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Water-soluble nanoconjugates of quantum dot-chitosan-antibody for *in vitro* detection of cancer cells based on "enzyme-free" fluoroimmunoassay

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ABSTRACT

Cancer remains one of the world's most devastating diseases with millions of fatalities and new cases every year. In this work, we attempted to develop a facile "enzyme-free" fluoroimmunoassay based on the novel nanoconjugates composed of CdS quantum dots (QDs) as the fluorescent inorganic core and an antibody-modified polysaccharide as the organic shell, modeling their possible application for the *in vitro* diagnosis of non-Hodgkin lymphoma (NHL) cancer. Chitosan was conjugated with an anti-CD20 polyclonal antibody (pAbCD20) by the formation of covalent amide bonds. In the sequence, these chitosan-antibody conjugates were utilized as direct ligands for the surface biofunctionalization of CdS QDs (CdS/chitosan-pAbCD20) using a single-step colloidal process in aqueous medium at room temperature. The most relevant physico-chemical properties of these nanoconjugates were assessed by morphological and spectroscopic techniques. The results indicated that CdS nanocrystals were produced with an average diameter of 2.5 nm and with cubic zinc blende crystalline nanostructure. The CdS-immunoconjugates (CdS/chitosan-pAbCD20) presented colloidal hydrodynamic diameter (H_D) of 15.0 ± 1.2 nm. In addition, the results evidenced that the "enzyme-free" QD-linked immunosorbent assay (QLISA) was effective for the *in vitro* detection against the antigen CD20 (aCD20) based on fluorescent behavior of the CdS nanoconjugates. Moreover, the CdS-immunoconjugates were successfully used for fluorescence bioimaging of NHL cancer cells. Finally, the cell viability results using different cell cultures based on LDH, MTT and Resazurin bio-assays have demonstrated no cytotoxicity of the new CdS-chitosan bioconjugates relative to the standard controls. Thus, CdS conjugates may offer a promising platform for the future development of *in vitro* and *in vivo* applications for the detection and diagnosis of NHL cancer cells.

1. Introduction

Despite undeniable advances in medicine, cancer remains one of the most lethal diseases of the current century in developed countries. To efficiently alter this scenario, nanomedicine, an emerging research area that integrates nanotechnology, biology, and medicine, has come into focus due to its potential to provide innovative diagnostic tools for the detection of primary cancers at their earliest stages and to offer improved therapeutic methods for effectively and selectively killing tumor cells. The most promising aspects of utilizing nanomaterials as diagnostic systems in oncology lie in their potential for bio-functionalization, which enables the materials to be targeted in a specific manner to the site of disease [1–4]. Among several alternatives of nano-dimensional materials, semiconductor nanocrystals, often referred to as quantum dots (QDs), have increasingly drawn the attention of scientists and professionals in all areas of science because of their outstanding optical, electronic, magnetic and chemical properties [1,5–7]. Compared to fluorescent organic dyes, QDs possess unique optical properties, such as high brightness, narrow and tunable spectrum emission, long-term stability, and simultaneous detection of multiple signals, which make them promising diagnostic tools for biomedical applications. However, QDs must be surface-modified to render them biocompatible and water-soluble, which is required for applications under biological microenvironments [1,2,5–7]. In that sense, an interesting approach to produce QDs may be combining the synthesis, stabilization and surface-functionalization with molecules of biological interest, such as polysaccharides, antibodies, peptides, enzymes, and nucleotides

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[2,6,7], to targeting diseased sites with high specificity, selectively and sensitivity for molecular imaging a specific tumor location [1,2,5].

Thus, chitosan and its derivatives have been selected to synthesize surface-modified conjugates for biomedical applications because it is one of the most abundant polysaccharides derived from natural sources that can be easily functionalized to improve some properties, such as water-solubility and biochemical specificity, and widen its range of bioapplications [3,8–11]. Nonetheless, few studies have been published combining the synthesis and direct bio-functionalization of QDs using chitosan and its derivatives in aqueous media [9–12]. To overcome the relative deficiency of the specific affinity of chitosan towards other biomolecules, which is mandatory for diagnosing and targeting cancer cells, other molecules with high bio-affinity, such as antibodies, RNA, and DNA, may be conjugated with chitosan. In this respect, antibodies (or immunoglobulins, IgG) offer a fascinating and flexible platform that should be exploited based on the strong affinity of antibody–antigen interactions, which constitute one of the most important natural defense mechanisms found in mammals [13,14]. Antibodies linked to polysaccharides and polymers are usually called immunoconjugates, which can be associated with fluorophores to be utilized for imaging and diagnosing cancer cells [13–15]. Immunoassays based on antibody–antigen reactions, such as Enzyme-Linked Immunosorbent Assay (ELISA), have been broadly used for decades for qualitative and quantitative diagnoses of practically all types of diseases. However, these methodologies often require expertise and specific equipment and usually involve a large number of wash and rinsing steps, which makes them time-consuming processes. The continuous efforts that are being made to improve bioassays using antibodies have led to the development of fluorescent-labeled immunosorbent assay [16] which uses chromophores such as organic dyes, fluorescent proteins and quantum dots [16–18]. The "enzyme-free" techniques based on the conjugation of fluorescent semiconductor nanoparticles with antibodies potentially offer several advantages over conventional immunoassay technologies (*e.g.* ELISA), such as speed, simplicity, and high signal enhancement for the rapid detection of disease-related proteins or biomarkers [19–22]. Only recently, it was reported by our research group [23] the possibility of using QD-immunoconjugates for developing a laser light scattering immunoassay (LIA) and also for directly bioimaging of cancer cells.

Thus, in the present work some advances in this theme are reported for the first time, where a qualitative and semi-quantitative "enzyme-free" fluorescent immunoassay was designed and performed using immunoconjugates made of CdS-QDs directly stabilized by chitosan-antibody (QLISA) for possible detection *in vitro* of the B-cell cancer biomarker CD20. Moreover, this study presents three cytotoxicity assays (LDH, MTT, Resazurin) of the CdS-chitosan bioconjugates based on different cell cultures (normal fibroblast and cancer cells) as a preliminary assessment of future applications for the *in vitro* and *in vivo* detection of NHL cancer cells.

2. Materials and methods

2.1. Materials

All of the reagents and precursors, cadmium perchlorate hydrate (Aldrich, USA, $Cd(ClO_4)_2 \cdot 6H_2O$), sodium sulfide (Synth, Brazil, >98%, $Na_2S \cdot 9H_2O$), sodium hydroxide (Merck, USA, ≥99%, NaOH), acetic acid (Synth, Brazil, ≥99.7%, CH_3COOH), 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride (EDC, Sigma, USA, ≥98%, $C_8H_{17}N_3 \cdot HCl$, $M_W = 191.7\ g \cdot mol^{-1}$), and N-hydroxysulfosuccinimide sodium salt (sulfo-NHS, Aldrich, USA, ≥98%, $C_4H_4NNaO_6S$, $M_W = 217.1\ g \cdot mol^{-1}$), were used as received. Chitosan powder (Aldrich Chemical, USA, catalog#448869, low molecular weight, M_W = 50–190 kDa, lot supplied = 60–70 kDa; degree of deacetylation DD ≥ 75.0%, lot supplied = 96.1%; viscosity 20–300 cPoise, lot supplied = 35 cPoise, 1 wt.% in 1% acetic acid) was used as the reference polysaccharide ligand. Anti-CD20 polyclonal antibody (pAbCD20) and B-lymphocyte antigen CD20 (aCD20) were supplied by Abcam (Cambridge, MA, USA). Unless indicated otherwise, deionized water (DI-water, Millipore Simplicity™) with a resistivity of 18 MΩ · cm was used to prepare the solutions, and the procedures were conducted at room temperature (RT, 23 ± 2 °C).

2.2. Bioconjugation of antibody (pAbCD20) to chitosan

The bioconjugation of chitosan with the antibody was based on the procedure published by our group [23]. In order to avoid redundancy, only major relevant experimental aspects will be presented. In brief, the antibodies (pAbCD20) were bioconjugated to the chitosan polysaccharide polymer using EDC as a "zero-length" crosslinking agent in the presence of sulfo-NHS [24,25]. Chitosan solution (1%, w/v) was prepared by adding chitosan powder (2.59 g) to a 250 mL aqueous solution (2%, v/v) of acetic acid. The solution was placed under constant moderate stirring overnight at RT, until complete solubilization occurred (pH ~ 3.6). The pH value of this chitosan acetate solution was adjusted to 5.5 ± 0.1 with NaOH ($0.1\ mol \cdot L^{-1}$), resulting in a sodium acetate-buffered solution, referred to as "chitosan_5.5". EDC (10 mg, 1.0 wt%) and sulfo-NHS (20 mg, 2.0 wt.%) was dissolved in phosphate saline buffer at pH 7.4 (1.0 mL). Anti-CD20 polyclonal antibody (pAbCD20) was used as received ($0.9\ mg \cdot mL^{-1}$). Bioconjugation was performed as follows: 95 μL of pAbCD20 was added to a reaction flask with 150 μL of EDC solution and 150 μL of sulfo-NHS solution, and then, the solution was magnetically stirred for 15 min at 6 ± 2 °C. Under continuous stirring, 11 mL of chitosan solution ("chitosan_5.5") was introduced into the flask, and then, the system was incubated at 6 ± 2 °C overnight, yielding to conjugates of chitosan and antibodies linked by stable amide bonds (RC(O)NR′R″) [23,26] referred to as chitosan-immunoconjugates ("chitosan-pAbCD20"). Chitosan and chitosan-immunoconjugate solutions (pH = 5.5 ± 0.1) were analyzed by Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) method (Thermo Fischer, Nicolet 6700) over the range of 400 to 4000 cm^{-1} using 64 scans and a resolution of 2 cm^{-1}. The samples were prepared by placing a droplet of the solutions on KBr powder and drying at 60 ± 2 °C for 24 h. Conjugation between the antibody and chitosan was also confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis (SDS-PAGE) as reported [23].

2.3. Synthesis of CdS/polysaccharides immunoconjugates

CdS nanoparticles stabilized by chitosan and immunoconjugates were synthesized *via* an aqueous route in a reaction flask at room temperature using the procedure developed by our group [23]. In brief, 7.5 mL of "chitosan-pAbCD20 solution" or "chitosan_5.5" and 39.5 mL of DI water were added to the reaction vessel (pH = 5.5 ± 0.1). Under moderate magnetic stirring, 4.0 mL of Cd^{2+} precursor solution ($Cd(ClO_4)_2 \cdot 6H_2O$, $8 \times 10^{-3}\ mol \cdot L^{-1}$) and 2.5 mL of S^{2-} precursor solution ($Na_2S \cdot 9H_2O$, $1.0 \times 10^{-2}\ mol \cdot L^{-1}$) were added to the flask (S:Cd molar ratio was kept at 1:2) and stirred for 3 min. The CdS QD dispersions produced, referred to as CdS/chitosan-pAbCD20 or CdS/chitosan (reference), depending on the capping ligand used for the synthesis and biofunctionalization, were clear, homogeneous and yellowish. The CdS QD colloids were purified using an ultracentrifuge with a 50,000 molecular mass (M_W) cut-off cellulose membrane (Amicon filter, Millipore). Centrifugation was conducted for 90 min (15 mL, 6 cycles × 15 min per cycle, at 8000 rpm, 23 ± 2 °C) using a centrifuge (Quimis, Brazil). After the first cycle, the QDs were washed 5 times with 5 mL of sodium acetate buffer (pH = 5.5 ± 0.1). Centrifugal forces caused the removal of excess reagents through the membrane into a filtrate vial. After the last cycle, the total volume of the QD suspensions was then filled to 15 mL using sodium acetate buffer and sampling aliquots of 3.0 mL were collected to evaluate the stability of the colloids, which was measured using UV–Visible spectroscopy (UV–Vis). After purification, CdS QD dispersions were stored at 6 ± 2 °C (in dark) for further use.