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Research paper

Nanoparticles decorated with proteolytic enzymes, a promising strategy to overcome the mucus barrier

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ABSTRACT

The intestinal mucus gel layer represents a stumbling block for drug adsorption. This study is aimed to formulate a nanoparticulate system able to overcome this barrier by cleaving locally the glycoprotein substructures of the mucus. Mucolytic enzymes such as papain (PAP) and bromelain (BRO) were covalently conjugated to poly(acrylic acid) (PAA). Nanoparticles (NPs) were then formulated via ionic gelation method and characterized by particle size, zeta potential, enzyme content and enzymatic activity. The NPs permeation quantified by rotating tube studies was correlated with changes in the mucus gel layer structure determined by pulsed-gradient-spin-echo NMR (PGSE-NMR), small-angle neutron scattering (SANS) and spin-echo SANS (SESANS). PAP and BRO functionalized NPs had an average size in the range of 250 and 285 nm and a zeta potential that ranged between −6 and −5 mV. The enzyme content was 242 μg enzyme/mg for PAP modified NPs and 253 μg enzyme/mg for BRO modified NPs. The maintained enzymatic activity was 43% for PAP decorated NPs and 76% for BRO decorated NPs. The rotating tube technique revealed a better performance of BRO decorated NPs compared to PAA decorated NPs, with a 4.8-fold higher concentration of NPs in the inner slice of mucus. Addition of 0.5 wt% of enzyme functionalized NPs to 5 wt% intestinal mucin led to c.a. 2-fold increase in the mobility of the mucin as measured by PGSE-NMR indicative of a significant break-up of the structure of the mucin. SANS and SESANS measurements further revealed a change in structure of the intestinal mucus induced by the incorporation of the functionalized NPs mostly occurring at a length scale longer than 0.5 μm. Accordingly, BRO decorated NPs show higher potential than PAP functionalized NPs as mucus permeating drug delivery systems.

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1. Introduction

Among all the administration routes the oral one is the most favorable; however, the low oral bioavailability of numerous drugs is a major issue and strategies able to improve drug solubility and adsorption are needed. One of the main causes of low drug oral bioavailability is the presence of a barrier that covers all the surface of the GI tract, namely the mucus barrier. This continuous gel layer is a complex matrix formed mainly by mucin, a polypeptide with highly glycosylated negatively charged regions, hydrophobic regions and cysteine rich regions interconnected via disulfide bonds. Mucus is a semipermeable barrier that guarantees the exchange of nutrients, gases and water and prevents the permeation of many pathogens and foreign particles [1]. In recent years many attempts have been undertaken to formulate drug delivery systems (DDSs) able to overcome this barrier. A strategy that showed potential was a nanoparticulate system bearing mucolytic agents able to cleave the mucus substructure in front of them and therefore to cross this barrier. An *in vivo* study showed a prolonged residence time, compared to nonfunctionalized NPs, in the small intestine of rats [2]. As this preliminary study showed great potential, it is worth to continue working in this direction to further improve such systems and the analytical methods needed behind. Accordingly, it was the aim of this study to compare the features of the papain (PAP) conjugated nanoparticles (NPs) with a new carrier bearing bromelain (BRO). The chosen enzymes are stable in simulated intestinal fluid as demonstrated by *in vitro*

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studies and their maintained mucolytic activity and ability to permeate through the intestine was demonstrated by several *in vivo* studies [3–6]. However the enzymes are digested in gastric environment and therefore enteric coat is necessary for *in vivo* application. Both enzymes are known to decrease the intestinal mucus viscosity [7] but, to our knowledge, a systematic comparison between PAP and BRO modified NPs has not been performed yet. BRO was selected for the formulation of functionalized NPs in order to improve the tolerability of the carrier. In fact, beside the known occupational allergy caused by both enzymes (means a long term work related exposure) [8–11], just one case of allergic reaction associated with BRO ingestion could be found in the literature [12], while several cases of non-occupational allergic sensitization were reported for PAP [13,14]. Moreover, a series of clinical trials were performed in the past decade, last one published in 2013, illustrating the good tolerability to BRO ingestion by patients [15–18]. Poly(acrylic) acid was chosen as polymeric backbone for this DDS as it shows weak mucoadhesive properties [19] and bears carboxylic groups to which enzymes can be conjugated via carbodiimide chemistry [20]. The reaction can take place at the amino terminal group of both enzymes and at lysine residues, which are 10 for PAP and 15 for BRO [21,22]. To further investigate and gain a deeper understanding of the enhancing permeation properties of this new DDS, the results obtained with previously established techniques were correlated with the results obtained exploiting innovative techniques (such as pulsed-gradient spin-echo NMR, small-angle neutron scattering and spin-echo small angle neutron scattering) yielding information on the effect of these NPs on the structure of mucus at small length scales.

2. Materials and methods

2.1. Materials

Poly(acrylic) acid (PAA, solution 35 wt% in H_2O, average molecular weight 100 kDa), papain from carica papaya 3.6 U/mg (EC 3.4.22.2), bromelain from pineapple steam 3.4 U/mg (EC 3.4.22.32), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), N-hydroxysuccinimide (NHS), ethylenediaminetetraacetic acid (EDTA), casein, L-cysteine, trehalose, trichloroacetic acid (TCA), resazurin and all other salts and solvents at analytical grade were purchased from Sigma–Aldrich (Vienna, Austria). Lumogen red was purchased from Kremer pigmente GmbH & Co. KG (Aichstetten, Germany). Bicinchoninic acid kit (BCA) was purchased from Thermo Scientific (Vienna, Austria). Minimum essential medium (MEM) was purchased from Biochrome AG (Berlin, Germany). Intestinal mucin (Imucin) and intestinal mucus (Imucus) were obtained from Jeff Pearson (Newcastle University, Institute for Cell and Molecular Biosciences, Newcastle upon Tyne, United Kingdom).

2.2. Polymer synthesis

3.26 g of PAA solution, corresponding to 1 g of polymer (MW 100 kDa, 0.01 mmol), was diluted in 1 L of water, pH adjusted to 6, to obtain a 0.1 wt% solution. 5 g of EDAC (191.7 Da, 26.08 mmol) and 3 g of NHS (115.09 Da, 26.07 mmol) were dissolved in 100 mL of water and added to the polymer solution. The reaction mixture was incubated for 1 h under vigorous stirring. Then, 1 g of PAP (theoretical molecular weight 23.4 kDa, 0.043 mmol [23]) or 1.42 g of BRO (approximate molecular weight 33 kDa, 0.043 mmol [24]) was dissolved in 500 mL of water and slowly added to the reaction mixture and stirred for 24 h at 10 °C. The obtained enzyme–polymer conjugate was dialyzed against demineralized water for 3 days at 10 °C. The solutions were then lyophilized yielding a white powder referred to PAA-PAP and PAA-BRO, respectively [2].

2.3. Nanoparticles formulation

100 mg of PAA or enzyme–polymer conjugate was dissolved in 10 mL water to obtain a 1 wt% solution and the pH was adjusted to 8. This mixture was slowly added to 7.5 mL of Lumogen acetone solution 0.07 mg/mL. After 10 min equilibration 1 mL of $CaCl_2$ 5 mg/mL dissolved in water was added drop by drop and stirred for 30 min. The suspension was then centrifuged at 5500 rpm (4966*g*) for 25 min (MiniSpin centrifuge, Eppendorf), the supernatant discarded and the pellet washed with 50 vol% acetone/water mixture for three times. Finally the washed pellet was resuspended in 1 wt% trehalose/water solution by means of probe sonication for 10 s and then lyophilized. The obtained products were referred to PAA NPs when PAA was used for the formulation, PAA-PAP NPs when PAA-PAP was used for the formulation and PAA-BRO NPs when PAA-BRO was used for the formulation.

2.4. Nanoparticles characterization

Nanoparticle particle size and charge characterization were carried out by using a NICOMPTM 380 ZLS PSS (Particle Sizing Systems, CA, USA). Particle size was recorded at a scattering angle of 90° for 10 min at room temperature. Zeta potential was recorded under electric field strength of 5 V/cm for 3 min at room temperature.

2.5. Enzyme quantification

The amount of enzyme conjugated to polymers and nanoparticles was determined by micro BCA protein assay, following the provider's instruction. The samples were dissolved in 0.1 M NaOH solution containing 1.5 wt% of sodium dodecyl sulfate to obtain a concentration of 0.1 mg/mL. The samples were incubated at 25 °C in a thermomixer (Thermomixer Conform; Eppendorf, Hamburg, Germany) under constant shaking, 1000 rpm for 2 h. Thereafter, 150 µL of sample was mixed with 150 µL of working reagent in a 96 well plate and incubated for further 2 h at 37 °C. Finally the absorbance was detected at 562 nm by using a microplate reader (TECAN Infinite M200, Austria GmbH). The amount of enzyme was extrapolated by fitting the data to a calibration curve obtained via analyzing solution with different concentration of PAP or BRO. The enzyme content of polymers and NPs was reported on a weighted base as the ratio between the amount of conjugated enzyme and the amount of polymers or NPs. The conjugation efficacy onto the polymers was calculated as weight percent of the amount of conjugated enzyme relative to the initial amount of enzyme used.

2.6. Enzymatic activity assay

The remaining enzymatic activity of conjugated enzyme was detected via casein assay [25]. The polymers were dissolved and the particles were dispersed at a concentration between 0.5 and 1 mg/mL in PBS 50 mM pH 8 containing 2 mM EDTA and 5 mM L-cysteine. The solutions were then diluted with 2 mL of PBS 10 mM pH 8 and further with 1 mL of 2 wt% casein solution. The reaction mixtures were incubated for 30 min at 37 °C in a thermomixer under constant shaking, 750 rpm. After incubation 120 µL of 100 wt% TCA solution was added in order to stop the reaction. The samples were then centrifuged for 10 min at 13,400 rpm (12,100*g*) and the absorbance of the supernatant was detected at 280 nm. The assay was performed in parallel with solutions presenting the same enzyme concentration of each sample. The remaining enzymatic activity of the enzyme-conjugated samples was calculated as percentage comparing the absorbance of the samples and the pure enzyme solutions.

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