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Chitosan based nanoparticles functionalized with peptidomimetic derivatives for oral drug delivery

Research Paper

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The goal of this study was to develop an optimized drug delivery carrier for oral mucosa applications able to release *in situ* bioactive molecules by using biopolymeric materials. Among them chitosan and poly(lactic-co-glycolic acid) (PLGA) have gained considerable attention as biocompatible carriers able to improve the delivery of active agents. The formulation of such vehicles in the form of nanoparticles (NPs) could permit to exploit the peculiar properties of nanomaterials in order to enhance the efficacy of active agents. Chitosan (CS) and PLGA chlorexidine dihydrochloride (CHX)-loaded NPs were synthesized by ionotropic gelation and osmosis based methodology respectively. In order to facilitate NPs adhesion on human dental surfaces, two different strategies were employed: PLGA particles with an external shell of CS to produce a positive surface charge allowing CHX loaded PLGA NPs to interact with the negative charged dental surfaces, while CS particles were functionalized with peptidomimetic derivative glutathione (GSH). The morphology was investigated by scanning electron microscopy. A sustained release profile of CHX from CS NPs was achieved. CS-based NPs adhered on human tooth surfaces in a simulated brushing and rinsing process and their *in vitro* toxicity evaluation on Human Gingival Fibroblasts (HGFs) was between 20 and 60% in all experimental conditions. Thanks to their adhesion properties and low cytotoxicity, the synthesized CS-based formulations may be efficiently exploited for therapy purposes or to enhance *in vivo* dental care (i.e. preparation of toothpastes or other cosmetics for daily oral care).

Introduction

The need to control the proliferation of bacterial flora in the oral cavity in order to prevent pathologies such as dental caries and periodontal diseases has prompted researchers in the odontoiatric field to seek molecules with such abilities. Chlorhexidine (CLX) is the gold standard of oral antiseptics because it is characterized by both bactericide and bacteriostatic activities and it is used in bacterial plaque control since 1959 [1,2].

However, CLX is poorly soluble in water, therefore it has been used in saline form, as acetate, digluconate or dihydrochloride (CHX) [3]. CLX salts alter the polarity of the anionic bacterial cell membrane, leading to potassium ions leakage and cell death [4–6].

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Currently, CLX salts, which cause alterations of bacterial adherence to teeth, are used in the prevention and treatment of caries, in secondary infections after oral surgical procedures, and in the maintenance of peri-implant tissues health [2].

Moreover, CLX salts show a relevant anti-plaque activity thanks to their interaction with negative moieties present in oral structures (teeth, restorative materials and mucosa) [7,8] and for this reason such compounds could cause the formation of stains on the tooth surface [9].

Substantivity is an important characteristic that can be implemented through the use of devices that allow a continuous and controlled release of CLX over time and hinder its adhesion to the tooth surface, thus reducing the formation of anti-aesthetic stains.

Natural and synthetic polymers are interesting materials for developing controlled release systems of bioactive molecules being vehicles than can release encapsulated drugs by surface or bulk erosion, by diffusion or swelling, in a time- or environmental condition-dependent manner [10]. In general, in such controlled-release systems, drug stability, adsorption and therapeutic concentration within the target tissue can be greatly improved [11–13]. Moreover, delivery systems can be tailor made, for example by surface functionalization, to achieve tissue- or organ-specific targeting [14].

Chitosan has been applied widely in the medical and pharmaceutical fields [15] on the basis of the fact that it contains many amino and hydroxyl groups, thus it can bind effectively to negatively charged substances via electrostatic interactions or hydrogen bonding. Therefore, chitosan is considered extremely useful as a vehicle for mucoadhesive drug delivery and also it is able to accelerate wound healing, inhibit bacterial growth, and alleviate pain [16,17]. Since the tooth surface is negatively charged, we consider positively charged chitosan as one of the best candidate materials for the formulation of toothpaste actives delivery vehicles.

Chitosan and its derivatives have been diversely employed in the field of drug delivery [18–25], however chitosan NPs have so far been scarcely used as delivery vehicles for toothpaste actives.

Recently, different chitosan-chlorhexidine materials have been developed as antimicrobial agents for both oral and extraoral sites [26,27] and some examples include chitosan-based hydrogels [28] and polymeric films [29]. Such formulations are of interest for clinical applications, however CHX-based formulations are also used in cosmetics, such as toothpastes and mouthwashes. For such applications, NP-based smart materials may have a strong potential as drug-loaded polymeric nanovectors able to afford a controlled *in situ* release with the aim to obtain enhanced formulations of commercial products.

The present study focuses on the development and on the *in vitro* evaluation of chitosan-based NPs as delivery vehicles for the controlled release of antibacterial molecules for oral mucosa applications. Peptidic residues bearing thiol groups (i.e. glutathione, GSH) have been chemically conjugated to polymeric NPs with the aim to improve and target CLX salts release. In fact such thiol groups, mimicking the N-terminal residues of common salivary phosphopeptides such as staterin (involved in teeth mineralization), could enhance the adhesion of NPs on dental surfaces. Moreover, thiolated derivatives of chitosans have been proved to improve their mucoadhesive properties by the formation of disulfide bonds between thiol groups of the thiomers and cysteine-rich subdomains of glycoproteins present in the oral mucosa layer. Different formulations were prepared and characterized by dynamic light scattering and zeta-potential measurements. *In vitro* drug release profiles were evaluated in phosphate buffer (PBS) at different pH values, followed by a cytotoxicity assessment on Human Gingival Fibroblasts (HGFs) using MTT assay [30,31].

Materials and methods

Materials

Chitosan (CS, MW 50 and 150 kDa), chlorhexidine dihydrochloride (CHX, ≥98%), L-glutathione (GSH, ≥98%) and sodium tripolyphosphate (TPP, 85%) were purchased from Sigma (St. Louis, Missouri, U.S.A.) and used as received. All other culture grade and reagent grade chemicals and solvents were purchased form Carlo Erba (Milan, Italy) and used without further purification.

CS depolymerization

Low molecular weight CS was prepared by oxidative degradation with NaNO2 according to previously published procedures [32]. A 1% (w/w) CS solution (MW 150 kDa) was prepared in 1% acetic acid. A known amount of 0.1 M NaNO2 was then added to the CS solution, with a CS/NaNO2 molar ratio of 0.01. The mixture was magnetically stirred for 3 hours at room temperature, after which the pH was changed to 8 by the addition of NaOH 1 N, in order to precipitate CS from the solution. The solid fraction was recovered by centrifugation (14,000 rpm, 15 min, 25°C), washed with deionized water and freeze dried. The molecular weight of the prepared sample was determined by Gel Permeation Chromatography. The calibration curve was generated by using three 1 mg/mL aqueous solutions of Pullulan standards of known molecular weight (5.0, 12.2 and 48.0 kDa). The measures were carried out at a flow rate of 0.8 mL/min and at room temperature.

CS functionalization with GSH

CS was functionalized with GSH by EDC chemistry [33]. 5 mL of a DMSO solution containing 0.049 mL of 1-(3-ehtyldimethylaminopropyl)-3-ethylcarbodiimide (EDC), 6.45 mg of N-hydroxysuccinimide (NHS) and 17.21 mg of GSH were added to a 1% (w/v) CS (MW 50 kDa) solution in acetate buffer. The mixture was incubated at room temperature and under magnetic stirring for 16 hours, after which CS was precipitated by adding NaOH 1 M until pH 9 was reached.

The suspension was dialyzed against phosphate buffer for 3 days and then against deionized H_2O for further 3 days. GSH-functionalized CS (CS-GSH) was recovered by centrifugation and freeze dried.

NMR spectrometry

CS functionalization rate was determined by ^{1}H NMR by analyzing samples dissolved in D_2O acidified with DCl. All spectra were recorded at 298 K on a Bruker AVANCE III spectrometer at 9.4 T operating at the hydrogen frequency of 400.13 MHz and equipped with a Bruker multinuclear *z*-gradient inverse probe head.

The ^{1}H spectra were acquired employing the standard presat pulse sequence for solvent suppression.

The scan number is 64 transients, the recycle delay is 9.5 s, the spectral width is 15 ppm and 64 K data points for a total acquisition time of 15 s.