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Preparation and characterization of an amphoteric chitosan derivative employing trimellitic anhydride chloride and its potential for colon targeted drug delivery system

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ABSTRACT

Trimellitic anhydride chloride was used to generate a water soluble amphoteric chitosan derivative (CTAA). CTAA was combined with alginate to facilitate direct targeting of 5-FU to the colon. The structure of CTAA was characterized by FTIR, $^{1}H/^{13}C$ NMR and HSQC. The physical properties of the CTAA hydrogel were analyzed by scanning electron microscopy, X-ray diffraction and thermogravimetry. Inhibition of a pancreatic enzyme's activity and cytotoxicity studies of CTAA were also carried out. Sodium alginate was mixed with CTAA to form a polyelectrolyte complex film and resulted in improved controlled drug release. Swelling characteristics of the films as a function of pH were also investigated. An optimized formulation was exposed to conditions simulating the stomach, small intestine, and colon. The CTAA/alginate formulation used in this preliminary study presents potential for at least 28% of ingested 5-FU to arrive at the colon ready for release by colonic enzymes. Films were chosen in the study to severely stretch the capabilities of the CTAA/alginate system. The system nevertheless will need modifying for in vivo delivery to the colon.

1. Introduction

Colorectal cancer manifests as cancerous growths in the colon, rectum and appendix and is reported as one of the most frequent causes of cancer deaths [1,2]. Various drugs and combination of drugs have been exploited for the chemotherapy of colorectal cancer [3,4]. 5-Fluorouracil (5-FU) is a hydrophilic drug that has been the most widely used drug for the treatment of colorectal cancer for many decades [5,6]. It can be administered either orally or intravenously. To achieve successful oral colonic delivery, a drug needs to be protected from absorption and/or the environment of the upper gastrointestinal tract (GIT) and then be abruptly released into the proximal colon, which is considered the optimum site for colon targeted delivery of drugs. Oral colon-specific drug delivery of 5-FU can be mediated by means of prodrugs, azopolymers, time, pH, and pressure-sensitive polymers, as well as microbial activated approaches [7,8]. Among the various hydrogels, including natural, synthetic and natural/synthetic hybrid hydrogels, chitosan has attracted significant attention in the field of colon delivery, due to its unique polymeric cationic character, biocompatibility and its degradation by anaerobic microflora present in the colon [9–11]. Chitosan is a hydrophilic polyelectrolyte polysaccharide composed of glucosamine and N-acetyl glucosamine units linked by β(1-) glycosidic bonds. Chitosan is produced by alkaline N-deacetylation of chitin, a major component of the shells of crabs, shrimps, and krill [12]. The poor solubility of chitosan in water is a major limiting factor for some applications [13]. Therefore, a special emphasis has been placed on the chemical modifications employed to prepare chitosan derivatives with improved water solubility [14–22].

The aim of this paper was to introduce and characterize a new amphoteric chitosan derivative. Also the potential for this derivative for colon selective delivery of drugs was studied using 5-FU as a model anti-cancer drug. Few modified chitosan based films have been developed for drug delivery applications mainly due to difficulty in dissolving modified chitosan matrices or requirement of acidic media in order to prepare the films. The main potential for drug delivery using chitosan films logically lies with topical applications. For example, chitosan films have been studied for their potential for topical drug (ondasetron) release [23], wound dressing [24] and artificial skin [25]. One could also logically argue that

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films are not particularly applicable to gastrointestinal tract (GIT) drug delivery. Yet as discussed below both Chen et al. and Shu et al. prepared films with delivery to the GIT as their aim [26]. We acknowledge that films, even cut into small pieces are not ideal for patient acceptance for GIT delivery unless encapsulated at least for patient comfort and delivery to the stomach. Using an acidic environment in the film preparation process may alter some drugs' characteristics such as peptide or protein drug denaturation. Therefore in this study, a film as a drug delivery device was chosen using trimellitic anhydride as the crosslinker which produces a water soluble chitosan. Also to overcome the problem of immediate drug release due to the high solubility of the CTAA film in the acidic environment of the stomach, sodium alginate, was complexed with the chitosan derivative to facilitate directed targeting of 5-FU to the colon. The film approach was chosen in this study to present a wide surface area for potentially the fastest, worst sustained release compared for example, to tablets. The film approach in our study nevertheless showed sustained release and indeed retention of some 5-FU under all three simulated GIT conditions.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Acros Organics (Geel, Belgium). The molecular weight of chitosan was also determined to be 198 kDa using the Markwink viscometry method in a solvent of 0.1 M acetic acid/0.2 M NaCl maintained at 25 °C. Low viscosity alginic acid sodium salt (100–300 cP as a 2% solution at 25 °C), Trimellitic anhydride chloride (TMAC), 5-fluorouracil (5-FU), trypsin from porcine pancreas, α-chymotrypsin from bovine pancreas pepsin, pancreatin, almond β-glucosidase, N-α-benzoyl-L-arginine p-nitroanilide (BAPNA) and N-benzoyl-L-tyrosine ethyl ester solution (BTEE) were obtained from Sigma–Aldrich (Auckland, New Zealand).

2.2. Preparation of chitosan trimellitic amic acid (CTAA)

A total of chitosan (2 g, 0.0124 mol of glucosamine residues) was first suspended in glacial acetic acid (50 ml) under vigorous stirring at room temperature. Then TMAC (2.61 g, 0.0124 mol anhydride) dissolved in DMF (50 ml) was added. The mixture was stirred at room temperature for 24 h. The crosslinked chitosan powder was filtered off, washed with methanol and then dried in vacuum.

2.3. Characterization of the CTAA

The FTIR spectra of the films were recorded using Nicolet 5700 FTIR spectrometer in the range of 4000–400 cm^{-1}. ^{1}H, ^{2}D and ^{1}H–^{13}C HSQC spectra were recorded at 298 K on a Bruker Avance 700 MHz spectrometer equipped with a cryoprobe. The ^{1}H NMR spectra for the CTAA were recorded using a Bruker Avance 500 MHz NMR spectrometer. The concentration of the sample was about 10 mg/ml in D_2O. Solid-state ^{13}C magic angle spinning (MAS) NMR spectra were also obtained at a ^{13}C frequency of 50.3 MHz on a Bruker (Rheinstetten, Germany) DRX 200 MHz spectrometer. The X-ray diffraction patterns were recorded on a Rigaku SPIDER curved image-plate detector using a multi-metal-layer Osmic confocal optic to monochromate and focus the Cu Kα radiation produced by a Rigaku MM007 micro-focus rotating-anode generator. Elemental analysis was performed with a Carlo Erba Elemental Analyser EA 1108 using a flash combustion technique (Campbell Microanalytical Laboratory, Otago University, Dunedin, New Zealand). A TA Instruments SDT Q600 instrument was used for simultaneous DTG and TGA data acquisition. Data were analyzed using TA Universal Analysis software. Samples were loaded into an aluminum oxide crucible and heated at a rate of 5 °C/min to 600 °C under a dynamic nitrogen atmosphere.

2.4. Enzyme inhibitory effect

2.4.1. Trypsin inhibition study

The enzymatic inhibitory activity of chitosan and CTAA toward trypsin was evaluated with BAPNA as the substrate [27]. Chitosan and CTAA powder were dispersed in Tris-buffer (1 ml, 0.05 M, pH 8.2) containing $CaCl_2$ (0.02 M) to reach a final concentration of 0.1% (w/v) solutions. After adding 30 U of trypsin solution (0.3 ml in 10 mM HCl), the mixture was incubated at 37 °C. Thereafter, the BAPNA solution (2.1 ml, dissolved in DMF) was added and incubation continued for 15 min at 37 °C. After stopping the enzymatic action with 1% trichloroacetic acid solution, the nitroaniline formed was analyzed at 405 nm using a UV/visible spectrometer.

2.4.2. α-Chymotrypsin inhibition study

The assay of α-chymotrypsin inhibition by chitosan and CTAA matrices was performed with N-benzoyl-L-tyrosine ethyl ester solution (BTEE) as the substrate [28]. Chitosan and CTAA powder were dispersed in Tris–HCl buffer (1 ml, pH 7.8) containing $CaCl_2$ (0.02 M) to get a final concentration of 0.1% (w/v) solutions. After 15 min incubation at 25 °C, chymotrypsin solution (0.3 ml, 2 mg/ml in 1 mM HCl) was added. Thereafter, BTEE (1.4 ml, 37 mg dissolved in 63 ml of methanol and 37 ml of demineralized water) was added and the mixture was incubated at 37 °C for 5 min. After stopping the enzymatic action with 1% trichloroacetic acid solution, absorbance at 256 nm (resulting from the hydrolysis of benzoyl-L-tyrosine ethyl ester) was recorded.

2.5. Cell cultures

Crandall Rees feline kidney (CRFK) cells were maintained in an advanced Dulbecco modified medium (Adv DMEM, Invitrogen) supplemented with 2% (growth medium) or 1% (maintenance medium) fetal bovine serum (Thermo Scientific, MyClone), 1% antibiotic solution to a final concentration of 100 U/ml of penicillin and 100 μg/ml of streptomycin (PenStrep, Invitrogen), and 1% glutamax (Invitrogen). The cells were seeded into preselected wells of 96-well tissue culture plates (Thermo Scientific, Nunc) at a concentration of 5×10^4 cells/well in a 100 μl volume. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 overnight.

2.6. Cytotoxicity test

WST-1 assays were carried out to test the cytotoxicity of chitosan and CTAA. Briefly, the suspended complex (10 mg/ml) was subjected to serial 2-fold dilutions in the maintenance medium. The growth medium was removed from pre-seeded 96-well plates after approximately 24 h, when the monolayers were 95% confluent, and replaced with 200 μl of each complex's dilution, from 0.16 mg/ml to 0.005 mg/ml in duplicate (test wells). For each complex dilution, an equal amount (200 μl) was also added in duplicate to wells that were not pre-seeded with cells (control wells). The growth medium was similarly replaced in 4 cell control wells (pre-seeded with cells, without addition of any complex) and added to 4 medium control wells (empty wells not pre-seeded with cells). Plates were incubated at 37 °C, 5% CO_2 humidified atmosphere for 24 h. The cultures were inspected visually for signs of toxicity using an inverted microscope. In addition, the number of viable cells in each well was quantified using a colorimetric cell viability assay (WST-1 reagent, Roche), according to the manufacturer's instructions. The % cell viability as compared to control cells was calculated separately for each dilution of each compound based on the optical