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Research paper

S-preactivated thiolated glycol chitosan useful to combine mucoadhesion and drug delivery

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ABSTRACT

This work describes S-preactivated *N*-acetylcysteine (NAC)- and glutathione (GSH)-glycol chitosan (GC) polymer conjugates engineered as potential mucoadhesive platform. Preactivated thiomers (GC-NAC-MNA, GC-GSH-MNA) were synthesized by bond formation between GC-NAC or GC-GSH and 2-mercaptonicotinic acid (MNA) used as ligand. The presence of protected thiol moieties on this new class of thiolated GC made them not subject to oxidation. The structural modifications of the resulting derivatives were confirmed by proton Nuclear Magnetic Resonance (^{1}H NMR) and Size Exclusion Chromatography (SEC). The conjugates displayed 91.2% and 90.1% of S-preactivation for GC-NAC-MNA and GC-GSH-MNA, respectively. The polymers were tested in ex-vivo and in vitro for their mucoadhesive properties and toxicity. The results showed that the preactivation of GC-NAC and GC-GSH increased their mucoadhesive abilities compared to their thiolated precursors by 1.4-, 4.4-fold in time of adhesion evaluated using rotating cylinder method, 1.6-, 1.5-fold in total work of adhesion (TWA) and 2.0-, 1.3-fold in maximum detachment force (MDA) determined using tensile studies, respectively. Moreover, water-uptake studies showed an improved in weight indicating water-uptake strongly dependent on derivations, before erosion occurred, whereas disintegration took place for the thiolated polymers within the first hour. The S-preactivated modification did not affect the cell viability of Caco2 cells exposed to the polymers. The release of the model drug sodium naproxen from tablets prepared with a lyophilized mixture of drug and polymer was studied via dissolution apparatus revealing that the preactivation on GC-GSH and GC-NAC involves a slowdown in the drug release rate. The results shown that the novel preactivated thiolated GC-derivatives can be considered promising excipients for the development of mucoadhesive drug delivery systems.

1. Introduction

The ability of synthetic or natural polymers to adhere on the surface of mucosal tissues is most commonly defined as mucoadhesion and the use of mucoadhesive drug delivery systems is advantageous in terms of extended residence time of the formulation at the site of interest, consequent increased bioavailability of the loaded drug and less frequent dosing. [1–3]. Therefore, mucoadhesive drug delivery systems are considered useful approaches for the selective release of drugs to particular mucosal tissues. Toward this end, different mucoadhesive natural polymers have been explored as drug delivery systems [3–5]. Among them, chitosan is an interesting mucoadhesive cationic polymer characterized by the presence of different functional groups such as amino and hydroxyl functions on the polymer chains which in turn make it chemically active, facilitating the introduction of various biofunctional groups. Moreover, the poor water solubility of chitosan under physiological conditions limits its practical use. Chitosan has mucoadhesive and permeation enhancing properties, because can interact with epithelial cells or anionic substructures in mucous layer through ionic interaction with the positive charges of its amino groups. However, the amino groups of chitosan have pKa values of 5.5–6.5. Hence, at physiological pH values, chitosan display loss of surface charge and aggregation, which limits its utility as a carrier for drug delivery. For these reasons, several chemical modifications have been introduced on chitosan to optimize the its physico-chemical and biological properties. For instance, a chemically modified chitosan with an ethylene glycol portion, namely glycol chitosan, characterized by water solubility at the entire pH ranges and positive charge retention at

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physiological pH values, is more suitable for pharmaceutical and biomedical applications. [6]. Thiolated polymers, or thiomers, have been developed to further improve the mucoadhesive properties of well-established mucoadhesive polymers such as chitosan and its derivatives. This synthetic approach is based on the introduction to the polymeric backbone of small molecules bearing a thiol group. This type of chemical modification is carried out because it is known that thiol groups of the polymer are able to form covalent disulfide bonds with thiol groups of cysteine-rich sub-domains of the mucus [7]. Although thiomers show this advantage, they also present an important disadvantage such as low stability in aqueous solutions and in the presence of oxygen, because they are subject to thiol oxidation at pH 6 unless sealed under inert conditions [8]. In fact, it has been reported that the glycol chitosan derivatives conjugate with *N*-acetylcysteine- and glutathione-glycol chitosan, show some mucoadhesive properties, but are characterized by high amounts of disulfide groups for oxidation of thiols [6]. This too-early oxidation of thiol groups prior to get into contact with the mucus layer limits the interactions, lowering the thiomer efficacy. To prevent this limitation, recently a second generation of thiomers, also known as preactivated thiomers, has been established [9]. The concept for preactivated thiomers is based on the formation of a covalent bond between the thiol-bearing polymer and pyridyl substructures, such as pyridyl disulfides that react quantitatively and rapidly with thiol moieties over a large pH range to form disulfide bonds. Therefore, if sulfhydryl groups of thiomers are completely changed in nicotinamidyl disulfides, they will be preserved from oxidation. For these reasons, S-preactivated thiolated polymers result more stable in water solutions and in presence of oxygen, over a large pH range, presenting a sharp advantage compared to first generation thiomers. Furthermore, based on theoretical considerations, preactivated thiomers are even more reactive then first generation thiomers.

Therefore, the aim of this study was to preactivate thiolated glycol chitosan and evaluate its potential for drug delivery. The impact of the preactivation of thiol groups of the glycol chitosan was investigated by comparison with thiolated ones. Resulting polymers were characterized in terms of chemical structure and mucoadhesive properties. Finally, tablets were prepared and loaded with naproxen sodium, used as model drug, to asses if preactivate thiolated glycol chitosans can be considered tools for the preparation of mucoadhesive drug delivery system.

2. Materials and method

Glycol chitosan (degree of polymerization P400; GC), glutathione (GSH), *N*-acetylcysteine (NAC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 5,5′-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent), *N*-hydroxysuccinimide (NHS), 2-mercaptonicotinic acid (2MNA), 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), sodium borohydride ($NaBH_4$) and the component for simulated intestinal fluid were purchased from Sigma-Aldrich, Italy. The dialysis tubing cellulose membrane (molecular weight cut-off of 3.5 kDa) were all purchased from Spectrum, Italy.

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

To obtain glycol-chitosan-glutathione (GC-GSH), the glycol-chitosan (GC) was further modified according to a method firstly described by our research group [6]. Glutathione (GSH) was coupled to glycol-chitosan by the covalent attachment between the carboxylic acid moieties of the sulfhydryl agent and the primary amino group of GC to form amide bonds. Briefly, 1 g of GC was hydrated in 20 mL of a hydrochloric acid solution (pH 5.0). The coupling reaction was mediated by EDAC in order to activate the carboxylic acid moieties of the subsequently added of GSH. Therefore, GSH (3 g, 9.6 mmol), NHS (1.2 g, 11.6 mmol), and EDAC (2.2 g, 33.64 mmol), were dissolved in 20 mL of the same acid solution. After 30 min, GC was added dropwise over the course of 10 min. The reaction mixture was incubated for 3 h at room temperature under constant stirring. After this time the synthesized polymer was washed using an Amicon Centrifugal Filtration Device (100 kDa MWCO regenerated cellulose membrane) in order to isolate polymer conjugate and to eliminate unbound GSH. The loaded device was centrifuged at 4000 rpm for 20 min, concentrating the GC-GSH polymer conjugate solution to approximately 1 mL. The polymer conjugated was washed three times, using centrifuge, under identical conditions, after dilution of sample it in another 10 mL of fresh Milli-Q water. The purified and concentrated GC-GSH solution was frozen at −20 °C and lyophilized (Christ Alpha 1–4 LSC) for 24 h under reduced pressure (0.016 mbar). The collect lyophilized polymer was stored under nitrogen and in desiccator at 4 °C until further use.

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

The synthesis of GC-NAC polymer conjugate was carried out with the same method used for GC-GSH conjugate above reported [6]. Briefly, 1 g of GC was hydrated in 20 mL of a hydrochloric acid solution (pH 5.0). After 30 min the solution of GC was added dropwise over the course of 5 min to the previously prepared solution of NAC (1.6 g, 9.6 mmol), NHS (1.2 g, 11.6 mmol), and EDAC (2.2 g, 11.6 mmol), dissolved in hydrochloric acid solution (pH 5.0). The reaction mixture was incubated for 3 h at room temperature under constant stirring. GC-NAC polymer conjugate was further purified as described above for the GC-GSH derivative.

2.3. Quantitative analysis of thiol and disulfide groups

To determine the total amount of attached thiol groups a method described previously was employed [10,11]. In order to calculate the amount of polymer free thiol groups, NAC and GSH were used as standards. To reduce the existing intra- and inter molecular disulfide bonds the polymer solutions were treated with $NaBH_4$, then the amount of thiol groups was determined photometrically using Ellman's reagent (5,5′-dithio-bis (2-nitrobenzoic acid)). The amount of disulphide bonds was calculated subtracting the quantity of free thiol groups from total thiol moieties present on the polymer. To guarantee complete purification via dialysis a TNBS test was carried out to determine remaining traces of unbound NAC and GSH according to a method described previously [12].

2.4. Preactivation of thiolated GC conjugates

S-preactivated thiolated GCs were obtained further modifying the thiolated conjugates according to a method previously presented by Perrone et al. [9]. The thiol group of synthetized polymers was bound covalently to the pyridinyl ligand 2-mercaptonicotinic acid (2MNA) via disulfide bond formation. Hence to form disulphide bonds between 2MNA molecules, a solution of 2MNA (1% m/v) at pH 8 (adjusted with NaOH) was treated with hydrogen peroxide to dimerize the ligands. When hydrogen peroxide was added in a final concentration of 1.4% (v/v), a change of the reaction mixture color from yellow to colorless was observed. The dimeric product, 2,2-dithiodinicotinic acid, was frozen at −20 °C and lyophilized (Christ Alpha 1-4 LSC) for 24 h under reduced pressure (0.016 mbar). Subsequently, the dimeric aromatic ligand was bounded covalently to the thiolated polymer trought a disulfide exchange reaction. Briefly, 400 mg of each thiolated GC conjugate were dissolved in 40 mL demineralized water. Then, 100 mg of 2MNA dimer were added to the polymer solution under stirring and the pH was adjusted to 8 using 5 M NaOH. The reaction mixture was stirred for 6 h. The synthetized polymer conjugates were dialyzed for 3 days using Spectra/Por 3 membrane with a molecular weight cut off of 3.5 kDa against 2.5 L of demineralized water pH 6.0 adjusted with NaOH, in order to eliminate unbound 2MNA and to isolate polymer