ACS APPLIED MATERIALS & INTERFACES

Research Article

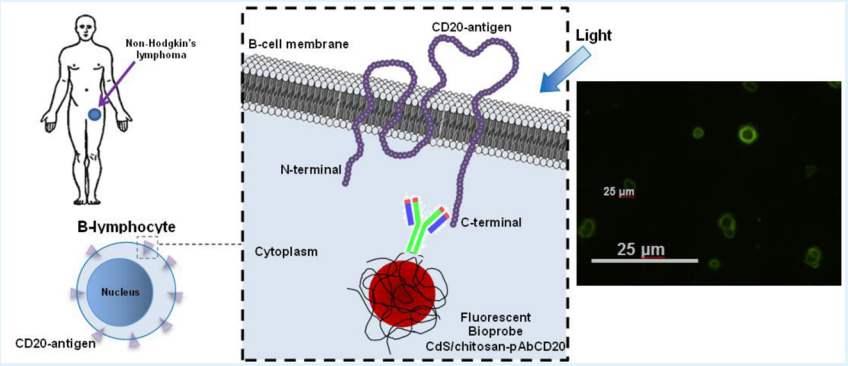
www.acsami.org

Fluorescent Nanohybrids Based on Quantum Dot–Chitosan–Antibody as Potential Cancer Biomarkers

Alexandra A. P. Mansur,[†] Herman S. Mansur,*[,†] Amanda Soriano-Araújo,[†] and Zélia I. P. Lobato[‡]

[†]Center of Nanoscience, Nanotechnology, and Innovation-CeNano[2]I, School of Engineering, Department of Metallurgical and Materials Engineering and [‡]Department of Preventive Veterinary Medicine, Veterinary School, Federal University of Minas Gerais, Av. Antônio Carlos, 6627, 31270-901, BeloHorizonte/MG, Brazil

Supporting Information



ABSTRACT: Despite undeniable advances in medicine in recent decades, cancer is still one of the main challenges faced by scientists and professionals in the health sciences as it remains one of the world's most devastating diseases with millions of fatalities and new cases every year. Thus, in this work, we endeavored to synthesize and characterize novel multifunctional immunoconjugates composed of quantum dots (QDs) as the fluorescent inorganic core and antibody-modified polysaccharide as the organic shell, focusing on their potential applications for in vitro diagnosis of non-Hodgkin lymphoma (NHL) cancer tumors. Chitosan was covalently conjugated with anti-CD20 polyclonal antibody (pAbCD20) via formation of amide bonds between amines and carboxyl groups. In the sequence, these biopolymer–antibody immunoconjugates were utilized as direct capping ligands for biofunctionalization of CdS QDs (CdS/chitosan–pAbCD20) using a single-step process in aqueous medium at room temperature. The nanostructures were characterized by UV–vis spectroscopy, photoluminescence spectroscopy (PL), FTIR, and transmission electron microscopy (TEM) with selected area electron diffraction. The TEM images associated with the UV–vis optical absorption results indicated formation of ultrasmall nanocrystals with average diameters in the range of 2.5–3.0 nm. Also, the PL results demonstrated that the immunoconjugates exhibited "green" fluorescent activity under ultraviolet excitation. Moreover, using in vitro laser light scattering immunoassay (LIA), the QDs/immunoconjugates have shown binding affinity against antigen CD20 (aCD20) expressed by lymphocyte-B cancer cells. In summary, innovative fluorescent nano-immunoconjugate templates were developed with promising perspectives to be used in the future for detection and imaging of cancer tumors.

KEYWORDS: *nanomaterials, quantum dots, bionanoconjugates, nanotechnology, nanohybrid*

1. INTRODUCTION

Despite undeniable progress in medicine over the past few decades, cancer remains one of the deadliest diseases of the 21st century, second only to cardiovascular diseases in developed countries.[1,2] To effectively alter this scenario, nanomedicine, an emerging research area that integrates nanotechnology, biology, and medicine, has come into the focus owing to its potential to provide innovative diagnostic tools for detection of primary cancers at their earliest stages and offer improved therapeutic methods for effectively and selectively killing tumor cells.[1−6]

The most promising aspects of utilizing nanodimensional materials as diagnostic and therapeutic (or theranostic) systems in oncology lie in their potential biofunctionalization, which enables the materials to localize (or be targeted) in a specific manner to the site of disease and reduce or even eliminate numerous challenging side effects.[1,2,4,6] In the vast realm of nanomaterials designed for biomedical applications, semiconductor nanocrystals or quantum dots (QDs) have received an outstanding increase in interest from scientists and professionals in all areas of science because of their exceptional optical, electronic, and chemical properties.[1,3,7,8] Essentially, QDs are nanometer-sized luminescent semiconductor nano-

Received: April 1, 2014
Accepted: June 23, 2014
Published: June 23, 2014

crystals that possess unique optical properties, such as high brightness, narrow spectrum emission, long-term stability, simultaneous detection of multiple signals, and tunable emission spectra, which make them appealing as prospective diagnostic and therapeutic systems in oncology.[1−3] When conjugated with molecular agents of biological interest, such as antibodies, peptides, enzymes, nucleotides, and other molecules,[2,7,8] QD-based bioprobes can be used to target cancer cells with high specificity, selectively, and sensitivity for molecular imaging a specific tumor location.[1−3] However, to be used under biological conditions, QDs must exhibit compatibility with the water-based physiological medium and retain their functional activity in a microenvironment where several natural macromolecules exist. Therefore, surface chemical engineering of QDs is required to render the particles water soluble and biocompatible.[1,3,7,8] In view of the large number of biomolecules available to produce surface-modified conjugates for biomedical applications, chitosan (poly-$\beta(1 \rightarrow 4)$-2-amino-2-deoxy-D-glucose) and its derivatives have often been employed.[5,9−11] Chitosan is one of the most abundant polysaccharides derived from natural sources that can be chemically functionalized to improve important properties, such as water solubility and biochemical specificity, and broaden its range of bioapplications.[5,9−12] Surprisingly, studies on direct biofunctionalization of QD surfaces with chitosan and its derivatives in aqueous media have scarcely been reported in the literature.[5,9−11]

In addition to biocompatibility, high biological affinity is another crucial feature to consider in designing surface-modified nanosystems for diagnosis and targeting specific cancer sites, which are usually found in biomacromolecules such as enzymes, proteins, antibodies, RNA, DNA, and, to a lower extent, polysaccharides. In this respect, antigen−antibody interactions offer a fascinating and flexible platform to be exploited. Essentially, antibodies or immunoglobulins (IgG) are a group of glycoproteins that constitute one of the most important specific defense mechanisms in vertebrate animals.[13,14] All antibodies (IgG) share a very similar structure: a "Y"-shaped formation composed of bifunctional molecules with two identical domains for antigen recognition (Fab fragment, N-terminal, highly specific) and two identical domains with effector functions (Fc fragment, carboxyl terminal).[13,14] Combinations of antibodies linked to other molecules such as polysaccharides and polymers are usually referred to as immunoconjugates, which may be associated with fluorophores or radioisotopes to be utilized for imaging, diagnostic, and cancer treatment purposes, such as in lymphomas.[13−15] Thus, quantitative and qualitative diagnostic methods based on antibody−antigen reactions (called immunoassays) have been widely used for decades for detection of practically all types of diseases.[13,16,17] However, these methodologies require expertise and specific equipment and usually involve a large number of washing and rinsing steps, which makes them laborious and time-consuming processes. Thus, development of new technologies offering rapid, reliable, and reproducible immunoassays at relative low cost is highly desirable.[16,17]

Dynamic light scattering (DLS) is a technique widely used for analyzing the particle size and size distribution of polymers, proteins, colloids, and nanoparticles in solutions and dispersions.[16] Recently, it was demonstrated that DLS can measure the interactions between antibody-coated nanoparticles with target analytes, referred to as laser light scattering immunoassay (LIA). The method potentially offers several advantages such as speed, simplicity, high signal enhancement, and sensitivity over conventional immunoassays technologies for detection of disease-related proteins or biomarkers.[16,18,19] Hence, the conjugation of fluorescent semiconductor nanoparticles with antibodies combines the exceptional optical properties of the nanocrystals themselves with the specific and selective recognition ability of the antibody to the antigen.

Thus, in this research, a novel multifunctional colloidal nanohybrid system was designed and engineered for the first time based on the combination of fluorescent CdS quantum dots with chitosan−antibody (anti-CD20) immunoconjugates as bioaffinity ligands for potential applications in the detection of non-Hodgkin lymphoma (NHL) cancer. In this study, we demonstrated that the CdS−immunoconjugates were effective for detection of the antigen CD20 using a relatively rapid in vitro method based on laser light scattering immunoassay. Moreover, the CdS−immunoconjugates behaved as biomarkers using fluorescent microscopy for diagnosis and imaging of lymphoma cancer cells.

2. EXPERIMENTAL SECTION

2.1. Materials. All 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 (Sigma, USA, ≥ 98%, $C_8H_{17}N_3{\cdot}HCl$), and *N*-hydroxysulfosuccinimide sodium salt (Aldrich, USA, ≥ 98%, $C_4H_4NNaO_6S$), were used as received. Chitosan powder (Aldrich Chemical, USA, catalog no. 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 SimplicityTM) with a resistivity of 18 MΩ·cm was used to prepare the solutions and procedures were conducted at room temperature (23 ± 2 °C).

2.2. Bioconjugation of Antibody (pAbCD20) to Chitosan. The antibodies (pAbCD20) were bioconjugated to the chitosan polysaccharide using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC, M_w = 191.7 g·mol^{-1}) as a "zero-length" cross-linking agent in the presence of *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS, M_w = 217.1 g·mol^{-1}).[20,21]

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 room temperature until complete solubilization occurred (pH ≈ 3.6). The pH of this chitosan acetate solution was adjusted to 5.5 ± 0.1 with NaOH (0.1 mol·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 %) were dissolved in phosphate saline buffer at pH 7.4 (1.0 mL). Anti-CD20 polyclonal antibody (pAbCD20) was used as received (0.9 mg.mL^{-1}). Bioconjugation was carried out 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 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 the system was incubated at 6 ± 2 °C overnight. EDC in the presence of sulfo-NHS converts the carboxyl groups on pAbCD20 to amine-reactive sulfo-NHS esters. These esters commonly react with available amine groups in chitosan, yielding conjugates of chitosan and antibodies linked by stable covalent amide bonds (RC(O)NR′R′′) referred to as chitosan−immunoconjugates ("chitosan−pAbCD20").[22]

Conjugation between antibody and chitosan was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE). Antibodies (pAbCD20) were separated by electrophoresis on 4−12% gradient polyacrylamide SDS gel (Amersham ECL

Gel, GE Healthcare, Uppsala, Sweden). Gels were stained with Coomassie Blue to identify the antibody (Coomassie Brilliant Blue G-250).

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 as follows: 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 ($Cd(ClO_4)_2{\cdot}6H_2O$, 8×10^{-3} mol L^{-1}) and 2.5 mL of S^{2-} precursor solution ($Na_2S{\cdot}9H_2O$, 1.0×10^{-2} mol L^{-1}) were added to the flask (S:Cd molar ratio was kept at 1:2) and stirred for 3 min. The CdS QDs 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) cutoff 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 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−vis spectroscopy. After purification, QD dispersions were stored at 6 ± 2 °C for further use.

2.4. Characterization of CdS/Polysaccharides Immunoconjugates. UV−vis spectroscopy measurements were conducted using PerkinElmer equipment (Lambda EZ-210) in transmission mode in a quartz cuvette over a wavelength range from 600 to 190 nm (from visible to ultraviolet). Absorption spectra were used to monitor formation of CdS QDs during the reaction and their relative colloidal stability in the media. On the basis of the "absorbance onset" of the curve, it was possible to estimate the average nanoparticle size and their optical properties. All experiments were conducted in triplicate ($n = 3$) unless specifically noted. Statistical analysis of results was performed assuming the mean and standard deviation where necessary.

Photoluminescence (PL) characterization of the CdS/polysaccharides conjugates and intermediates systems was performed based on spectra acquired using the Nanodrop 3300 fluorospectrometer (Thermo Scientific, blue LED with $\lambda_{excitation}$ = 470 ± 10 nm). All photoluminescence spectra were collected at room temperature, and measurements of fluorescence intensities were reported in relative fluorescent units (RFU). All tests were conducted using a minimum of four repetitions ($n \geq 4$). Additionally, QD colloidal media were placed inside a "darkroom chamber" where they were illuminated by a UV radiation emission bulb ($\lambda_{excitation}$ = 254 and 365 nm, 6 W, Boitton Instruments). Digital color images were collected when the QDs fluoresced in the visible range of the spectra.

Nanostructural characterization of the CdS/chitosan−pAbCD20, based on the images and selected area electron diffraction patterns (SAED), was conducted using transmission electron microscopy (TEM) with a Tecnai G2−20-FEI microscope at an accelerating voltage of 200 kV. Energy-dispersive X-ray spectra (EDX) were collected for chemical analysis. In all TEM analyses, samples were prepared by dropping the colloidal dispersion onto a holey carbon grid after the purification procedure developed in this study. QD sizes and distribution data were obtained based on the TEM images by measuring at least 100 randomly selected nanoparticles using an image processing program (ImageJ, version 1.44, public domain, National Institutes of Health).

CdS/polysaccharides dispersions were analyzed by the diffuse reflectance infrared fourier transform spectroscopy (DRIFTS) method (Thermo Fischer, Nicolet 6700) over the range from 400 to 4000 cm^{-1} using 64 scans and a resolution of 2 cm^{-1}. Samples were prepared by placing a droplet of the dispersions on KBr powder and drying at 60 ± 2 °C for 24 h.

Zeta-potential measurements were performed on QD colloidal water media using a ZetaPlus instrument by applying the laser light diffusion method (Brookhaven Instruments). This instrument uses the laser Doppler electrophoresis technique (35 mW red diode laser at λ = 660 nm). All tests were performed using a minimum of three replicates (n = 3), and the values were averaged.

2.5. Immunological Assays for Detection of Cancer Biomarkers. As proof of the concept, a combination of fluorescent and immunobiochemical methods was utilized to characterize in vitro the activity and affinity of the new nanohybrid systems, based on chitosan−antibody−quantum dot immunoconjugates. It should be highlighted that for in vivo detection of cancer cells further characterization of biological properties of the immunoconjugates will be performed in future research.

2.5.1. Polymer−Antibody−Quantum Dot Immunoassay with DLS Method. In this study, an innovative assay was used often referred to as laser-light scattering immunoassay (LIA) as the immunodiagnostic method for detection of antibody using dynamic light scattering (DLS) as probe. To improve the reliability of the results, CdS/polysaccharides conjugates stabilized with chitosan bioconjugated with B-lymphocyte antigen CD20 (chitosan−aCD20) were also prepared in a similar procedure as described in sections 2.2 and 2.3. The main difference is with regard to the pH of bioconjugation and synthesis (pH = 4.7 ± 0.1) selected based on the estimated isoelectric point of antigen (~5.0). The QDs colloids prepared with chitosan−aCD20 ligand were referred to as CdS/chitosan−aCD20. The following "control" samples were analyzed using DLS: CdS/chitosan, CdS/chitosan−pAbCD20, and CdS/chitosan−aCD20. Formation of CdS/antibody:antigen assemblies (referred to as CdS/chitosan−pAbCD20:CdS/chitosan−aCD20) were studied from the system obtained from a mixture of CdS/chitosan−pAbCD20 and CdS/chitosan−aCD20 after incubating at 37 ± 2 °C for 2 h.

Dynamic light scattering analyses were performed using a Brookhaven ZetaPlus instrument with a laser light wavelength of 660 nm (35 mW red diode laser) and a thermostat with temperature stabilization. Standard square acrylic cells with a volume of 4.5 mL were used. For the DLS of the QDs, the colloidal solutions (5 mL) in the presence of 0.025 mol·L^{-1} NaCl (2 mL) were filtered four times through a 0.45 μm aqueous syringe filter (Millex LCR 25 mm, Millipore) to remove any possible dust. Samples were measured at 25 ± 2 °C, and light scattering was detected at 90°. Each required approximately about 3 min, and 3 measurements were obtained for each system and averaged.

2.5.2. Immunohistochemistry Assay−Fluorescence Imaging of Lymphoma Cells Labeled with QD Bioconjugates. Human B-cell lymphoma cell line expressing CD20 antigen in surface (TOLEDO CRL2631, American Type Culture Collection (ATCC), Manassas, USA) were maintained at 37 °C and 5% CO_2 in ATCC-formulated RPMI-1640 Medium (catalog no. 30-2001) supplemented with fetal bovine serum (Sigma-Aldrich, St Louis, USA) to a final concentration of 10%.

Colloidal suspensions of CdS QDs at a concentration of approximately 2.0 μmol L^{-1} were used in the fluorescence imaging experiments. TOLEDO cells expressing the CD20 antigen (3×105 cells·mL^{-1}) were incubated with CdS/chitosan-pAbCD20 conjugates (1:1) in microfuge tubes for 1 h at 37 °C (referred to as immunoconjugates). For the negative control experiment, TOLEDO cells were incubated with mock conjugated CdS/chitosan quantum dots. As these cells were grown in suspension, after adding 500 μL of cells with quantum dots on the glass microscopy slides, they were centrifuged (800 rpm for 5 min) for the sedimentation and adherence of the cells onto the solid support (cytocentrifuge apparatus with cyto chamber 2 mL and chamber cyto slide carrier, Universal 320R Centrifuge, Hettich Lab Technology, Kirchlengern, Germany). The sample of CdS/chitosan−pAbCD20 without cells was referred to as "blank". The supernatant was discarded to remove any unbound CdS quantum dots, and the cells that remained attached to the substrate (solid phase) were fixed using 4% paraformaldehyde in phosphate buffer for 30 min. After removing the fixative, CdS quantum dot immunoconjugates bound to TOLEDO cells were examined by inverted fluorescence microscopy (fluorescence microscope Ti-U, Nikon, illuminating system 130 W/Hg lamp, objective 20×, eyepiece 10×) coupled to a refrigerated CCD camera (DS Qi1-U3, Nikon) with

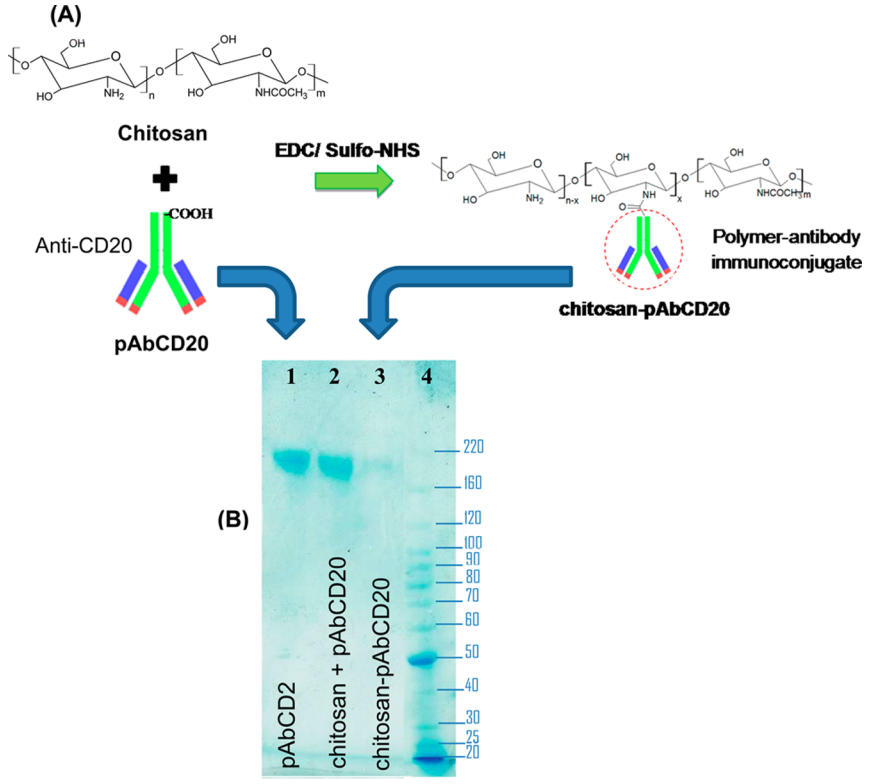


Figure 1. (A) Structure of the immunoconjugates based on the reaction of chitosan and anti-CD20 antibody (pAbCD20) using EDC/sulfo-NHS as a cross-linker. (B) Conjugation of pAbCD20−chitosan confirmed by SDS-PAGE (lane 1, pAbCD20; lane 2, physical mixture of pAbCD20 and chitosan unbound; lane 3, "chitosan-pAbCD20" covalently linked conjugates; lane 4, protein marker, BenchMark Protein Ladder; units kDa).

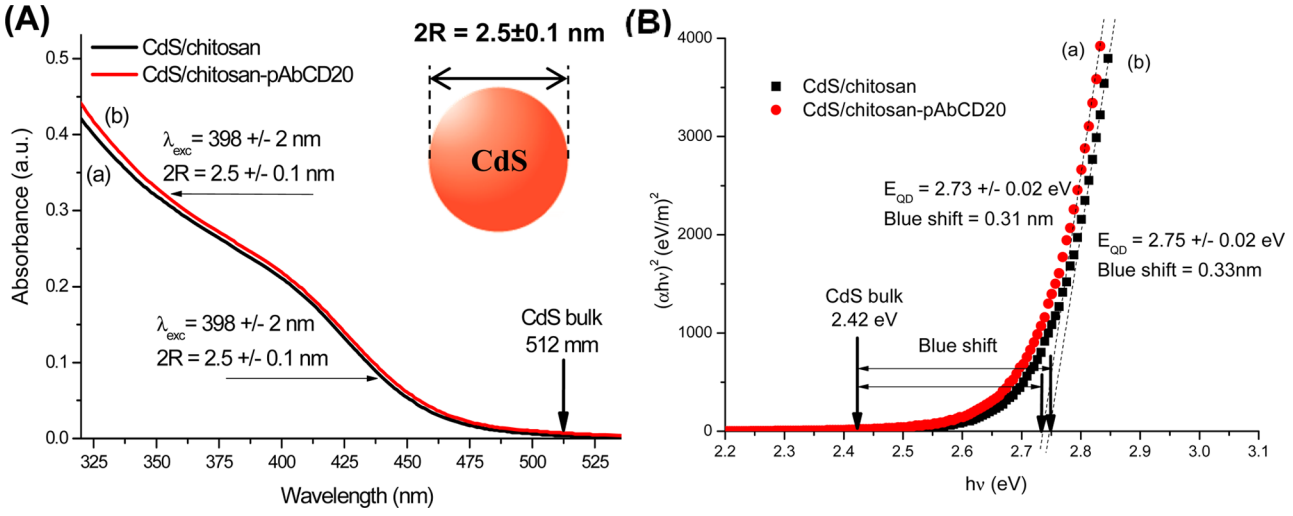


Figure 2. (A) UV−vis spectra and (B) optical band gap, determined using the "TAUC" relation of CdS/chitosan (a) and CdS/chitosan−pAbCD20 conjugates.

1280 × 1024 resolution (1.5 Mpixels). The filter cubes FITC (excitation filter, 460−500 nm; emission filter, 510−560 nm) and TRITC (excitation filter, 530−560 nm; emission filter, 590−650 nm) were used for green and orange-red emissions, respectively. Intensity fluorescence profiles were obtained using imaging software NIS-Elements, Nikon, at the following conditions: FTIC filter-N/D lamp control, 1; exposure time, 353 ms; auto gain, 1.7×; and TRITC filter N/D lamp control, 2; exposure time, 60 ms; auto gain, 1.7×. For each system, 15 measurements were collected at the same conditions and averaged.

3. RESULTS AND DISCUSSION

3.1. Synthesis of Polymer−Antibody Immunoconjugates. In this study, a novel polymer−antibody immunoconjugate was designed and synthesized with the objective of combining a set of physicochemical, biochemical, and immunological properties into a single system. The polyclonal antibody anti-CD20 (pAbCD20) was covalently coupled with the chitosan biopolymer, leading to formation of the immunoconjugates (Chi-pAbCD20). The carboxyl-reactive EDC was utilized as the cross-linker in the presence of sulfo-NHS (catalyst) for covalently coupling the carboxylic group from pAbCD20

dx.doi.org/10.1021/am5019989 | ACS Appl. Mater. Interfaces 2014, 6, 11403−11412

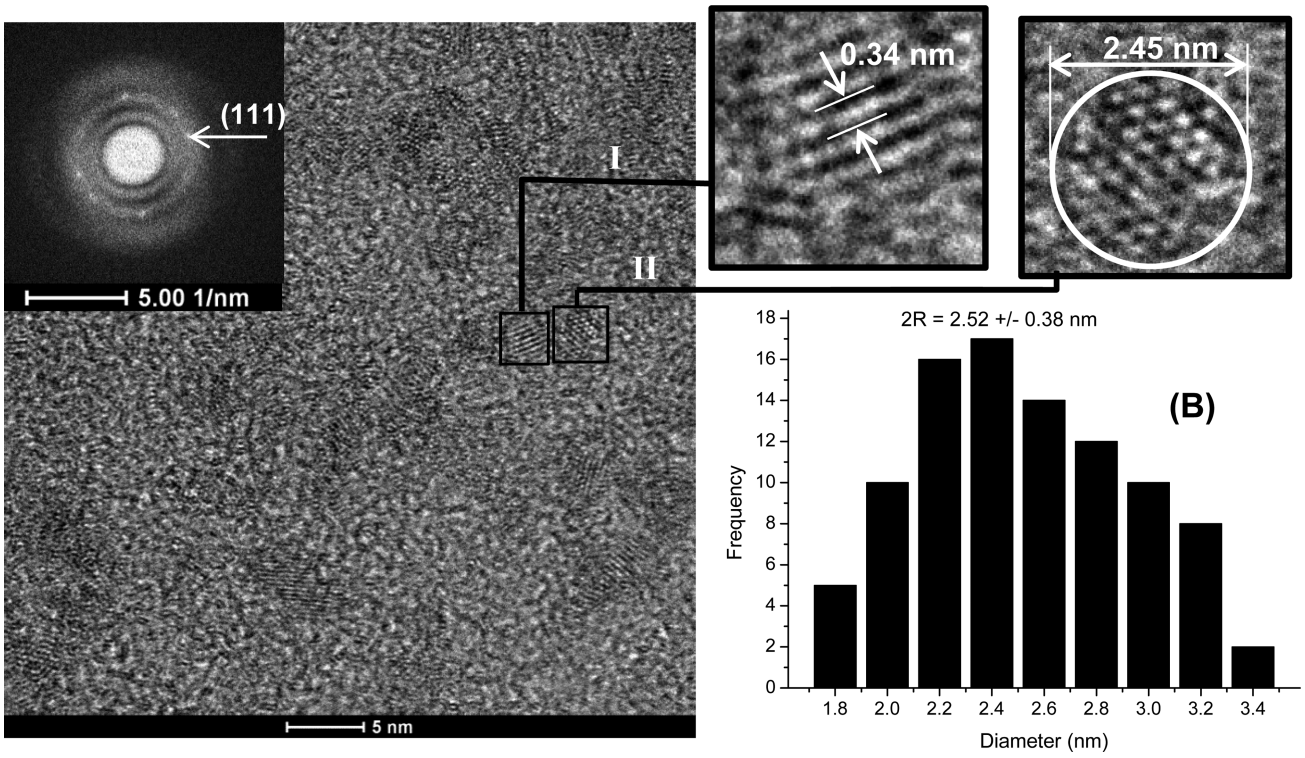


Figure 3. (A) TEM image and (B) histogram of nanoparticle size distribution of CdS/chitosan−pAbCD20. (Inset) Diffraction ring attributed to the (111) plane of the cubic lattice of CdS. Details: (I) Nanocrystal plane spacing; (II) Nanoparticle size.

(known as the Fc; region of antibodies, IgG) to the available amine groups present in the chitosan polysaccharide structure ($R-NH_2$).[20−22] Moreover, EDC is a water-soluble, zero-length cross-linker that is not incorporated into the conjugated network by the reaction, and therefore, there are no concerns about its toxicity relative to that of other cross-linkers (glutaraldehyde, formaldehyde, epichlorohydrin, etc.).[22] The structure of the immunoconjugate designed and synthesized based on pAbCD20 and chitosan is schematically represented in Figure 1A.

SDS-PAGE analysis was performed in order to confirm formation of the chitosan−pAbCD20 immunoconjugates (Figure 1B). The major reference band associated with the free antibody is observed in lane 1. This corresponding band can be clearly observed in lane 2 when a physical mixture of chitosan and antibody was tested (without EDC/sulfo-NHS), indicating the lack of conjugates in the medium. On the other hand, the band associated with the free antibody almost disappeared in lane 3 after the EDC-mediated carbodiimide reaction, demonstrating that pAbCD20 was chemically conjugated to chitosan. The occurrence of antibody cross-linking prior to the reaction with chitosan was also evaluated through SDS-PAGE, and the results indicated no detectable Ab−Ab species (Supporting Information, Figure S1).

3.2. Characterization of CdS Quantum Dots. *3.2.1. Characterization of CdS Quantum Dot Immunoconjugates by UV−Vis Spectroscopy.* In Figure 2A, UV−vis spectra of the conjugate CdS/chitosan and CdS/chitosan−pAbCD20 colloidal suspensions are shown after stabilization. The curve exhibits a broad absorption band between 350 and 450 nm, with the exciton absorption peak transition (λ_{exc}) at approximately 400 nm. This behavior is attributed to the nucleation/growth of CdS nanoparticles within the "quantum confinement regime" based on the so-called blue shift observed for the curve relative to the "bulk" value (Figure 2A, arrow at λ = 512 nm). Therefore, the optical band gap energy of a QD (E_{QD}) is greater than that of the original bulk material (E_g).[3]

The average CdS nanoparticle size was determined based on Henglein's empirical model,[21] which relates the diameter of the CdS nanoparticle (2R) to the exciton absorption peak (λ_{exc}) in UV−vis spectra according to eq 1. Therefore, CdS QDs with an estimated diameter (2R) of 2.5 ± 0.1 nm were produced and stabilized.

$$2R(\text{nm}) = \frac{0.1}{0.1338 - 0.0002345\lambda_{exc}} \quad (1)$$

The optical band gap (absorbance onset) and the blue-shift values were determined from the absorption coefficient data as a function of wavelength using the TAUC relation[23] extracted from the UV−vis spectra. Results are summarized in Figure 2B and support formation of CdS QDs in the chitosan media because the band gap energy of the semiconductor (~2.74 eV) was significantly greater than that of the CdS bulk (i.e., 2.42 eV, blue shift ≈ 0.32 eV).[21] These results provide strong evidence that CdS QDs were effectively produced and stabilized by chitosan−antibody conjugates in aqueous medium. It should be noted that, based on a literature review, despite several studies reporting the synthesis of CdS QDs, there have been no previous studies that have developed CdS QDs with chitosan−antibody (anti-CD20) immunoconjugates.

3.2.2. TEM Morphological Analysis. TEM images and SAED patterns of CdS QDs capped by the carbohydrate−antibody are shown in Figure 3A. The CdS/chitosan−pAbCD20 nanoparticles exhibited a spherical shape with a reasonable monodisperse distribution and average size of 2.52 ± 0.38 nm (size distribution histogram shown in Figure 3B). Lattice fringes with an interplanar distance of approximately 0.34 ± 0.01 nm can be assigned to the (111) plane of cubic CdS (ICCD-89-0440).

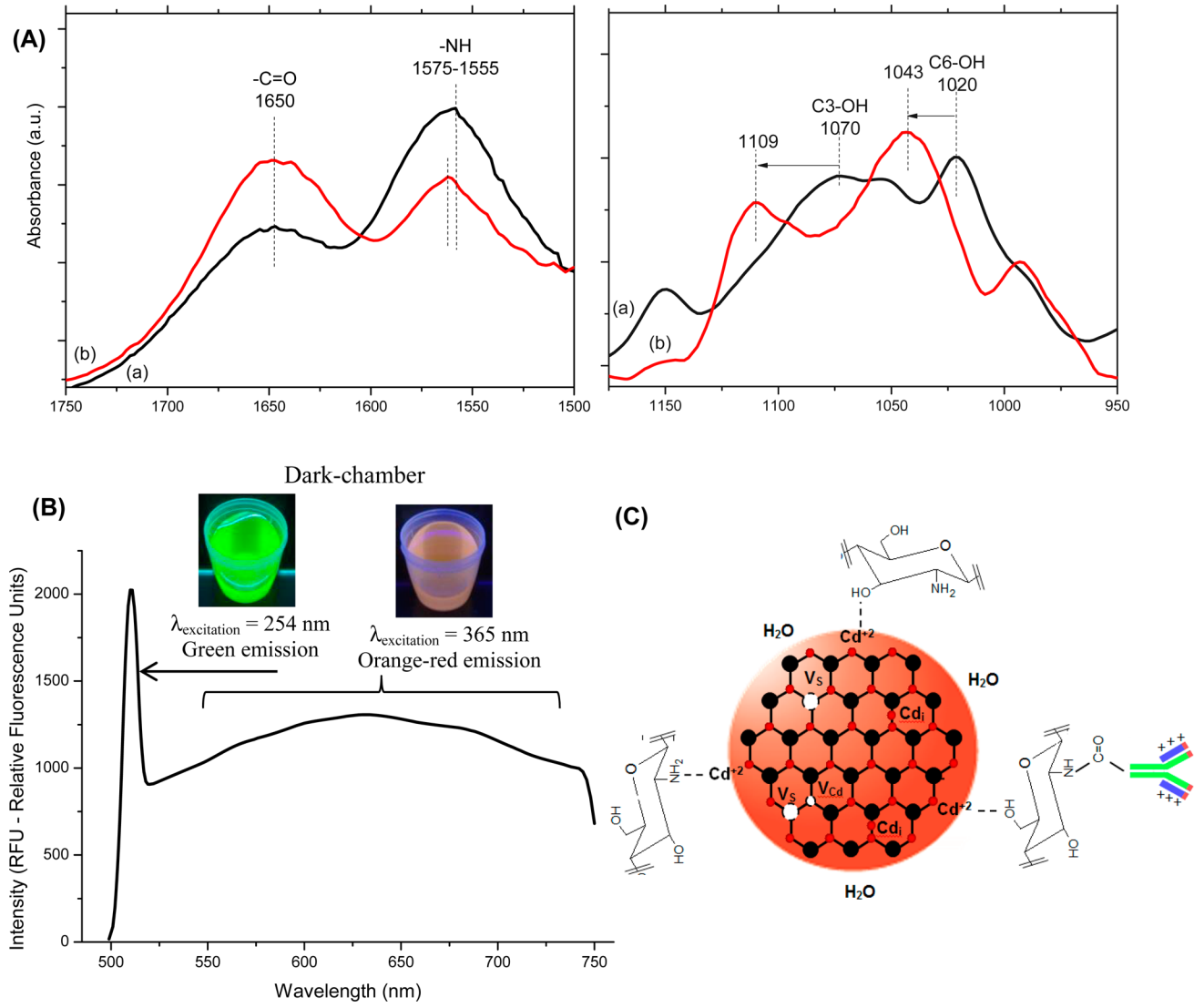


Figure 4. (A) FTIR spectra of chitosan−pAbCD20 polymer−antibody (a) and CdS/chitosan−pAbCD20 (b). (B) PL spectra of CdS/chitosan−pAbCD20. (C) Schematic representation of defects in the nanocrystal structure and some interactions at CdS/chitosan−pAbCD20 interfaces (not to scale).

This result was confirmed by the X-ray diffraction pattern obtained from CdS (Supporting Information, Figure S2). Therefore, the TEM results demonstrated that CdS quantum dots were properly stabilized by chitosan covalently bonded to pAbCD20, which is in reasonable agreement with the values obtained from the UV−vis optical absorbance shown in Figure 2.

3.2.3. Characterization of CdS Quantum Dot Immunoconjugates by FTIR Spectroscopy. FTIR spectra of chitosan−pAbCD20 ligand (a) and CdS/chitosan−pAbCD20 conjugates (b) are shown in Figure 4A. In the spectrum of the QD immunoconjugates, the bands ranging from 1575 to 1555 cm^{-1}, associated with the N−H bending of amines,[9] are slightly shifted toward a lower energy (i.e., wavenumber). On the other hand, no considerable changes were detected in the position of the amide I band (1650−1640 cm^{-1}) assigned to the carbonyl stretching of the acetamides.[9] A significant change was observed in the bands at 1070 and 1020 cm^{-1}, which are associated with secondary (C3−OH) and primary (C6−OH) alcohols, respectively.[9] The C3−OH stretching vibration was red shifted (shifted toward a lower wavenumber/energy) by 39 cm^{-1}, whereas the primary alcohol band shifted its position to a lower energy by 23 cm^{-1}. Additionally, the broad peak of the chitosan−pAbCD20 ligand at 3420 cm^{-1}, corresponding to the stretching vibration of $-NH_2$ and −OH groups,[9] became significantly narrower after stabilization, indicating the reduction of "free" amine groups in CdS/chitosan−pAbCD20.[24] The differences between the FTIR spectra of the chitosan immunoconjugates, before and after conjugation with CdS nanocrystals, may be attributed to formation of a coordination complex between chitosan and cadmium ions (predominantly Cd^{2+} on the surfaces of the QDs) under the effects of the amino and hydroxyl functional groups.[24]

Characterization of CdS Quantum Dot Immunoconjugates by Photoluminescence Spectroscopy (PL). Typical room-temperature PL spectra of the CdS nanoparticles stabilized by chitosan and chitosan−antibodies are presented in Figure S3(d), Supporting Information, and Figure 4B, respectively. From a general perspective, band edge recombination (E_{exc}, blue luminescence) was not detected, and other bands could be observed at approximately 509 nm (green) and within the 600−750 nm range (orange-red), in agreement with the literature.[25] The bright green emission (inset in Figure 4B) is favored by the synthesis of nanoparticles under an excess of metal atoms that

dx.doi.org/10.1021/am5019989 | ACS Appl. Mater. Interfaces 2014, 6, 11403−11412

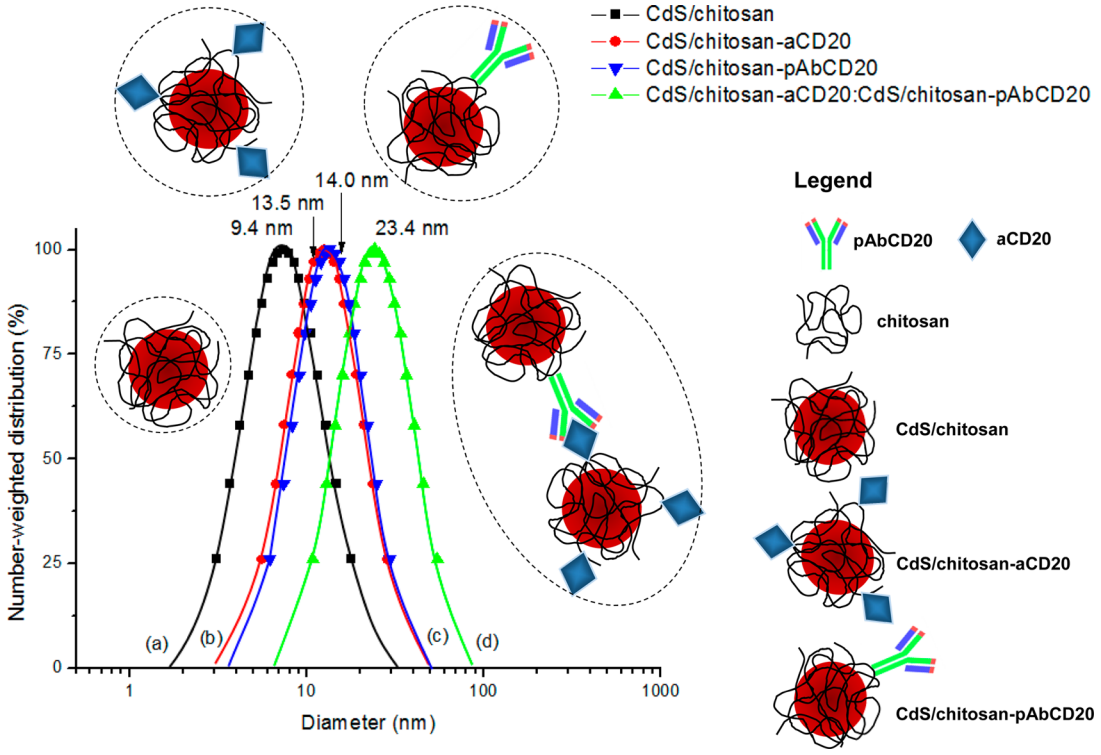


Figure 5. DLS-measured size distribution of fluorescent bioconjugates: (a) CdS/chitosan; (b) CdS/chitosan−CD20 antigen; (c) CdS/chitosan−anti-CD20 antibody; (d) affinity nanocomplexes of CdS/chitosan−CD20 antigen:CdS/chitosan−anti-CD20 antibody. (Inset) Illustration of the immunoconjugates before and after successful formation of the affinity nanocomplexes measured by DLS assay (LIA).

enter the lattice at interstitial sites (Cd_i), compatible with the procedure used in this work using a stoichiometric molar ratio of cation:anion = 2:1 (Cd:X, X = S).[25−27] The luminescence related to orange-red bands (inset in Figure 4B) is typically detected in CdS nanocrystals that contain a certain concentration of intrinsic defects. Orange luminescence (590−620 nm) is observed when interstitial atoms of the metal are present in the semiconductor lattice, and a red band (620−750 nm) of PL is typically observed for nanoparticles that contain a certain concentration of intrinsic defects of the V_{Cd}−V_S (divacancies, i.e., cadmium V_{Cd} and sulfur V_S)[26] or V_S type.[27]

3.2.5. Zeta-Potential Analysis. The zeta potentials (ZPs) calculated for CdS/chitosan and CdS/chitosan−pAbCD20 at pH 5.8 ± 0.1 were +1.4 ± 0.3 and +15.4 ± 0.4 mV, respectively, indicating that these conjugates were not predominantly electrostatically stabilized but relied on the steric hindrance of the polymer chains adsorbed physically and chemically to prevent close contact between the fluorescent QDs.[28] Furthermore, these results indicated that the CdS/chitosan−pAbCD20 immunoconjugates possessed higher positive surface charges than the CdS QDs stabilized with the reference ligand (i.e., chitosan). Hence, these results suggest that the antibodies were conjugated to chitosan with positive charges facing outward in the polymeric "shell". It was not hidden by the conjugation and is available for antigen affinity recognition. Figure 4C summarizes the results of the zeta-potential measurements, with a schematic representation (not to scale) of the interactions that occurred at the interfaces and intrinsic defects, based on FTIR and PL results.

3.3. Immunoassays of CdS Quantum Dot/Chitosan Conjugates Based on Dynamic Light Scattering. In this study an innovative homogeneous immunoassay method was designed and employed for qualitative detection of cancer biomarkers using dynamic light scattering (DLS-LIA). The utility of the DLS technique lies in its ability to distinguish individual nanoparticles, dimers, oligomers, or aggregates in colloidal suspensions because of their differences in size, commonly referred to as "hydrodynamic diameter" (H_D). This feature makes DLS a suitable analytical tool for developing immunoassays involving formation of antigen−antibody complexes in aqueous colloidal media.[18]

The size distributions of the conjugates measured by DLS and the average H_D values are shown in Figure 5. It can be observed that the CdS chitosan conjugates used as the initial reference presented H_D values of 9.4 ± 1.2 nm and that the CdS−immunoconjugates, synthesized with the polyclonal anti-CD20 antibody (CdS/chitosan−pAbCD20) and with the CD20 antigen (CdS/chitosan−aCD20), showed H_D values of 14.0 ± 1.2 and 13.5 ± 1.2 nm, respectively. As expected, the H_D value for the CdS chitosan conjugates was smaller than the H_D values measured for both types of CdS−immunoconjugates. This result may be attributed to the relatively larger "excluded volume" of the polymer−protein immunoconjugates (chitosan−antibody and chitosan−antigen) compared with that of the polymer ligand (chitosan) alone. Additionally, the H_D measurements are compatible with the expected dimensions of colloidal nanohybrids with a "core−shell" structure, i.e., a CdS QD "inorganic core" capped by ligands forming an "organic shell". Thus, to the average diameter of the CdS QDs ("core") estimated in previous sections by UV−vis and TEM analyses, the shell of the ligand is added, i.e., chitosan (low molecular weight, ~60−70 kDa), or the conjugates chitosan−antibody (chitosan−pAbCD20) and chitosan−antigen (chitosan−aCD20), compatible with the H_D results measured by DLS.

Moreover, as far as the use of DLS as an immunoassay is concerned, the most important result could be clearly observed (Figure 5d) when both immunoconjugates (CdS/chitosan−

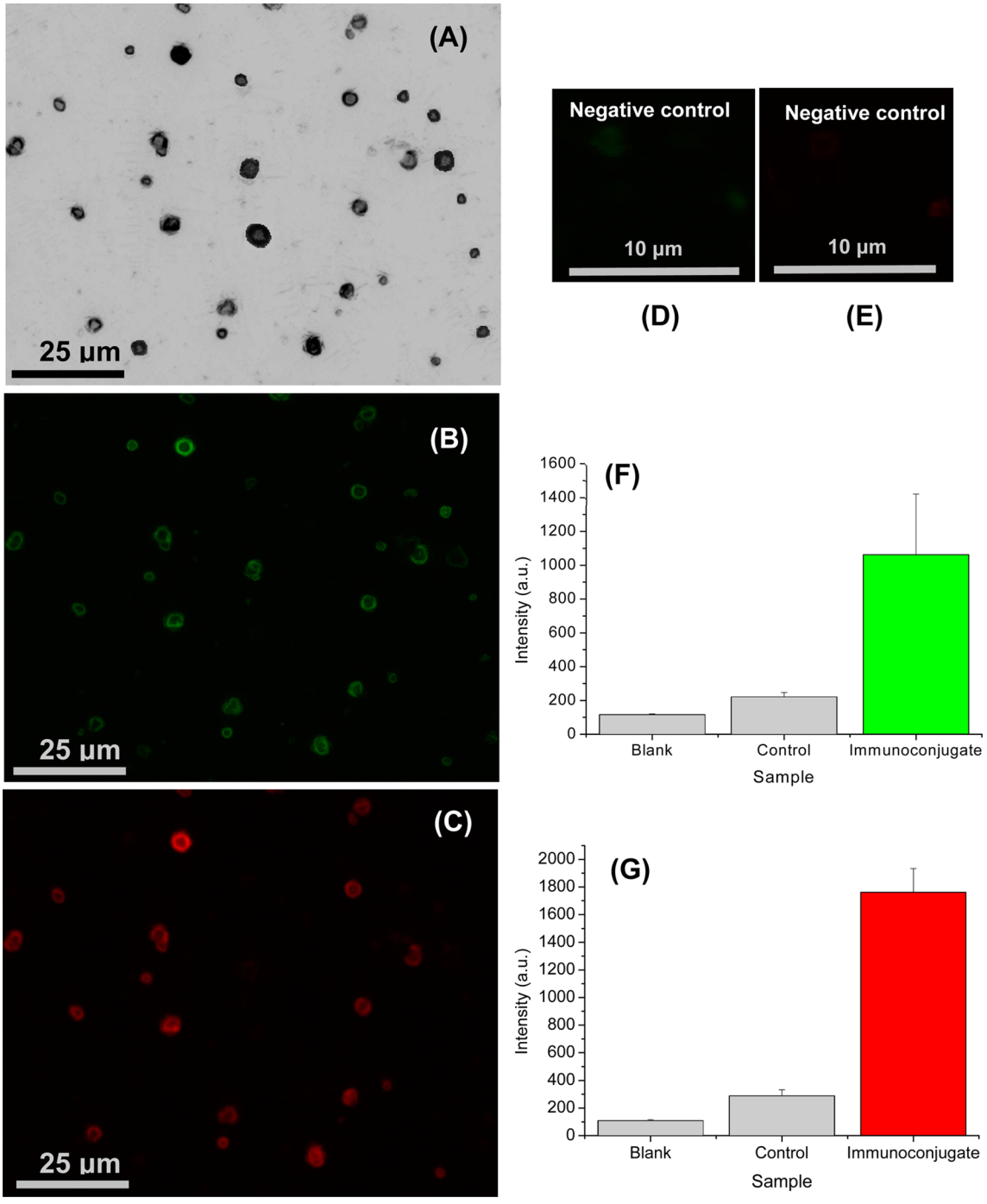


Figure 6. Detection of the B-cell cancer biomarker CD20 internalization through immunofluorescence: (A) bright field microscopy; (b) green (FITC filter) and (C) orange-red (TRITC filter) fluorescence images of immunoconjugates (CdS/chitosan−pAbCD20 with lymphoma cells); (D) green and (E) orange-red fluorescence of negative controls; (F) green and (G) orange-red fluorescence intensity profiles of blank, negative control, and immunoconjugates.

pAbCD20 and CdS/chitosan−aCD20) colloidal dispersions were mixed, leading to H_D values of 23.4 ± 3.3 nm. This increase in H_D may be attributed to the sum of the relative contributions from both immunoconjugates, CdS/chitosan−pAbCD20 and CdS/chitosan−aCD20. These findings can be interpreted by considering the high affinity between antibody anti-CD20 and antigen CD20 in aqueous colloidal media. Thus, in the presence of antigen CD20 in solution, the immunoconjugates CdS/chitosan−pAbCD20 and CdS/chitosan−aCD20 coupled together into pairs through formation of antibody−antigen complexes, which was quantitatively monitored by DLS measurements. These results demonstrate the feasibility of the DLS method to serve as an immunoassay using QD nanoparticles functionalized with immunoconjugates.

As this point it is appropriate to discuss the potential contribution of the produced nanoimmunoconjugates in the field of cancer diagnosis and treatment. Non-Hodgkin lymphomas (NHL) account for approximately 4% of cancers, ranks fifth in cancer mortality, and is the leading cause of cancer-related deaths for patients between 20 and 40 years of age.[29]

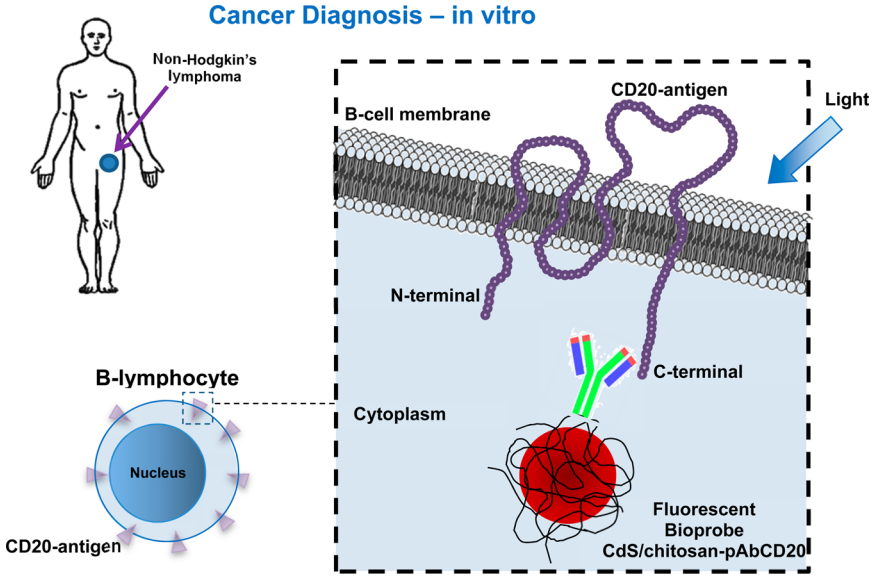


Figure 7. Schematic representation of the hypothesized nanohybrid system (not to scale). CdS/immunoconjugates may behave simultaneously in the detection and targeting of NHL cancers, based on the binding affinity between the antigen CD20 at the B-cell biomembrane and the anti-CD20–antibody.

CD20 is a nonglycosylated 33–37 kDa phosphoprotein (297 amino acids sequence) expressed on more than 95% of normal and neoplastic B cells, such as in NHL. Moreover, the CD20 receptor is exclusively expressed on B cells, and it has not been detected on other normal human tissues.

In consideration of the aforementioned reasons, it is of paramount importance that NHL cancers be detected as early as possible by rapid, efficient, accurate diagnosis based on CD20 as the B-cell-specific antigen. Currently, diagnosis of NHL malignancies relies on a combination of morphologic findings and immunohistochemical stains usually from lymph node biopsy specimens, which is labor intensive and time consuming, taking hours to days to complete. Consequently, in view of the results of the present study, the LIA method can be envisioned as a rapid and reliable assay for detection of the CD20 cancer biomarkers. It was verified that the LIA method was successful in detecting the antibody–antigen nanocomplexes, based on the high-affinity reaction between CD20 antigen and the anti-CD20 antibody, using a rapid, single-step homogeneous immunoassay in aqueous colloidal media.

3.4. Immunoassays of CdS Quantum Dot Conjugates Based on Fluorescence Microscopy. CD20 is a specific membrane receptor known to be overexpressed in lymphoma B cells.[30,31] Thus, TOLEDO cells (a lymphoma B-cell line) were selected for the cellular uptake assay. Figure 6A shows the bright-field phase image of lymphoma cells with typical morphology.[32] Figure 6B and 6C indicates the green and orange-red fluorescence of B-cell cancer biomarker CD20 (CdS/chitosan–pAbCD20) in the tested cell line. The fluorescence microscopy images indicate that the majority of tumor cells were specifically labeled with quantum dot conjugates with anti-CD20 antibodies. The maximum fluorescence intensity is observed at cell membrane as expected once CD20 is a transmembrane protein with C-terminal domain, the binding site of pAbCD20, occurring on the cytoplasmic side of cell.[31] The control samples, treated with CdS/chitosan, showed only minor and unspecific fluorescence (Figure 6D and 6E). Quantitative results of fluorescence intensity measured at the cell membranes (Figure 6F and 6G) indicated that the CdS/chitosan–pAbCD20 immunoconjugates specifically targeted to CD20 proteins. It was observed that the fluorescence intensities associated with the immunoconjugate nanoparticles (CdS/chitosan–pAbCD20) were 4.8- (green) and 6.1-fold (orange-red) higher as compared to the samples with no conjugation of antibodies (CdS/chitosan without pAbCD20). To evidence that another cell line is not labeled nonspecifically with antibodies anti-CD20, an assay containing CD20 negative cells (nontumor cell line, VERO, and a tumor cell line, HeLa) was prepared and tested at the same conditions and no fluorescence was detected (Supporting Information, Figures S4 and S5). This confirms the immunoconjugate specificity and selectivity. The possibility of emission/detection of two colors of the visible spectrum (green and orange-red) allows a larger number of fluorescent biomarkers to be distinguished simultaneously.

In the present study, it was clearly demonstrated that novel nanohybrids based on CdS/immunoconjugates behaved simultaneously as fluorescent probes for imaging and cancer biomarker detection. The entire envisioned application of the nanohybrid system developed in this study is schematically depicted in Figure 7. The CdS/immunoconjugates will simultaneously serve as probes for detection, targeting, and treatment of B-cell NHL cancers based on the binding affinity between the antigen CD20 and the anti-CD20–antibody. Certainly, several studies should be performed by scientists, oncologists, and other specialists to exploit the large number of in vitro and in vivo biomedical applications afforded by the novel fluorescent biomarkers developed in this work.

4. CONCLUSIONS

In this study, the synthesis of CdS QDs directly capped with chitosan–anti-CD20 antibody immunoconjugates was demonstrated for the first time using "single-step" aqueous colloidal chemistry, and these conjugates exhibit multifunctional properties as fluorophore probes whose in vitro biological affinity can be exploited for detection and imaging of antigen CD20 present in NHL cancer. This strategy may be potentially applied to avail of the numerous opportunities and address the challenges presented in the fields of oncology and nanotechnology.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures and relevant protocols as described. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +55 31-3409-1843 Fax: +55 31-3409-1815. E-mail: hmansur@demet.ufmg.br.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge financial support from CAPES, FAPEMIG, and CNPq. We express our gratitude to the staff from the Microscopy Center/UFMG for TEM analysis.

■ REFERENCES

(1) Mansur, H. S.; Mansur, A. A. P. In *Encyclopedia of Biomedical Polymers and Polymeric Biomaterials*; Mishra, M., Ed.; CRC Press: Boca Raton, FL, 2015; 7 vol. set.

(2) Fang, M.; Peng, C. W.; Pang, D. W.; Li, Y. Quantum Dots for Cancer Research: Current Status, Remaining issues, and Future Perspectives. *Cancer Biol. Med.* **2012**, *9*, 151−163.

(3) Mansur, H. S. Quantum Dots and Nanocomposites. WIREs Nanomed. *Nanobiotechnol.* **2010**, *2*, 113−129.

(4) Ferrari, M. Cancer Nanotechnology: Opportunities and Challenges. *Nat. Rev. Cancer* **2005**, *5*, 161−171.

(5) Mansur, H. S.; Mansur, A. A. P.; Curti, E.; de Almeida, M. V. Functionalized-Chitosan/Quantum Dots Nano-Hybrids for Nanomedicine Applications: Towards Biolabeling and Biosorbing Phosphate Metabolites. *J. Mater. Chem. B* **2013**, *1*, 1696−1711.

(6) Ma, X.; Zhao, Y.; Liang, X.-J. Theranostic Nanoparticles Engineered for Clinic and Pharmaceutics. *Acc. Chem. Res.* **2011**, *44*, 1114−1122.

(7) Mansur, A. A. P.; Saliba, J. B.; Mansur, H. S. Surface Modified Fluorescent Quantum Dots with Neurotransmitter Ligands for Potential Targeting of Cell Signaling Applications. *Colloids Surf., B* **2013**, *111*, 60−70.

(8) Mansur, H. S.; González, J. C.; Mansur, A. A. P. Biomolecule-Quantum Dot Systems for Bioconjugation Applications. *Colloids Surf., B* **2011**, *84*, 360−368.

(9) Ramanery, F. P.; Mansur, A. A. P.; Mansur, H. S. One-Step Colloidal Synthesis of Biocompatible Water-Soluble ZnS Quantum Dot/Chitosan Nanoconjugates. *Nanoscale Res. Lett.* **2013**, *8*, 512.

(10) Tan, W. B.; Huang, N.; Zhang, Y. Ultrafine Biocompatible Chitosan Nanoparticles Encapsulating Multi-Coloured Quantum Dots for Bioapplications. *J. Colloid Interface Sci.* **2007**, *310*, 464−470.

(11) Mansur, H. S.; Mansur, A. A. P.; Curti, E.; de Almeida, M. V. Bioconjugation of Quantum-Dots with Chitosan and N,N,N-Trimethyl Chitosan. *Carbohydr. Polym.* **2012**, *90*, 189−196.

(12) Alves, N. M.; Mano, J. F. Chitosan Derivatives Obtained by Chemical Modifications for Biomedical and Environmental Applications. *Int. J. Biol. Macromol.* **2008**, *43*, 401−414.

(13) Scott, A. M.; Wolchok, J. D.; Old, L. J. Antibody Therapy of Cancer. *Nat. Rev. Cancer* **2012**, *12*, 278−287.

(14) Arruebo, M.; Valladares, M.; Gonzalez-Fernandez, A. Antibody-Conjugated Nanoparticles for Biomedical Applications. *J. Nanomater.* **2009**, *2009*, No. 439389.

(15) Koshkaryev, A.; Sawant, R.; Deshpande, M.; Torchilin, V. Immunoconjugates and Long Circulating Systems: Origins, Current State of the Art and Future Directions. *Adv. Drug Delivery Rev.* **2013**, *65*, 24−35.

(16) Antony, T.; Saxena, A.; Roy, K. B.; Bohidar, H. B. Laser Light Scattering Immunoassay: An Improved Data Analysis by CONTIN Method. *J. Biochem. Biophys. Methods* **1998**, *36*, 75-.

(17) Tang, D.; Cui, Y.; Chen, G. Nanoparticle-Based Immunoassays in the Biomedical Field. *Analyst* **2013**, *138*, 981−990.

(18) Liu, X.; Dai, Q.; Austin, L.; Coutts, J.; Knowles, G.; Zou, J.; Chen, H.; Huo, Q. A One-Step Homogeneous Immunoassay for Cancer Biomarker Detection Using Gold Nanoparticle Probes Coupled with Dynamic Light Scattering. *J. Am. Chem. Soc.* **2008**, *130*, 2780−2782.

(19) Yang, D.; Ma, J.; Zhang, Q.; Li, N.; Yang, J.; Raju, P. A.; Peng, M.; Luo, Y.; Hui, W.; Chen, C.; Cui, Y. Polyelectrolyte-Coated Gold Magnetic Nanoparticles for Immunoassay Development: Toward Point of Care Diagnostics for Syphilis Screening. *Anal. Chem.* **2013**, *85*, 6688−6695.

(20) Dixit, C. K.; Vashist, S. K.; MacCraith, B. D.; O'Kennedy, R. Multisubstrate-Compatible ELISA Procedures for Rapid and High-Sensitivity Immunoassays. *Nat. Protoc.* **2011**, *6*, 439−445.

(21) Mansur, H. S.; Mansur, A. A. P.; González, J. C. Synthesis and Characterization of CdS Quantum Dots with Carboxylic-Functionalized Poly (Vinyl Alcohol) for Bioconjugation. *Polymer* **2011**, *52*, 1045−1054.

(22) Hermanson, G. T. *Bioconjugate Techniques*, 2nd ed; Elsevier Inc.: Amsterdam, 2008.

(23) Tauc, J.; Menth, A. States in the Gap. *J. Non-Cryst. Solids* **1972**, *8*−*10*, 569−585.

(24) Cooper, J. K.; Franco, A. M.; Gul, S.; Corrado, C.; Zhang, J. Z. Characterization of Primary Amine Capped CdSe, ZnSe, and ZnS Quantum Dots by FT-IR: Determination of Surface Bonding Interaction and Identification of Selective Desorption. *Langmuir* **2011**, *27*, 8486−8493.

(25) Skobeeva, V. M.; Smyntyna, V. A.; Sviridova, O. I.; Struts, D. A.; Tyurin, A. V. Optical Properties of Cadmium Sulfide Nanocrystals Obtained by the Sol-Gel Method in Gelatin. *J. Appl. Spectrosc.* **2008**, *75*, 576−582.

(26) Smyntyna, V.; Skobeeva, V.; Malushin, N. The Nature of Emission Centers in CdS Nanocrystals. *Radiat. Meas.* **2007**, *42*, 693−696.

(27) Ramsden, J. J. Gratzel, Photoluminescence of Small Cadmium Sulphide Particles. M. *J. Chem. Soc., Faraday Trans.* **1984**, *80*, 919−933.

(28) Jiang, J.; Oberdörster, G.; Biswas, P. Characterization of Size, Surface Charge, and Agglomeration State of Nanoparticle Dispersions for Toxicological Studies. *J. Nanopart. Res.* **2009**, *11*, 77−89.

(29) Jazirehi, A. R.; Bonavida, B. Cellular and Molecular Signal Transduction Pathways Modulated by Rituximab (Rituxan, Anti-CD20 mAb) in Non-Hodgkin's Lymphoma: Implications in Chemosensitization and Therapeutic Intervention. *Oncogene* **2005**, *24*, 2121−2143.

(30) Ginaldi, L.; De Martinis, M.; Matutes, E.; Farahat, N.; Morilla, R.; Catovsky, D. Levels of Expression of CD19 and Cd20 in Chronic B Cell Leukaemias. *J. Clin. Pathol.* **1998**, *51*, 364−369.

(31) Maloney, D. G. Anti-CD20 Antibody Therapy for B-Cell Lymphomas. *N. Engl. J. Med.* **2012**, *366*, 2008−2016.

(32) Miranda, R. N.; Briggs, R. C.; Kinney, M. C.; Veno, P. A.; Hammer, R. D.; Cousar, J. B. Immunohistochemical Detection of Cyclin D1 Using Optimized Conditions Is Highly Specific for Mantle Cell Lymphoma and Hairy Cell Leukemia. *Mod. Pathol.* **2000**, *13*, 1308−1314.