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Mucoadhesive nanostructured polyelectrolytes complexes modulate the intestinal permeability of methotrexate

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ABSTRACT

Nanostructured polyelectrolytes complexes (nano PECs) loaded with methotrexate (MTX) were obtained by the polyelectrolyte complexation of chitosan (CS) and hyaluronic acid (HA), further incorporating hypromellose phthalate (HP). The mean diameter of nano PECs ranged from 325 to 458 nm, with a narrow size distribution. Zeta potential was close to + 30 mV, decreasing to + 21 mV after the incorporation of HP, a range of values that favour the physical stability of system as the interaction with cationic biological membranes. The electrostatic interactions between the different components were indicated by the FTIR data. The mucoadhesiveness of nano PECs was demonstrated and MTX and HP influenced this property. The cell viability assays showed the biosafety of the isolated polymers and nano PECs in intestinal HT29-MTX and Caco-2 cell lines at 4 h of test. The permeability values of MTX loaded in CS/HA nano PECs were 7.6 and 4-fold higher than those of CS/HA/HP nano PECS and free drug, respectively, in the Caco-2 monoculture. In mucus secreting co-culture cell model these values were 3 and 6.5 fold, respectively. Such features indicate that nano PECs developed in this work can be promising carriers for MTX in the treatment of local or systemic diseases.

1. Introduction

Methotrexate (MTX) is an antifolate cytotoxic drug, in higher doses is currently used in the treatment of several solid tumours such as those in colorectal and, in lower doses MTX has shown a promise activity as an immunomodulatory drug, being used successfully in the treatment of systemic inflammatory diseases (Gaies et al., 2012; Higano and Livingston, 1989; Jachens and Chu, 2008; Jain et al., 1979; Martins and Yamamoto, 2008). Several dosing regimens can be adopted in MTX therapy, by oral route, for the treatment of such diseases (Gorlick et al., 1997; Widemann and Adamson, 2006). MTX is classified as class III in the Biopharmaceutical Classification System (high solubility and low permeability), a limiting property when the therapy requires the systemic absorption. Another issue is the low oral bioavailability of MTX due to the action of the P-glycoprotein (P-gp) as an efflux pump (Barrueco et al., 1992; Borst et al., 1999; Huber et al., 2010). To overcome these problems, an excess of drug is usually administered but the side effects are thus exacerbated (Barrueco et al., 1992; Thiebaut et al., 1987).

The pharmaceutical nanotechnology is a relevant technological tool that enables the building of new nanostructures with different and improved properties, allowing the protection of drug against premature degradation by the gastrointestinal tract (GIT) enzymes or low pH value of gastric fluid and the modulation of the drug release rates or the targeting of drug for specific organs or tissues (Hamidi et al., 2008; Janes et al., 2001). Nanostructured polyelectrolytes complexes (nano PECs) are obtained by the polyelectrolyte complexation technique; which adds important technological advantages as the simplicity of execution and relative low cost process. An important property of the nano PECs intended to the oral route of administration is the mucoadhesiveness. This property may prolong the nano PEC residence time at the absorption site, promoting a closer contact with the epithelial barrier, favouring the local interaction with the site of action or the systemic absorption. The mucoadhesive property of natural polymers as chitosan (CS), hypromellose phthalate (HP) and hyaluronic acid (HA), in the GIT, have been explored in the design of nano PECs (Ensign et al., 2012; Pedreiro et al., 2016). CS is a biocompatible and biodegradable cationic polysaccharide; due to the deprotonation of their

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amino groups, composed by D-glucosamine and *N*-acetyl-D-glucosamine units linked by β-1,4 linkages (Rinaudo, 2011; Wang et al., 2012). The ability of CS to open the cellular tight junctions improving the drug permeability has been extensively reported (Sarmento et al., 2007; Vllasaliu et al., 2010; Wang et al., 2017). For oral route, the high solubility of CS in acidic pH is a drawback, so that the association with HP, an enteric coating polymer, could be a rational way to prevent the drug premature release. Besides, HP is able to form polyelectrolytes complexes with cationic polyelectrolytes (Meehan, 2006). The HA is an anionic polysaccharide, biocompatible and biodegradable with high molecular weight, composed by D-glucuronic acid and *N*-acetyl-D-glucosamine. HA is an important component of many tissues and extracellular matrix and has the ability to interact specifically with CD44 receptors, expressed in the surface of intestinal epithelial cells, responsible for the transmission of internalization signals (Becker et al., 2009; Liao et al., 2005; Ponta et al., 2003). Thus, the incorporation of HA into the nano PECs could contribute to the mucoadhesiveness, improving the biological interaction of the system.

In this work CS/HA nano PECs with or without HP, loaded with MTX, were obtained and characterized by physical-chemical properties and drug association efficiency. The biological interaction of the systems was evaluated by mucoadhesiveness and intestinal *in vitro* permeability assays; using monoculture model of Caco-2 cells and mucus producing triple co-culture model of Caco-2:HT29-MTX:Raji B cells.

2. Materials and methods

2.1. Materials

2.1.1. Chemical materials

Low molecular weight chitosan (Mw ≈ 200 kDa; deacetylation degree of 90%) was obtained from Sigma Aldrich® (St. Louis, MO, USA). Hypromellose phthalate (phytalyl content of 31%) from Shin-Etsu® (Tokyo, Japan). High molecular weight sodium hyaluronate (Mw ≈ 1000 kDa; glucuronic acid content of 47%) was obtained from ViaFarma (São Paulo, Brazil) and methotrexate from Fagron (São Paulo, Brazil). All the other materials used were of analytical grade and obtained from commercial suppliers.

2.1.2. Cell line and culture

C2BBe1 clone of Caco-2 was obtained from American Type Culture Collection (ATCC, USA), HT29-MTX and Raji B were provided by Dr. T. Lesuffleur (INSERMU178, Villejuif, France) and by Dr. Alexandre Carmo (Cellular and Molecular Biology Institute – IBMC, Porto, Portugal), respectively. The cells were cultured with Dulbecco's modified Eagle medium (DMEM) from Lonza (Verviers, Belgium), supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 1% (v/v) penicillin (100 U/mL) and streptomycin (100 µg/mL), 1% (v/v) non-essential amino acids (NEAA). All these supplements were purchased from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). The reagents 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich® (St. Louis, MO, USA) and Triton X-100 from Spi-Chem (West Chester, PA, USA). Paraformaldehyde (PFA) was purchased from Merck Millipore (Billerica, MA, USA), Alexa Fluor® 546 Phalloidin and Goat anti-rabbit Alexa-Fluor 488® secondary antibodies was purchased from Molecular Probes® (Life Technologies S.A., Madrid, Spain). Rabbit occludin primary antibody was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Fluorescence mounting medium was purchased from Dako (Peterborough, UK). The culture flasks and 96-well tissue culture plates were purchased from Corning Inc., (Steuben County, NY, USA) and the 6-wells plates Transwell™ (PET membrane, pore size of 3 µm) was obtained from Corning, Madrid, Spain. The cells were maintained in a conventional incubator (Binder® CB 210, Germany) at 37 °C and 5% CO_2 in a water-saturated atmosphere.

2.2. Determination of molecular weight of polymers

The molecular weight (*Mw*) and second virial coefficient (A_2) of CS, HA and HP were estimated by the static light scattering technic on a Zetasizer Nano ZS® (Malvern Instruments, Worcestershire, UK) equipment, using the Zetasizer nano ZS v7.11 software (Worcestershire, UK). Polymers dispersions, in different concentrations (0.02–0.9 mg·mL^{-1}) at 25 °C were analysed implementing the Rayleigh equation, that describes the intensity of light scattered from the polymer structure in solution (Ferreira et al., 2017). The experiment was conducted using toluene, previously filtered through a polytetrafluoroethylene membrane (PTFE, 0.2 µm), as an equipment internal standard and ultra-pure water filtered through a cellulose acetate membrane (RC, 0.2 µm), as the blank. CS, HA and HP were dispersed in filtered acetic acid (0.1 M), ultra-pure water and sodium hydroxide (0.1 M), respectively. Applied refractive index increments (dn/dc) were 0.181 mL·g^{-1} for the CS, 0.152 mL·g^{-1} for the HP and 0.167 mL·g^{-1} for the HA (Fukasawa and Obara, 2003; Hokputsaa et al., 2003; Schatz et al., 2003). For each concentration, the scattering intensity was measured, in triplicate.

2.3. Preparation of nanostructured polyelectrolytes complexes

Nano PECs composed by CS/HA and CS/HA/HP were obtained by the polyelectrolyte complexation technic (Makhlof et al., 2011; Pedreiro et al., 2016). CS was first dispersed overnight in acetic acid 0.1 M (0.5 mg/mL) and added slowly in an aqueous dispersion of HA (0.1 mg/mL), under magnetic stirring for 15 min (1:0.2, w/w). Afterwards, for the samples with HP, the polymer dispersed in sodium hydroxide 0.1 M (0.5 mg/mL) was added to the preformed particles (1.0:0.2:0.2, w/w/w), under magnetic stirring for 30 min. Before the complexation process, the pH values of all polymers dispersions were adjusted to 5.5. Drug loaded nano PECs were prepared by the previously MTX solubilisation in sodium hydroxide 0.1 M (2.0 mg/mL) and it addition into the CS dispersion, to the final concentration of 5% (w/w). Nano PECs were labelled according to the polymeric content as NpHA for CS/HA in the composition and NpHP for CS/HA/HP in the composition. The suffixes -MTX and -0 and were used for samples containing or not drug, respectively.

2.4. Characterization of nanostructured polyelectrolytes complexes

The average hydrodynamic diameter and zeta potential (ZP) analyses of NpHA-0, NpHP-0, NpHA-MTX and NpHP-MTX were performed by the dynamic light scattering technic (DLS) and electrophoretic light scattering, at 25 °C, in a detection angle of 173° using the equipment Zetasizer Nano ZS®. Analyses were performed in triplicate and the results were expressed by the average of 10 determined values and its standard deviation. The nano PECs morphology was also analysed by field emission gun scanning electron microscopy (FEG-SEM; JEOL JSM-7500F, Japan). Samples were diluted (1:30, v/v), placed on a metallic holder and dried at room temperature (RT). After, samples were covered with carbon and photomicrographs at different magnification were taken. In order to evaluate polymer-polymer and polymer-drug interactions, the FTIR (Fourier transform infrared spectroscopy) of isolated polymers, free MTX and nano PECs were recorded at RT with a VERTEX 70 spectrometer BRUKER (Massachusetts, USA) and ATR accessory, by the attenuated total reflection method (diamond crystal). For each sample, 64 scans were recorded between 3600 and 400 cm^{-1}.

To determine the %AE, a known volume of the NpHA-MTX and NpHP-MTX dispersions were centrifuged at 14,000 rpm in an ultracentrifuge (ThermoScientific, Massachusetts, USA), for 30 min. The supernatant was filtered through a cellulose acetate membrane (0.20 µm) and analysed by spectrophotometry in a multimode plate reader (Biotek Synergy 2, Winooski, VT, USA) at 303 nm. Tests were performed in triplicate, and the %AE was calculated according the Eq. (1).