Carbohydrate Polymers 117 (2015) 577–584

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Thiolated nanocarriers for oral delivery of hydrophilic macromolecular drugs

S. Dünnhaupt[a], J. Barthelmes[a], S. Köllner[a], D. Sakloetsakun[b], G. Shahnaz[c], A. Düregger[d], A. Bernkop-Schnürch[a,*]

[a] University of Innsbruck, Institute of Pharmacy/Pharmaceutical Technology, CCB—Centrum of Chemistry and Biomedicine, Innrain 80-82, 6020 Innsbruck, Austria
[b] Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand
[c] Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 54320, Pakistan
[d] Medical University of Innsbruck, Department of Experimental Urology, Anichstrasse 35, 6020 Innsbruck, Austria

ARTICLE INFO

Article history:
Received 27 January 2014
Received in revised form
22 September 2014
Accepted 22 September 2014
Available online 7 October 2014

Keywords:
Polymer synthesis
Nanoparticles
Permeation enhancement
Adhesion
Cytotoxic effects

Chemical compounds studied in this article:
Chitosan (PubChem CID: CID 21896651)
Thioglycolic acid (PubChem CID: 1133)
L-Cysteine hydrochloride (PubChem CID: 60960)
Dithionitrobenzoic acid (PubChem CID: 6254)
Thiazolyl blue tetrazolium bromide (MTT) (PubChem CID: 64965)
Sodium borohydride (PubChem CID: 22959485)

ABSTRACT

It was the aim of this study to investigate the effect of unmodified as well as thiolated anionic poly(acrylic acid) (PAA) and cationic chitosan (CS) utilized in free-soluble form and as nanoparticulate system on the absorption of the hydrophilic compound FD_4 across intestinal epithelial cell layer with and without a mucus layer. Modifications of these polymers were achieved by conjugation with cysteine to PAA (PAA-Cys) and thioglycolic acid to CS (CS-TGA). Particles were prepared via ionic gelation and characterized based on their amount of thiol groups, particle size and zeta potential. Effects on the cell layer concerning absorption enhancement, transepithelial electrical resistance (TEER) and cytotoxicity were investigated. Permeation enhancement was evaluated with respect to in vitro transport of FD_4 across Caco-2 cells, while mucoadhesion was indirectly examined in terms of adsorption behaviour when cells were covered with a mucus layer. Lyophilized particles displayed around 1000 μmol/g of free thiol groups, particle sizes of less than 300 nm and a zeta potential of 18 mV (CS-TGA) and −14 mV (PAA-Cys). Cytotoxicity studies confirmed that all polymer samples were used at nontoxic concentrations (0.5% m/v). Permeation studies revealed that all thiolated formulations had pronounced effects on the paracellular permeability of mucus-free Caco-2 layers and enhanced the permeation of FD_4 3.0- to 5.3-fold. Moreover, polymers administered as particles showed a higher permeation enhancement than their corresponding solutions. However, the absorption-enhancing effect of each thiolated formulation was significantly ($p < 0.05$) reduced when cells were covered with mucus layer. In addition, all formulations were able to decrease the TEER of the cell layer significantly ($p < 0.05$). Therefore, both thiolated polymers as nanoparticulate delivery systems represent a promising tool for the oral administration of hydrophilic macromolecules.

1. Introduction

Based on their hydrophilic nature and molecular mass, macromolecular drugs exhibit a poor permeability across mucosal membranes resulting in overall insufficient bioavailability. Accordingly, at present most of these drugs are primarily administered via the parenteral route as just a limited portion of the dose reaches the plasma to generate its pharmacological effect when administered orally (Aungst, 2000). The design of a suitable drug delivery system for oral administration of macromolecular drugs and their absorption to therapeutic levels is therefore a major aim. Promising systems may comprise of excipients providing the drug entire to the specific site of absorption, prolonging its residence time and increasing the permeability for an easier transport to the systemic circulation. Therefore, reversible modifications of epithelial barrier structure by permeation enhancers are required. Low molecular weight enhancers generally have physicochemical characteristics favouring their own absorption, whereas polymeric enhancers are not absorbed, whereby the risk of systemic toxicity is minimized. One example for a suitable polymeric enhancer are thiolated polymer or designed thiomers, which have been developed as a category of mucoadhesive polymers with reactive thiol groups

* Corresponding author. Tel.: +43 512 507 58600; fax: +43 512 507 58699.
E-mail address: Andreas.Bernkop@uibk.ac.at (A. Bernkop-Schnürch).

http://dx.doi.org/10.1016/j.carbpol.2014.09.078
0144-8617/

immobilized on the polymeric structure (Bernkop-Schnürch, Schwarz, & Steininger, 1999). They can tightly adhere to the intestinal mucus layer for a prolonged time through covalent bonding with mucin glycoproteins via thiol-disulfide exchange reactions. Hence, they provide a steep drug concentration gradient at the absorption sites. In addition, thiomers demonstrated already a strong permeation enhancing effect for the uptake of poorly absorbed drugs from mucosal membranes (Clausen, Kast, & Bernkop-Schnürch, 2002; Clausen & Bernkop-Schnürch, 2001). The mechanism responsible for this permeation enhancing effect has been discovered within the last few years and shows a reversible opening of the tight junctions and the role of glutathione as permeation mediator (Clausen et al., 2002). Based on this, thiomers might show even improved features, when being formulated to nanoparticles (NP). Nanoparticulate delivery systems have been extensively investigated as oral delivery vehicles for macromolecular drugs due to their ability to protect these drugs from degradation, facilitate drug contact with the absorption sites, and promote drug absorption through the intestinal mucosa (Samstein, Perica, Balderrama, Look, & Fahmy, 2008). So far, however the full potential for thiolated nanoparticles as absorption enhancer for poorly absorbed drugs on the intestinal epithelium has not been tested in detail.

It was therefore the aim of the present study to examine and compare the capabilities of thiolated nanoparticles and solutions with varying characteristics on the absorption of a poorly absorbed model drug (FD_4) on the intestinal epithelial cell layer Caco-2. This confluent monolayer of polarized epithelial cells has previously been used as a model for studying effects of various absorption enhancers on intestinal epithelium (Anderberg, Nyström, & Artursson, 1992). The study focused on the application of the anionic poly(acrylic acid) and the cationic chitosan in different formulations to open epithelial tight junctions, thus allowing for paracellular transport. In addition, the influence of their mucoadhesive properties through addition of a mucus layer to the Caco-2 cells on permeation enhancing effects was evaluated.

2. Materials and methods

2.1. Materials

Chitosan with an average molecular weight (M_w) of 150 kDa and a deacetylation degree of 85% as well as PAA with an average M_w of 100 kDa were obtained from Sigma Aldrich, Austria. Sodium tripolyphosphate pentabasic (TPP), 5,5′-dithiobis(2-nitrobenzoic acid) (Elman's reagent), 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT), 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDAC), Hank's balanced salts for Hank's balanced salts solution (HBSS), thioglycolic acid (TGA), sodium borohydride ($NaBH_4$) and porcine gastric mucin were purchased from Sigma Aldrich, Austria. LDH based CytoTox 96 kit was obtained from Roche. Fluoresceinisothio-cyanate-dextran (FD_4, 4400 Da, purity >95%) was supplied from TdB Consultancy AB (Uppsala, Sweden). Caco-2 cells were kindly donated by Prof. Pfaller, Institute of Physiology, Medical University of Innsbruck. Cell culture medium, penicillin/streptomycin solution and fetal calf serum (FCS) were purchased from PAA, Austria. ThinCert 12-well transwells with polycarbonate membrane and 0.4 μm pore size as well as toxicity plates (24-well) were obtained from Greiner, Austria.

2.2. Synthesis of polymer conjugates

2.2.1. Modification of chitosan with thioglycolic acid (CS-TGA)

Thiolated chitosan can be synthesized by derivatization of its primary amino groups with coupling reagents bearing thiol functions. Modification in this study was achieved via the covalent attachment of thioglycolic acid (TGA) to chitosan (CS) as described previously (Kast & Bernkop-Schnürch, 2001). Briefly, 1.0 g of CS was dissolved in acetic acid 0.05% (v/v). TGA was chemically treated with EDAC in a final concentration of 100 mM in order to activate the carboxylic acid moieties. Thereafter, 500 mg of activated TGA were added drop wise to CS solution and the reaction mixture was incubated for 3 h at room temperature under vigorous stirring. Unbound compounds were isolated by exhaustive dialysis for five times at acid pH conditions. The thiomer (CS-TGA) was lyophilized and kept at 4 °C for further use.

2.2.2. Modification of poly(acrylic acid) with cysteine (PAA-Cys)

The poly(acrylic acid)-cysteine (PAA-Cys) conjugate was synthesized according to a method described previously by our group (Greindl & Bernkop-Schnürch, 2006). First, 500 mg of PAA were hydrated in distilled water and the pH was adjusted to 4.5. Thereafter, EDAC in a final concentration of 100 mM was added drop wise in order to activate the carboxylic acid moieties of the hydrated polymer. After 20 min incubation at room temperature, 500 mg of cysteine were added and pH was readjusted to 4.5. Reaction mixture was incubated again for 24 h at room temperature under constantly stirring. The resulting conjugate was isolated by dialysis according to the method described previously (Greindl & Bernkop-Schnürch, 2006). Afterwards the frozen aqueous polymer solution was dried by lyophilization and stored at 4 °C until further use. Samples prepared in exactly the same way but omitting EDAC during the coupling reaction served as controls.

2.2.3. Determination of modification degree

The degree of modification as amount of thiol groups immobilized on the polymer was quantified by Ellman's method using a spectrophotometer (Wilson, Bayer, & Hupe, 1977). Briefly, 0.5 mg of modified polymers were dissolved in phosphate buffer, into which 500 μl of Ellman's reagent (pH 8) were added. Samples were incubated at 37 °C in a water bath and protected from light for 2 h. Subsequently, 100 μl of each sample solution were transferred to a microplate reader (FluoStar Galaxy, BMG, Offenburg, Germany) to determine the content of thiol groups at a wavelength of 450 nm. Moreover, disulfide contents were evaluated after reduction with $NaBH_4$ and as well determined by Ellman's reagent. The total amount of these moieties is represented by the summation of free and oxidized thiol groups in form of disulfide bonds (Werle & Hoffer, 2006). Cysteine and TGA served as standards to calculate the quantity of thiol groups immobilized on the polymer.

2.3. Particle preparation and characterization

Cationic CS is able to form nanoparticles (NP) with negatively charged TPP and anionic PAA with positively charged Ca^{2+} by ionic gelation (Calvo, Vila-Jato, & Alonso, 1997; Greindl & Bernkop-Schnürch, 2006). Therefore, 100 mg of unmodified and modified CS were dissolved in 20 ml of acetic acid 0.05% (v/v) pH 5.5. A 0.5% (m/v) TPP solution (pH 7) was drop wisely added to the chitosan solutions until turbidity occurred.

For unmodified and modified PAA, 100 mg of each polymer were dissolved in distilled water to obtain a 0.5% (m/v) solution and pH was adjusted to 8. $CaCl_2$ was dissolved in distilled water at 10 mg/ml and added to PAA or PAA-Cys solution under continuous stirring until turbidity occurred. Each particle suspension was stirred with 300 rpm for 1 h at room temperature. To remove the anionic and cationic cross linkers (TPP and Ca^{2+}) each nanoparticle suspension was centrifuged 3 times (4300 rpm, 30 min) and resuspended in water (PAA) or 0.05% of acetic acid (CS). To avoid any particle aggregation, trehalose in a concentration of 3% was