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Polyelectrolyte complex containing silver nanoparticles with antitumor property on Caco-2 colon cancer cells

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ABSTRACT

Polyelectrolyte complex (beads) based on *N,N,N*-trimethyl chitosan/alginate was successful obtained and silver nanoparticles (AgNPs) were loaded within beads. *In vitro* cytotoxicity assays using beads/silver nanoparticles (beads/AgNPs) provided results, indicating that this material significantly inhibited the growth of colon cancer cells (Caco-2). *In vitro* release studies showed that the beads stabilized AgNPs and repressed Ag^0 oxidation under gastric conditions (pH 2.0). On the other hand, at physiological condition (pH 7.4) the beads/AgNPs promoted releasing of 3.3 μg of Ag^+ per each beads milligram. These studies showed that the concentration of Ag^+ released (3.3 μg) was cytotoxic for the Caco-2 cells and was not cytotoxic on healthy VERO cells. This result opens new perspectives for the manufacture of biomaterials based on beads/AgNPs with anti-tumor properties.

1. Introduction

Q3 Chitosan (CS) and *N,N,N*-trimethyl chitosan (TMC) are used like carrier materials for drugs delivery on oral administration [1]. It was demonstrated that CS and TMC enhances drug penetration capacity on across mucosa of intestine [2]. CS is a polycationic polymer at acidic pH conditions, while TMC has cationic property at acid, neutral and alkaline pH conditions, because this polymer presents in its chain *N*-quaternized groups [$-^{+}N(CH_3)_3$] [3–6]. Such polymers have numerous applications in the cosmetic, food and pharmaceutical industries, whereas theses materials have some properties such as, low toxicity, stability, mucoadhesivity, biocompatibility and biodegradability [4]. On the other hand, the sodium alginate (ALG) is an anionic polysaccharide which can easily interact with CS and TMC for form polyelectrolyte complexes (PECs) *via* electrostatic interactions and intermolecular/intramolecular secondary forces [7]. PECs have received much attention in the last years, since they are used for the preparation of drug carriers and tissue engineering scaffolds [8,9].

Silver nanoparticles (AgNPs) have recently made their way into cancer therapies [10]. When tested on living cells, they have interestingly been shown to possess dual activity, inhibiting the growth and division of tumor cells and their nuclei, while being biocompatible with healthy cells [10]. However, the potential application of AgNPs is significantly dependent on their stability against aggregation, which less the active surface area on AgNPs structure [11]. Furthermore, after synthesis, the AgNPs requiring protection to prevent their oxidation, which is related to AgNPs toxicity [12].

Polymeric composites based on chitosan/AgNPs formulations (CS/AgNPs) were extensively studied and the Ag^+ releasing is very important for biomedical applications of such materials to reduce the potential toxicity effects toward healthy human cells [13–15]. CS contains amino functional groups, which are used as a protecting agent for AgNPs and, due to their extraordinary properties such as biocompatibility and biodegradation the CS/AgNPs composites have attracted greatly attention in the last years [13,16,17]. TMC also was used like stabilization reagent for AgNPs preparation, instead sodium citrate at neutral aqueous solution [17].

However, the CS/AgNPs and/or TMC/AgNPs composites have limitations when destined to biological applications. In an aqueous

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environment, the AgNPs undergo the following redox reaction and a burst release of Ag^+ ions occurs, according to Eq. (1) [18].

$$4Ag_{(s)} + O_{2(aq)} + 4H^+_{(aq)} \rightarrow 4Ag^+_{(aq)} + 2H_2O_{(aq)} \quad (1)$$

The Ag^0 oxidation is favored under acidic conditions [18]. When subjected to the gastric condition, at pH ≈ 2.0, such materials are not efficient to protect and inhibit the silver oxidation due to the easy polymer dissolution at acidic environment [18]. Furthermore, the possible cytotoxic effect on healthy and/or deleterious cells is related to the amount of Ag^+ released [19]. Therefore, the abrupt oxidation of AgNPs for Ag^+ in the gastric region may increase the cytotoxic effects on healthy cells. So, an important issue concerning silver ion release is its kinetics; fast or slow release, high or low dose, short or long-term action [18,19]. All of these points are of great interest for the development of new biomaterials based on AgNPs technologies, through controlled release formulations that employ hydrogel polymer matrices (PECs) as systems for specific delivery and application [12]. Therefore, it is important to form a dose control of the Ag^+ ion that allows the cytotoxic effects to be achieved on specific targets with no toxic effects to human health [19].

Generally the surfaces of metallic nanoparticles (NPs) of Au and Ag are charged [17,20]. So, they can interact with other compounds presents in the body and such interactions often result in the formation of aggregates, leading to rupture of the NPs structure [17]. Therefore, the preparation of polyelectrolyte complexes (PECs) with loaded-AgNPs can be a strategic alternative to promote the protection of AgNPs and also to avoid nonspecific interactions between AgNPs and biological tissues [12]. Thus, the aim of this work was to prepare hydrogel polyelectrolyte based on PECs (beads) of *N,N,N*-trimethyl chitosan/alginate (TMC/ALG) and incorporate AgNPs within these PECs. The beads/AgNPs cytotoxic effects on Caco-2 cells and on VERO cells were evaluated. Additionally, Ag^+ ions release studies from beads/AgNPs were evaluated at gastric and physiological conditions and the results were discussed in the light of the cytotoxicity test.

2. Experimental

2.1. Materials

N,N,N-trimethyl chitosan (TMC) with quaternization degree (DQ) of 15% and M_V of 26×10^3 g mol^{-1} was synthesized from chitosan (Supplementary Material, see Fig. S1) [3,21]. Sodium alginate (ALG) was purchased from Across Organics (NJ, USA) and the ratio of mannuronic acid to guluronic acid (M/G) of the ALG was 1.56, as stated by the manufacturer. It has already been reported that the values of the average number (Mn) and average-weight (Mw) molecular weights for this alginate are 339,000 g mol^{-1} and 1,073,000 g mol^{-1}, respectively [22]. Silver nitrate, sodium citrate, potassium dihydrogen phosphate and acetic acid were purchased from Sigma–Aldrich. All reagents were used as received, i.e., without any further purification. VERO (African green monkey kidney) cells and the Caco-2 cell line, which originated from a human colonic adenocarcinoma, were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco®, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco®) and 50 μg ml^{-1} gentamycin, in an incubator at 37 °C, with 5% CO_2 and 95% relative humidity. The cells were expanded when the monolayer reached confluence at day 3 ± 1. After reaching 80% confluence, cells were digested by using Trypsin/EDTA solution (0.25% trypsin – Gibco®, and 1 mmol l^{-1} EDTA).

2.2. Silver nanoparticles synthesis

The experimental procedure used for obtention of silver nanoparticles was published in details by Turkevich et al. [23] with some modifications, according to Martins et al. [24]. Silver nitrate solution (1.0 mmol l^{-1}) in a reflux system was preheated at 90 °C until boiling, and then, 2.5 ml of sodium citrate solution (0.30 mol l^{-1}) were added to the system. So, after 4 min of heating at boiling state, the system was off. Finally, AgNPs suspension was poured into an ice bath and stored in amber vial under refrigeration at 4 °C. The average diameter size of AgNPs was obtained from Transmission Electron Microscopy (TEM) images, using the program Statistic version 8 [24].

2.3. Beads preparation and silver nanoparticles loading

The methodology employed for the preparation of beads was based on the method published by Martins et al. [21,22,24]. Scheme 1 depicts the preparation of beads based on TMC/ALG. For this, TMC and ALG solutions were prepared separately, from the solubilization of both polymers in a 1.0% (v/v) acetic acid solution. The volume ratio of TMC-solution to ALG-solution was kept constant. Then, AgNPs-suspension (5.0 ml containing 0.54 mg of Ag^0) was added to 10 ml of a previously prepared ALG-solution (0.5% wt/v) and the mixture was stirred until homogenization occurred. So, the ALG-solution/AgNPs (15 ml) were slowly dropped into a respective TMC-solution aliquot (20 ml), under magnetic stirring at room temperature. Finally, the beads loaded with AgNPs (beads/AgNPs) were separated from the suspension following the same methodology as described previously by Martins et al. [21,22,24]. Acetone aliquots (≈5 ml) were dropped into the suspension (35 ml) containing the beads. The suspension was slowly stirred and allowed to stand for approximately 1.0 min, until complete decantation of the beads was achieved and then ≈20 ml of supernatant was removed. This process was repeated once more and the internal water content of the beads diminished substantially, leading the beads to shrivel up. Thus, the beads presented mechanical consistency and the liquid phase composed of water and acetone (≈25 ml) was easily removed from the suspension. This process prevented the beads from adhering to each other causing the collapse [21,22,24]. Finally, the beads were washed twice with acetone, transferred to a polystyrene Petri dish and separated from each other. The drying of beads was performed at room temperature for 48 h.

2.4. Release assays

In vitro silver ion release assays were performed in two different environments: buffer solution at pH 2.0 (acetic acid/sodium acetate solution) and buffer solution at pH 7.4 (100 ml of potassium dihydrogen phosphate aqueous solution 0.5 mol l^{-1}, 148 ml of NaOH aqueous solution 0.2 mol l^{-1} and 752 ml of distilled water). Thus, a certain amount of dried beads/AgNPs was deposited in a sealed flask with 110 ml of the previously prepared buffer solution (pH 2.0 or 7.4) at 37 °C. At a desired time interval, aliquots were removed from the flask in order to quantify the amount of Ag^+ ion released.

2.5. Cytotoxicity assays

Cytotoxicity of beads and beads/AgNPs against VERO and Caco-2 cells were determined by sulforhodamine B assay as described previously by Martins et al. [22]. The cells were seeded in 96-well tissue plates (TPP – Techno Plastic Products, Switzerland) at a density of 2.5×10^5 (VERO cell) and 8×10^5 cell ml^{-1} (Caco-2 cell) in 100 μl medium for 24 h in the CO_2 incubator. The beads were dissolved in water and were added to the medium at various concentrations after 8 h. Following incubation for 48 h, the cell monolayers were

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