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Research paper

Development and in vivo evaluation of papain-functionalized nanoparticles

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ABSTRACT

The aim of the present study was to develop a novel nanoparticulate delivery system being capable of penetrating the intestinal mucus layer by cleaving mucoglycoprotein substructures. Nanoparticles based on papain grafted polyacrylic acid (papain-g-PAA) were prepared via ionic gelation and labeled with fluorescein diacetate. In vitro, the proteolytic potential of papain modified nanoparticles was investigated by rheological measurements and diffusion studies across fresh porcine intestinal mucus. The presence of papain on the surface and inside the particles strongly decreases viscosity of the mucus leading to facilitated particle transition across the mucus layer. Results of the permeation studies revealed that enzyme grafted particles diffuse through mucus layer to a 3.0-fold higher extent than the same particles without enzyme. Furthermore, the penetration behavior of the nanocarriers along the gastrointestinal tract of Sprague Dawley rats was investigated after oral administration of nanoparticles formulated as enteric coated capsules. The majority of the papain functionalized particles was able to traverse across the mucus layer and remained in the duodenum and jejunum of the small intestine where drug absorption primarily occurs. Polymeric nanoparticles combined with mucolytic enzymes that are capable of overcoming intestinal mucus barriers offer an encouraging new attempt for mucosal drug delivery.

1. Introduction

In recent years, various attempts have been undertaken to explore new routes for the administration of pharmaceutical active agents, alternative to painful injections associated with poor patient compliance [1]. Particular mucosal surfaces are readily accessible and display a convenient target site for delivering drugs to the human body [2]. Anatomical sites such as lung, eyes, vagina, nose and upper gastrointestinal tract are covered by a mucus gel and can be considered for efficient drug delivery [3]. Mucosal dosage forms are aimed either for local treatment of inflammations and infections (mucosal delivery) or for systemic absorption by permeating the mucus layer (transmucosal delivery).

However, mucus is a dynamic gel which is continuously secreted, shed and digested. In many cases, the mucus clearance mechanism removes the drug carrier before the therapeutic agent reaches the underlying absorptive cells and enters the systemic circulation. The structure and viscoelastic properties of mucus are determined by a matrix of glycosylated protein chains called mucin [4]. Due to the tenacious and sticky network of mucin fibers diffusion of foreign particles is restricted by trapping and steric hindrance [5]. There is a high demand for drug delivery systems which rapidly penetrate the mucus layer especially in the upper small intestine where absorption of most orally administered drugs occurs.

According to the nature of mucus, a promising technique to reach the underlying cell layer is presented by cleavage of mucoglycoprotein substructures by proteases [6]. Former studies demonstrated that proteolytic enzymes reduce significantly the elastic properties of freshly scrapped porcine mucus through irreversible enzymatic breakdown of complex protein substances. Among all enzymes tested within this rheological comparison papain and proteinase revealed a significant reduction in mucus viscosity after 1 h of incubation [7]. Owing to this mucolytic efficiency, it was the aim of this study to immobilize proteases on nanocarriers to obtain a local and selective disruption of the mucus gel layer. Cleaving of mucoglycoprotein substructures makes the mucus leakier and facilitates the transport of nanocarriers to the target site. We present a novel mucolytic nanoparticulate delivery system that is supposed to quickly traverse the mucus barrier leading to improved particle transport rates and increased drug concentrations in the upper gastrointestinal tract. Papain, one of the most promising mucolytic enzymes [8], was incorporated into polyacrylic acid particles by covalent immobilization. The impact of papain on the diffusion behavior of particles was

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analyzed in vitro by rheological analysis and utilization of diffusion chambers. In addition, fluorescence labeled nanoparticles were administered to rats to further investigate their penetration capability in vivo.

2. Experimental part

2.1. Materials

Polyacrylic acid (molecular mass ~ 100 kDa), papain (from *Carica papaya*, 3.6 units/mg solid), Coomassie Brilliant Blue G, casein (from bovine milk), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), L-cysteine ethyl ester hydrochloride (CYS-OET), trehalose and fluorescein diacetate (FDA) were purchased from Sigma–Aldrich (Vienna, Austria). PCcaps™ capsules were obtained from Capsugel (Peapack, NJ, USA). All other reagents were of analytical grade and received from commercial sources.

2.2. Synthesis of nanoparticles and FDA labeling

Mucus permeating particles were developed by covalent attachment of papain to polyacrylic acid, followed by an ionic gelation method. First, polyacrylic acid was dissolved in demineralized water to obtain a 0.1% solution. After adjusting the pH to 6.0, 500 mg of EDAC and 300 mg of N-hydroxysuccinimide being dissolved in 10 mL demineralized water were added. The reaction mixture was incubated for 1 h under vigorous stirring. Then, 100 mg of papain dissolved in demineralized water was slowly added to the solution and stirred for 12 h at 10 °C. The resulting protein–polymer conjugate was dialyzed against demineralized water for 2 days at 10 °C. Afterward, the pH of the dialyzed solution was raised to 8.0 with 1 M NaOH. Nanoparticles were produced by ionotropic gelation utilizing Ca^{2+} as crosslinker. For this purpose, a calcium chloride solution (0.5%) was added dropwise to the polymer/papain conjugate until turbidity occurred. In order to enhance the enzymatic activity of the coupled cysteine protease papain, particles containing L-cysteine ethyl ester hydrochloride (CYS-OET) were also prepared. Therefore, the polymer/enzyme solution was treated with a 50 mM aqueous solution of L-cysteine ethyl ester hydrochloride before ionic crosslinking with Ca^{2+}. Unmodified polyacrylic acid nanoparticles were obtained spontaneously upon incorporation of 25 mL $CaCl_2$ solution (0.5%) to 100 mL polyacrylic acid solution (0.1%) at pH 8.0 under vigorous stirring.

All obtained nanoparticles were purified by centrifugation three times at 5500 rpm for 25 min with 1.0% trehalose in order to prevent particle aggregation. The supernatant was discarded and the pellet was resuspended in demineralized water. Finally, the nanoparticle suspensions were lyophilized at −77 °C, 0.01 mbar (Virtis Bench top freeze-drier, Bartelt, Graz, Austria) and kept at 4 °C until further use.

For diffusion and in vivo studies, the marker fluorescein diacetate (FDA) was incorporated into the obtained particles according to the method described by our research group [9]. First, 10 mL of particle suspension was transferred to 10 mL of a 0.5% (w/v) FDA solution in acetonitrile. The mixtures were incubated on a thermomixer at 15 °C for 1.5 h. Afterward the labeled nanoparticles were centrifuged, resuspended in demineralized water and lyophilized.

The amount of incorporated FDA was quantified by alkaline treatment. Sodium hydroxide quantitatively hydrolyzes fluorescein diacetate to the fluorescent sodium fluorescein which enables detection at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Therefore lyophilized particles (1 mg/mL) were dissolved in 5 M NaOH and incubated for 1 h at 37 °C under stirring conditions. Then, the reaction mixtures were centrifuged at 13,500 rpm for 15 min and the supernatant was collected for fluorescence measurement using a microplate reader (Infinite™ M200, Tecan, Grödig, Austria).

2.3. Particle characterization

Size distribution and zeta potential analysis of nanoparticle suspensions were performed by photon correlation spectroscopy using PSS Nicomp 380 ZLS particle sizer (Santa Barbara, CA, USA) with laser wavelength of 650 nm and an E-fields strength of 10 V/cm.

2.4. Quantification of immobilized enzyme

The amount of immobilized protein on polyacrylic acid was determined by the Bradford assay [10]. Briefly, 0.2 mL of particle suspension was treated with 1.0 mL Bradford reagent and incubated for 45 min. The resulting papain-dye complex was measured photometrically at 595 nm. Since polymers are known to increase the absorbance at 595 nm induced by aggregates, corrections were made by measuring the samples also at 850 nm [11].

2.5. Measurement of the enzymatic activity

According to the method described previously by Itoyama et al., casein was utilized to determine the enzymatic activity of immobilized papain [12]. Briefly, 2.0 mL of 10 mM PBS (pH 8.0), 1.0 mL of papain or nanoparticle suspension in 50 mM PBS containing 2 mM EDTA and 5 mM L-cysteine and 1.0 mL of 2.0% (m/v) casein solution were combined. The reaction mixtures were incubated for 30 min at 37 °C under permanent stirring. Then, the reaction was stopped by adding trichloroacetic acid in a final concentration of 10.0%. The samples were centrifuged at 13,400 rpm for 10 min and the absorbance of the supernatant measured at 280 nm. The enzyme activity of immobilized papain was defined as relative activity corresponding to native papain displaying 3.6 units/mg.

2.6. Preparation of porcine mucus

Intestines from freshly slaughtered pigs were collected on ice, cut into smaller segments and rinsed carefully with physiological saline (0.9% NaCl). After the washing process, intestinal mucus was isolated by gently scraping from underlying tissue. Subsequently the pH of the resulting scrapings was adjusted to 6.5 by addition of 0.1 M NaOH.

2.7. Rheological evaluation of nanoparticle/mucus mixtures

The mucolytic potential of papain modified nanoparticles was investigated by determination of decrease in mucus viscosity. Briefly, 20.0 mg of particle formulations or 20.0 mg of native papain was hydrated in 500 μL of demineralized water and incubated with 9.5 mL natural mucus at 37 °C for 6 h. At regular time intervals, the viscoelastic properties of samples were measured by adding 700 μL of the mixtures to a cone-plate viscometer (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany). Dynamic oscillatory tests within the linear viscoelasticity region were performed as described by Marschütz and Bernkop-Schnürch [13]. Mucus samples without addition of proteolytic enzymes were treated in the same way and served as references.