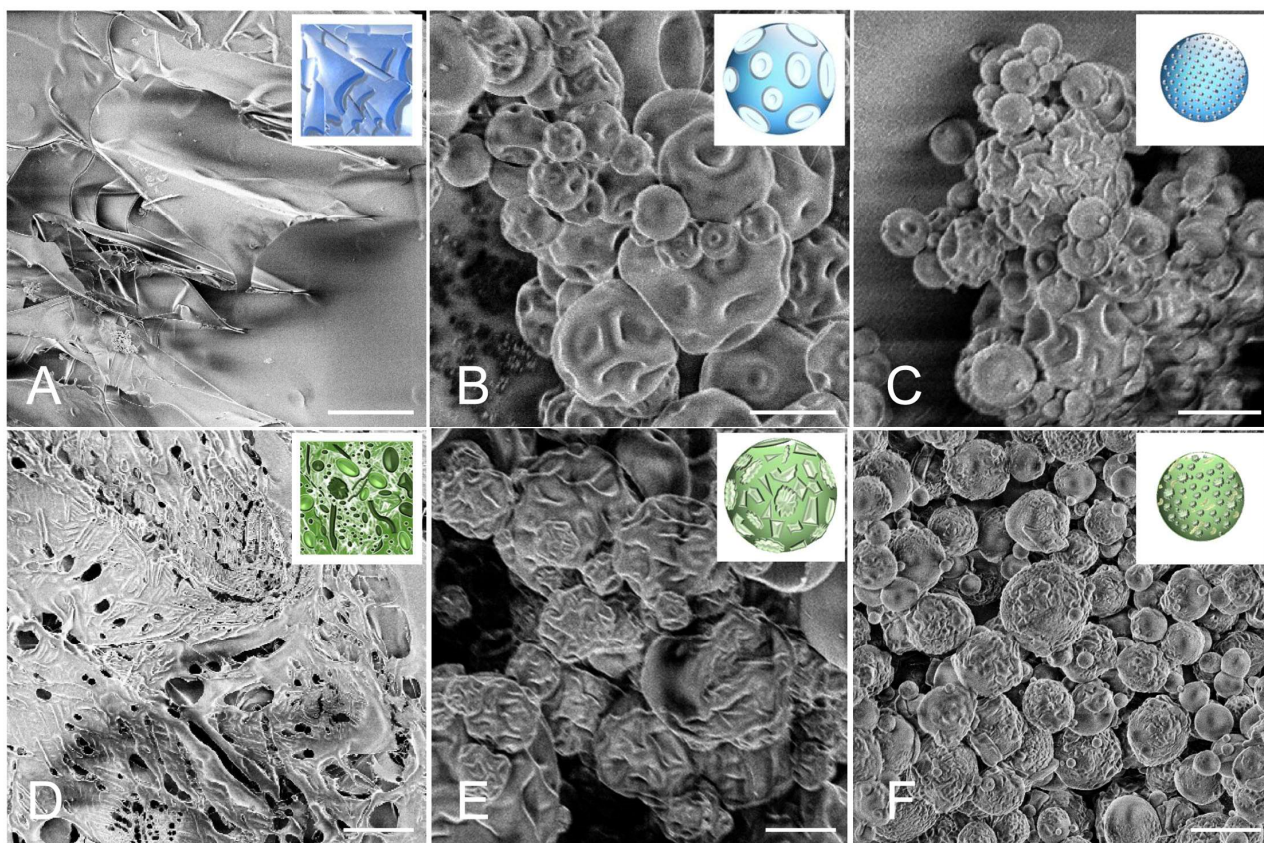


Fig. 8. SEM micrographs of pristine NAC-GC (A), NAC-GC MP (B), NAC-GC NP (C), pristine GSH-GC (D), GSH-GC MP (E), and GSH-GC NP (F). Scale bar 2 μm , accelerating voltage 1.5 kV.

The quantitative analysis of thiol and disulfide groups performed on GSH- and NAC-GC MP revealed that their surface is characterized by high amounts of oxidized thiol groups (Table 6). Indeed, about 20-25% of thiol groups, immobilized on the starting polymers NAC- and GSH-GC (see Table 1),

were converted to disulfide groups during the preparative process of the corresponding MP. Probably, the selected method promotes the thiol oxidation during the process due to the high inlet and outlet temperature (i.e. 145 and 80 °C) and oxygen rich air used as drying gas in the drying chamber. These problems were also reported by Barthelmes et al. [3]. On the contrary, the GSH- and NAC-GC NP produced with the new spray dryer were processed at low temperature (T_{inlet} 40 °C and T_{outlet} 32 °C) in the presence of N_2/CO_2 as the drying gas mixture. This mild operating condition substantially decreases the oxidation of the free thiol groups (Table 6).

As shown in Table 5, the EE% for MP were always lower than those observed for NP. These differences in EE% could be attributed to the poor water solubility of FDA. In fact, in the case of MP production the starting material was a suspension in water, which was replaced by a solution of water/acetonitrile for NP assembly leading to increased EE%.

Table 6. Amount of immobilized thiol groups in reduced and oxidized form for GSH- and NAC-GC MP and NP.

Polymer	Formulation	-SH	-S-S-	Σ -SH	-S-S-/ Σ -SH
		(mmol/g polymer)			(%)
NAC-GC	MP	5.0 ± 0.1	2.2 ± 0.3	7.2	30.5
	NP	6.0 ± 0.1	1.0 ± 0.1	7.0	14.3
GSH-GC	MP	2.7 ± 0.2	1.2 ± 0.1	3.9	30.8
	NP	3.5 ± 0.1	0.5 ± 0.2	4.0	12.5

Data are mean values of three separate experiments. -S-S- : disulfide bond; -SH: free thiol group; Σ -SH: total amount of thiol moieties. Data represents mean ± SD, $n=3$.

Similarly to other studies regarding bladder mucoadhesion of MP and NP, the fluorescent probe FDA was loaded on GSH- and NAC-GC MP and NP for detection purposes [3,5,21]. To further verify the mucoadhesive properties of GSH- and NAC-GC MP and NP, an *ex vivo* mucoadhesion assay was performed using FDA loaded MP and NP. The main difficulty of this test was to simulate the physiological condition of the bladder. To date, few studies address this topic. In previous studies, the emphasis of investigations of DDS for intravesical administrations was focused on the contact between the formulation and the absorption membrane as reported in Burjak et al. [43]. Recent studies have designed different mucoadhesion methods that involved the porcine urinary bladders *in toto* under continuous urine voiding [3,5] or piece of porcine bladder mucosa, which was placed into a

sloped channel, and artificial urine washed over the surface using a syringe pump [43], or bladder piece was incubated with formulation in artificial urine for 2 h and the piece was then rinsed three times with artificial urine [45]. In this study, to simulate the behaviour of MP and NP after intravesical instillation, the mucoadhesion study was designed in two steps: first it was realized a contact between MP or NP and vesical tissue in Franz cell (1 h) then, hourly three micturitions were simulated by washing with artificial urine the piece of tissue fixed on an inclined plane.

The obtained results evidenced a good adhesion of MP and NP on the mucosa bladder after 1 h of exposure. In detail, MP were less retained than NP (data not shown) probably due to the larger size and consequently to the lower specific surface. In fact, an elevated specific surface is important for mucosal contact and subsequent interaction. Additionally, MP are characterized by a thiol content lower than that presented on NP and this is detrimental for the formation of disulphide bridges between the carrier and cysteine rich domains of mucins present on the surface of the bladder as indicated in Cook et al. [44]. Among fluorescent NP, NAC-GC NP showed the best retention on bladder mucosa surface after 1 h (Fig. 9), and this result was significantly different from that observed for GSH-GC NP and GC NP ($p < 0.05$; Fig. 9), confirming the results achieved from mucoadhesion *in vitro* studies.

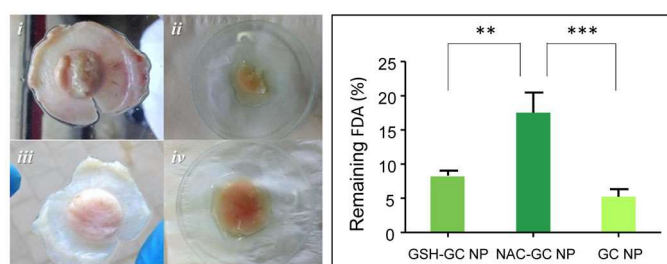


Fig. 9. Left panel, *ex-vivo* mucoadhesion of GSH- NAC-GC NP on pig bladder mucosa. Left panel, GSH-GC NP before (i), and after (ii) hydrolysis of FDA); NAC-GC NP before (iii), and after (iv) hydrolysis of FDA. Right panel, FDA (%) remaining of NP after *ex-vivo* mucoadhesion on pig bladder mucosa. Statistical values $p < 0.05$ (*) were estimated using one-way ANOVA and Bonferroni's Multiple Comparison Test. Data represents mean \pm SD, $n=3$.

4. Conclusion

In this study, GC thiomers, namely GSH- and NAC-GC, were synthesized by an optimized procedure. The newly synthesized thiomers resulted in less oxidized thiol moieties, and showed an advantageous improved water solubility, as well as, mucoadhesive properties, in a wide range of pH, compared to unmodified CS and GC, thus emerging as promising excipients to develop bladder formulations,

where significant variations in individual urine pH can occur. Furthermore, GSH- and NAC-GC MP and NP were obtained starting from parent polymers by spray-drying technique. In particular, instrumental and process parameters were explored, in order to achieve GSH- and NAC-GC polymer based NP by using a novel apparatus Nano Büchi B-90. The here prepared thiolated GC MP and NP, characterized by good mucoadhesive properties, represent effective and suitable candidates for the development of MDDS for intravesical treatment of local bladder diseases, since potentially able to increase the drug residence time in the bladder, and subsequently, the efficacy of therapy. In this perspective, further studies exploring the ability of these MDDS to deliver appropriate drugs for the treatment of bladder diseases are in progress and will be the future development of this work.

Acknowledgements

Authors acknowledge Angelini A.C.R.A.F. R&D for their technical support. Authors thank Mr. Antonio Palermo for his skillful technical assistance in recording NMR spectra. The University of Bari (Italy) and the Inter-University Consortium for Research on the Chemistry of Metal Ions in Biological Systems (C.I.R.C.M.S.B.) are also gratefully acknowledge for their financial support. This work was partially supported by PON project “Laboratorio per lo Sviluppo Integrato delle Scienze e delle Tecnologie dei Materiali Avanzati e per dispositivi innovativi-LABORATORIO SISTEMA”.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/xx.xxxx/j.actbio.xxxx.xx.xxx>.

References

- [1] A.K. Singla, M. Chawla, A. Singh, Potential applications of carbomer in oral mucoadhesive controlled drug delivery system: a review, *Drug Dev. Ind. Pharm.* 26 (2000) 913–924.
- [2] J.D. Smart, The basics and underlying mechanisms of mucoadhesion, *Adv. Drug Deliv. Rev.* 57 (2005) 1556–1568.
- [3] J. Barthelmes, S. Dünnhaupt, S. Unterhofer, G. Perera, W. Schlocker, A. Bernkop-Schnürch, Thiolated particles as effective intravesical drug delivery systems for treatment of bladder-related diseases. *Nanomedicine*, 8 (2013) 65–75.

- [4] A. Štorha, E.A. Mun, V.V. Khutoryanskiy, Synthesis of thiolated and acrylated nanoparticles using thiolene click chemistry: towards novel mucoadhesive materials for drug delivery, *RSC Adv.* 3 (2013) 12275–12279.
- [5] J. Barthelmes, G. Perera, J. Hombach, S. Dünnhaupt, A. Bernkop-Schnürch, Development of a mucoadhesive nanoparticulate drug delivery system for a targeted drug release in the bladder, *Int. J. Pharm.* 416 (2011) 339–345.
- [6] S. GuhaSarkar, R. Banerjee, Intravesical drug delivery: Challenges, current status, opportunities and novel strategies, *J. Control. Release*, 148 (2010) 147–159.
- [7] R.J. Evans, Intravesical therapy for overactive bladder, *Curr. Urol. Rep.* 6 (2005) 429–433.
- [8] A. Makhlof, M. Werle, Y. Tozuka, H. Takeuchi, Nanoparticles of glycol chitosan and its thiolated derivative significantly improved the pulmonary delivery of calcitonin, *Int. J. Pharm.* 397 (2010) 92–95.
- [9] K. Kafedjiiski, F. Föger, M. Werle, A. Bernkop-Schnürch, Synthesis and in vitro evaluation of a novel chitosan–glutathione conjugate, *Pharm. Res.* 22 (2005) 1480–1488.
- [10] A. Trapani, C. Palazzo, M. Contino, M.G. Perrone, N. Cioffi, N. Ditaranto, N. A. Colabufo, M. Conese, G. Trapani, G. Puglisi, Mucoadhesive properties and interaction with P-Glycoprotein (P-gp) of thiolated-chitosans and -glycol chitosans and corresponding parent polymers: a comparative study. *Biomacromolecules.* 15 (2014) 882–893.
- [11] G. Beamson, D. Briggs, *High Resolution XPS of Organic Polymers*, John Wiley and Sons Ltd, Chichester, England (1992)
- [12] X. Li, N. Anton, C. Arpagaus, F. Belletex, T.F. Vandamme, Nanoparticles by spray drying using innovative new technology: the Büchi nano spray dryer B-90, *J. Control. Release*, 147 (2010), pp. 304-310
- [13] R.G. Beri, J. Walker, E.T., Reese, J. E. Rollings, Characterization of chitosans via coupled size-exclusion chromatography and multiple-angle laser light-scattering technique, *Carbohydr. Res.* 238 (1993) 11–26.

- [14] M. Fee, N. Errington, K. Jumel, L. Illum, A. Smith, S.E. Harding, Correlation of SEC/MALLS with ultracentrifuge and viscometric data for chitosans, *Eur. Biophys. J.* 32 (2003) 457–464.
- [15] S. Nguyen, S. Hisiger, M. Jolicoeur, F.M. Winnik, M.D. Buschmann, Fractionation and characterization of chitosan by analytical SEC and ¹H NMR after semi-preparative SEC, *Carbohydr. Polym.* 75 (2009) 636–645.
- [16] T. Schmitz, V. Grabovac, T.F. Palmberger, M. H. Hoffer, A. Bernkop-Schnürch, Synthesis and characterization of a chitosan-N-acetyl cysteine conjugate. *Int. J. Pharm.* 347 (2008) 79–85.
- [17] B. Loretz, M. Thaler, A. Bernkop-Schnürch, Role of sulfhydryl groups in transfection? A case study with chitosan–NAC nanoparticles, *Bioconjug. Chem.* 18 (2007) 1028–1035.
- [18] D.F. Putnam, Composition and Concentrative Properties of Human Urine, NASA Contractor Report N° NASA CR-1802, 1971.
- [19] P. He, S.S. Davis, L. Illum, In vitro evaluation of the mucoadhesive properties of chitosan microspheres, *Int. J. Pharm.* 166 (1998) 75–68.
- [20] H. Takeuchi, J. Thongborisute, Y. Matsui, H. Sugihara, H. Yamamoto, Y. Kawashima, Novel mucoadhesion tests for polymers and polymer-coated particles to design optimal mucoadhesive drug delivery systems, *Adv. Drug Deliv. Rev.* 57 (2005) 1583–1594.
- [21] R. Vehring, Pharmaceutical particle engineering via spray drying, *Pharm. Res.* 25 (2008) 999–1022.
- [22] K. Albrecht, E.J. Zirm, T.F. Palmberger, W. Schollocker, A. Bernkop-Schnurch, Preparation of thiomers microparticles and in vitro evaluation of parameters influencing their mucoadhesive properties, *Drug Dev. Ind. Pharm.* 32 (2006) 1149–1157.
- [23] K. Albrecht, M. Greindl, C. Kremser, C. Wolf, P. Debbage, A. Bernkop-Schnurch, Comparative in vivo mucoadhesion studies of thiomers formulations using magnetic resonance imaging and fluorescence detection, *J. Control. Release.* 115 (2006) 78–84.
- [24] G.C. Overberger, J.J. Ferraro, Oxidation of monomeric and polymeric sulfhydryl compounds, *J. Org. Chem.* 27 (1962) 3539–3545.

- [25] S. Li, C. Schöneich, R.T. Borchardt, Chemical instability of protein pharmaceuticals: mechanism of oxidation and strategies for stabilization, *Biotechnol. Bioeng.* 48 (1995) 490-500.
- [26] S. Hauptstein, S. Bonejel, J. Griessinger, A. Bernkop-Schnürch, Synthesis and Characterization of pH Tolerant and Mucoadhesive (Thiol–Polyethylene Glycol) Chitosan Graft Polymer for Drug Delivery Pharmaceuticals, *J. Pharm. Sci.* 103 (2014) 594-601.
- [27] L. Cassettari, D. Vllasaliu, G. Mantovani, S.M. Howdle, S. Stolnik, L. Illum, Effect of PEGylation on the toxicity and permeability enhancement of chitosan, *Biomacromolecules.* 11 (2010) 2854-2865.
- [28] S. Dünnhaupt, J. Barthelmes, D. Rahmat, K. Leithner, C.C. Thurner, H. Friedl, A. Bernkop-Schnürch, S-protonated thiolated chitosan for oral delivery of hydrophilic macromolecules: evaluation of permeation enhancing and efflux pump inhibitory properties, *Mol Pharm.* 9 (2012) 133-1341.
- [29] N. Kubota, Y. Eguchi, Facile preparation of water-soluble N-acetylated chitosan and molecular weight dependence of its water-solubility, *Polymer J.* 29 (1997) 123-127.
- [30] D. Sakloetsakun, J. Iqbal, G. Millotti, A. Vetter, A. Bernkop-Schnürch, Thiolated chitosans: Influence of various sulfhydryl ligands on permeation-enhancing and P-gp Inhibitory properties, *Drug Dev. Ind. Pharm.* 37 (2011) 648-655.
- [31] S.A. Mortazavi, J.D. Smart, An investigation into the role of water movement and mucus gel dehydration in mucoadhesion, *J. Control. Release.* 25 (1993) 197–203.
- [32] S. Rossi, F. Ferrari, M.C. Bonferoni, C. Caramella, Characterization of chitosan hydrochloride-mucin interaction by means of viscosimetric and turbidimetric measurements, *Eur. J. Pharm. Sci.* 10 (2000) 251–257.
- [33] V. Grabovac, D. Guggi, A. Bernkop-Schnürch, Comparison of mucoadhesive properties of various polymers, *Adv. Drug Deliv. Rev.* 57 (2005) 1713–1723.
- [34] A. Bernkop-Schnürch, Thiomers: A new generation of muco-adhesive polymers, *Adv. Drug Deliv. Rev.* 57 (2005) 1569–1582.

- [35] V.M. Leitner, G.F. Walker, A. Bernkop-Schnürch, Thiolated polymers: evidence for the formation of disulphide bonds with mucus glycoproteins, *Eur. J. Pharm. Biopharm.* 56 (2003) 207–214.
- [36] C.M. Lehr, J.A. Bouwstra, E.H. Schacht, H.E. Junginger, In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers, *Int. J. Pharm.* 78, (1992) 43–48.
- [37] K. Deepak, M. Senthil Kumar, N. Mahadevan, Thiolated chitosan: modified advanced generation of mucoadhesive polymers, *Int. J. Recent Adv. Pharm. Res.*, 2 (3) (2012), pp. 31-41
- [38] X. Zhu, M. Su, S. Tang, L. Wang, X. Liang F. Meng, Y. Hong, Z. Xu, Synthesis of thiolated chitosan and preparation nanoparticles with sodium alginate for ocular drug delivery, *Mol. Vis.* 18 (2012) 1973–1982.
- [39] A. Bernkop-Schnürch, A.H. Krauland, V. M. Leitner, T. Palmbergerk, Thiomers: potential excipients for non-invasive peptide delivery systems, *Eur. J. Pharm. Biopharm.* 58 (2004) 253–63.
- [40] P. He, S.S. Davis, L. Illum, Chitosan microspheres prepared by spray drying, *Int. J. Pharm.* 187 (1999) 53–65.
- [41] M. Asada, H. Takahashi, H. Okamoto, H. Tanino, K. Danjo, Theophylline particle design using chitosan by the spray drying, *Int. J. Pharm.* 270 (2004) 167–174.
- [42] C. Draheim, F. de Crecy, S.Hansen, E.M. Collnot C.M. Lehr, A design of wxperiment study of nanoprecipitation and nano-microparticles with defined sizes and size distributions, *Pharm. Res.* 32 (2015) 2609-2624.
- [43] M. Burjak, M. Bogataj, M. Velnar, I. Grabnar, A. Mrhar, The study of drug release from microspheres adhered on pig vesival mucosa *Int. J. Pharm.* 224 (2001) 123–130.
- [44] M.T. Cook, S. A. Schmidt, E. Lee, W. Samprasit, P. Opanasopit, V.V. Khutoryanskiy, Synthesis of mucoadhesive thiol-bearing microgels from 2-(acetylthio)ethylacrylate and 2-hydroxyethylmethacrylate: novel drug delivery systems for chemotherapeutic agents to the bladder *J. Mater. Chem. B.* 3 (2015) 6599-6604.

- [45] Quan Zhang, Koon Gee Neoh, Liqun Xu Shengjie Lu, En Tang Kang, Ratha Mahendran, Edmund Chiong, Functionalized mesoporous silica nanoparticles with mucoadhesive and sustained drug release properties for potential bladder cancer therapy, *Langmuir* 30 (2014) 6151-6161.