Journal of Photochemistry and Photobiology B: Biology 142 (2015) 237–243

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

CdTe quantum dots conjugated to concanavalin A as potential fluorescent molecular probes for saccharides detection in *Candida albicans*

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ARTICLE INFO

Article history:
Received 24 July 2014
Received in revised form 7 November 2014
Accepted 10 November 2014
Available online 11 December 2014

ABSTRACT

Semiconductor colloidal quantum dots (QDs) have been applied in biological analysis due to their unique optical properties and their versatility to be conjugated to biomolecules, such as lectins and antibodies, acquiring specificity to label a variety of targets. Concanavalin A (Con A) lectin binds specifically to α-D-mannose and α-D-glucose regions of saccharides that are usually expressed on membranes of mammalian cells and on cell walls of microbials. *Candida albicans* is the most common fungal opportunistic pathogen present in humans. Therefore, in this work, this fungus was chosen as a model for understanding cells and biofilm-forming organisms. Here, we report an efficient bioconjugation process to bind CdTe (Cadmium Telluride) QDs to Con A, and applied the bioconjugates to label saccharide structures on the cellular surface of *C. albicans* suspensions and biofilms. By accomplishing hemagglutination experiments and circular dichroism, we observed that the Con A structure and biochemical properties were preserved after the bioconjugation. Fluorescence microscopy images of yeasts and hyphae cells, as well as biofilms, incubated with QDs-(Con A) showed a bright orange fluorescence profile, indicating that the cell walls were specifically labeled. Furthermore, flow cytometry measurements confirmed that over 93% of the yeast cells were successfully labeled by QD-(Con A) complex. In contrast, non-conjugated QDs or QDs-(inhibited Con A) do not label any kind of biological system tested, indicating that the bioconjugation was specific and efficient. The staining pattern of the cells and biofilms demonstrate that QDs were effectively bioconjugated to Con A with specific labeling of saccharide-rich structures on *C. albicans*. Consequently, this work opens new possibilities to monitor glucose and mannose molecules through fluorescence techniques, which can help to optimize phototherapy protocols for this kind of fungus.

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1. Introduction

Nanomedicine is developed to open new possibilities on research, prevention, and treatment of diseases through the innovative diagnostic imaging techniques and therapies [1]. Since the 1980s, quantum dots (QDs) have attracted considerable attention as a possible alternative approach for nanotechnology [2]. QDs are semiconductor nanocrystals that present unique properties such as: size-tunable emission, narrow symmetric emission bands, broad light absorbance, bright fluorescence, high quantum yield, high photostability. Their broad absorption band allows the analysis of multiple QDs with a single excitation light source reducing several experimental limitations and costs. Such features have raised attention from biomedical researchers [3]. Also, QDs are currently being applied in bioassays that may lead to diagnostic assays in the near future [4]. Therapeutic applications, such as photodynamic therapy, gene silencing, and drug delivery, as well as simultaneous diagnosis and treatment (theranostics) are also promising [2,5,6].

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http://dx.doi.org/10.1016/j.jphotobiol.2014.11.010
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QDs can be conjugated to biomolecules, such as lectins and antibodies, acquiring specificity to label a variety of targets. In fact, QDs represent a major advance in medical imaging and diagnosis as they have been used to label cancer and microbial cells to improve real-time fluorescence tracking and detection of molecular patterns in living cells and animals [7,8]. In this context, concanavalin A (Con A) is a lectin extracted from *Canavalia ensiformis* that has been broadly employed into molecular labeling since it binds specifically to α-D-mannose and α-D-glucose residues [9,10]. It can be applied for structural characterization of glycoconjugates and identification of carbohydrate rich structures in biological materials (e.g. body fluids, cells and tissues) [10].

C. albicans is a well-studied fungal model either in planktonic suspensions or biofilms with high relevance to biomedical research. In fact, *C. albicans* is the most common fungal opportunistic pathogen present in humans [11]. This fungus shows different cellular morphologies such as yeast and hyphae with a complex macromolecular cell wall structure presenting high concentrations of carbohydrates, making this species an easy target for Con A [12]. Besides, *C. albicans* is known to present a high antifungal resistance when organized in biofilms, where yeast and hyphae cells are encased in an extracellular matrix, protecting them against chemotherapeutic treatment [13]. The dimensions of *C. albicans* cells also allow real-time observations of the labeled structures under a confocal laser scanning microscope.

In this study we report an efficient methodology to obtain QD-(Con A) bioconjugates and applied them to label *C. albicans* suspensions and biofilms cultured *in vitro*. We show that cell wall and extracellular matrix of *C. albicans* can be labeled with CdTe-MSA (mercaptosuccinic acid)-(Con A) to achieve single-cell resolution.

2. Experimental procedures

2.1. Materials for CdTe-MSA QDs synthesis

Mercaptosuccinic acid (MSA), cadmium perchlorate ($Cd(ClO_4)_2$), sodium borohydride ($NaBH_4$), tellurium (Te) and concanavalin A (Con A) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Isopropyl alcohol and other routine chemicals were purchased from Vetec (Duque de Caxias, RJ, Brazil). All the chemicals used were at analytical grade and ultrapure water (18.2 MΩ) was used in all experiments.

2.2. General procedure for CdTe-MSA QDs synthesis and characterization

CdTe-MSA QDs were synthesized in aqueous colloidal dispersion according to the previously reported method with some modifications [14]. Briefly, QDs were prepared by the addition of Te^{2-} in a $Cd(ClO_4)_2$ solution of pH > 10 in the presence of MSA (mercaptosuccinic acid) as stabilizing agent in a 2:1:4.6 ratio of Cd:Te:MSA respectively. The Te^{2-} aqueous solution was prepared by reducing metallic tellurium with $NaBH_4$ at a high pH and under nitrogen saturated inert atmosphere. The reaction proceeded under constant stirring and heating at 90 °C during 5 h. At this moment a mercaptosuccinic acid solution (1.5 mmol) was added and the heating, at 90 °C, was performed for further 7 h.

After synthesized, QDs were structurally characterized by X-ray diffraction (XRD) and transmission electron microscopy (TEM). The powder XRD analysis was carried out with Siemens Nixdorf D5000 diffractometer with Cu Kα (λ = 0.15418 nm) radiation. The XRD pattern was obtained from CdTe-MSA QDs precipitated in an aqueous solution containing isopropyl alcohol. After XRD analysis we can estimate the average size of QDs by using Scherrer's equation.

$$0.9\lambda = d\ B\cos\theta$$

where λ is the X-ray wavelength (λ = 0.1542 nm), B is the width at half the maximum intensity (FWHM) of the more intense diffraction peak, θ is the Bragg angle and d will be the average diameter of the nanoparticles.

On the other hand, the average diameter (d) of the QDs can also be estimated using the wavelength at the first absorption peak (λ) and the empirical equation of Dagtepe et al. [15]:

$$d = \frac{1.3845 - 0.00066\lambda}{1 - 0.00121\lambda}$$

Furthermore, by using the average size obtained above and the Lambert–Beer's law, and after calculating the extinction coefficient (ε) of the CdTe QDs by using Yu et al. [16], we can estimate QDs concentration in the suspension.

$$\varepsilon = 10043(d)^{2.12} \quad \text{and } A = \varepsilon CL$$

where A is the absorption at the first peak which should be less than 0.1, C will be the estimated concentration and L is the size of the light path in cuvette, which is normally 1 cm. TEM images were obtained on a Tecnai G2 20 FEI 2006 microscope operating at 200 kV. The samples were prepared dropping diluted solutions of CdTe-MSA nanocrystals onto 200-mesh holey carbon-coated copper grids with the excessive solvent immediately evaporated. Optical characterization was carried out by absorption spectra on a spectrophotometer Evolution 600 UV–Vis, Thermo Scientific, and emission spectra were recorded using a fluorescence spectrometer LS 55, Perkin Elmer (λ_{exc} = 365 nm).

2.3. CdTe-MSA QDs conjugation to Con A

The CdTe-MSA QDs were conjugated to Con A by adsorption applying a ratio of 1000:1 (QDs:Con A). The QDs solution pH was adjusted at 8.0 and incubated with Con A for 2 h, at room temperature (RT), with low stirring. For 3 mL solution containing QDs concentration at 5×10^{-6} mol L^{-1}, Con A was added at 280 μg mL^{-1}. The QDs bioconjugated were optically characterized by absorption and emission spectra.

2.4. Con A structural study and determination of protein activity

To evaluate Con A secondary structure, circular dichroism (CD) spectra (free Con A and bioconjugated Con A) were recorded on Jasco (J-815) spectropolarimeter over a wavelength range of 185–250 nm, under constant N_2 purging. The CD profiles were obtained at 25 °C in 1 cm path length cuvettes by employing a scan speed of 50 nm min^{-1} and response time of 8 s and averaged over 10 scans to eliminate signal noise. The structural study was carried with free lectin and its conjugates at 2×10^{-9} mol L^{-1} protein concentration diluted in phosphate buffer 10 mM (pH 7.2).

Furthermore, the protein activity was evaluated by hemagglutination assay. For hemagglutination assay, the lectin, the QDs and the conjugates were serially diluted in 96 well microplates and incubated with 2% (v/v) trypsinated rabbit erythrocyte cell suspension at 150 mM NaCl for 45 min, at RT. The hemagglutination activity was expressed in hemagglutination units (HU).

2.5. Cell culture and biofilm assay

C. albicans (ATCC 10231) cells were cultivated in Sabouraud dextrose broth for 24 h at 37 °C and orbital shaking at 75 rpm. Fungal cells were centrifuged at 3500 rpm for 3 min, and the resulting pellet was resuspended in phosphate buffered saline (PBS). Cell concentration was adjusted by turbidity measured in a spectrophotometer. We used the optical density of 0.16 at 540 nm in an optical path length of 1 cm to obtain suspensions with 1×10^6 to

2×10^6 CFU mL^{-1}. The inocula concentrations were confirmed by colony forming units (CFU) count in Sabouraud agar culture.

Biofilms were grown on hydrogel (Acuvue 2®) composed of 42% poly(methyl methacrylate) and 58% water slabs that were pretreated overnight in fetal bovine serum (Sigma). After washing with 300 μL of PBS, slabs were placed in 96-wells microplates. Two hundred μL of yeast cells suspension in PBS were added into each well. The microplate was then incubated for 90 min at 37 °C to allow the cells to decant over the slabs and encourage adhesion. Supernatant solution was carefully replaced by 200 μL of fresh yeast nitrogen base dextrose broth (Difco™) and biofilms were then grown during 48 h at 37 °C.

2.6. Labeling C. albicans with QDs-(Con A)

The yeast cell suspension and biofilm labeling were evaluated by confocal laser scanning microscopy (Olympus IX81 and Leica TCS-SP8, respectively) to confirm the QDs-(Con A) labeling specificity for cell wall and extracellular matrix. QDs-(Con A), bare QDs and QDs-(inhibited Con A) systems were tested. The cell suspensions (10^6 CFU/mL) were incubated with 200 μL QDs (at 5×10^{-6} mol L^{-1}, obtained according to Section 2.2) at volume proportion of 1:1 for 1 h at RT. To remove non-binding QDs, all suspensions were centrifuged at 3500 rpm for 3 min, washed and resuspended in PBS. For biofilm label, broth was removed and the biofilm remaining was washed with 200 μL of PBS for non-adhered cells exclusion. A volume of 200 μL of each system was added to biofilm wells and incubated for 1½ h at RT. Biofilm samples were removed from wells and washed with 200 μL of PBS to remove non-binding QDs. To compose three and two-dimensional biofilm images, Imaris® 7.4.2 (Bitplane) and ImageJ 2.0 (NIH) were used to compile a sequence of 85 image stacks.

Furthermore, to confirm the specificity of the labeling results, as well as the efficiency of the bioconjugation, lectin binding inhibition assays were performed by incubating QDs-(Con A) with methyl-α-D-mannopyranoside (0.3 mol L^{-1}, Sigma) for 30 min. Then, the incubation with cell suspensions and biofilms remained during 1 h.

The percentage of labeled yeast cells in suspension was also observed by flow cytometry (FACSCalibur™, Becton Dickinson). Around 20,000 events were acquired using fluorescence excitation at 488 nm and measuring emission with band pass filter 585/20 nm. The collected data were processed by Cell Pro Software (Cell Quest™, Becton–Dickinson).

3. Results and discussion

3.1. Characterization of CdTe-MSA QDs

Structural and optical characterizations were used to analyze the size and crystallinity of QDs. The XRD pattern, presented in Fig. 1, showed three diffraction peaks observed at 2θ values 24.2°, 40.02° and 46.42°, respectively, corresponding to the (1 1 1), (2 2 0) and (3 1 1) CdTe crystalline planes. This result is consistent with bulk cubic (zinc blende) CdTe structure (JCPDS No. 75-2086) [17]. Following literature methods [18], the particle diameter was estimated at $d = 3.0$ nm according to Scherrer equation, using the peak at 24.2°. The TEM image of CdTe-MSA QDs (Fig. 2) shows that QDs size is around 3 and 4 nm. The optical analyzes revealed an absorption peak around $\lambda = 547$ nm and emission band maximum at $\lambda = 600$ nm, as shown in Fig. 3. According to Section 2.2, the dimension of the CdTe-MSA QDs (considering the wavelength at the maximum of the first absorption band) was estimated to be around $d = 3$ nm [15,19], which is in agreement with the size estimated from the crystallography data and TEM analysis.

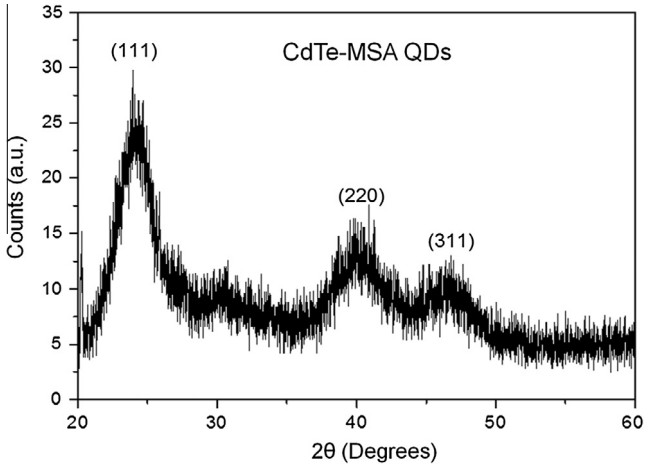

Fig. 1. Powder XRD pattern for as prepared CdTe-MSA QDs.

3.2. Characterization of CdTe-MSA QDs-(Con A) conjugates

The optical characterization of the CdTe-MSA QDs-(Con A) bioconjugates is shown in Fig. 3. The QDs emission spectra before and after conjugation did not show alterations in photoluminescence intensity (as shown in Fig. 3). Thus, the quantum yield (QY) can be considered the same for bare QDs and for conjugates. The QY for bare QDs was estimated as ~52% by using thermal lens technique [20].

The Con A protein activity was analyzed before and after CdTe-MSA QDs conjugation by hemagglutinating assay. Bare QDs did not show hemagglutinating activity. However, the CdTe-MSA QDs-(Con A) conjugate displayed an activity equivalent to Con A controls as exhibited in the Table 1, suggesting a biological activity preservation of the lectin in the conjugate. The Con A CD spectra exhibited a negative band in the UV wavelength region at 220–230 nm and a positive one at 190–200 nm. These bands are characteristic of a β-strands structure of the protein and represents $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions of peptide bonds [21,22]. The CD spectrum in the far UV region indicates no disruption of the secondary structure, with [θ] 222.5 being −4.6 mdeg as compared to −4.87 mdeg for the native protein (Fig. 4).

3.3. Labeling C. albicans with QDs-(Con A)

The labeling of *C. albicans* by QDs-(Con A), bare QDs and QDs-(inhibited Con A) was evaluated separately for cell suspensions and also for biofilms. In fungal suspension, an intense fluorescence was observed on cell surface in confocal imaging (Fig. 5A) after incubation with QDs-(Con A). The analysis also confirmed that bare QDs and QDs-(inhibited Con A) did not label *C. albicans* in suspensions and in biofilms (Fig. 5B and C). This event shows that the inhibitor (methyl-α-D-mannopyranoside) occupies Con A binding sites, disallowing subsequent binding of QDs-(Con A) to the fungal cell. This observation also shows a specific QDs-(Con A) labeling of *C. albicans* cell wall. Carbohydrates, mainly consisting of β-glucans (glucose polysaccharide), chitin (N-acetylglucosamine polysaccharide) and mannoproteins (glycoprotein composed of amino acids, N-acetylglucosamine and mannose), represent approximately 80–90% of fungal cell wall composition [23]. This high content of mannose and glucose represents an expressive number of suitable sites available for QDs-(Con A) binding. Fluorescence images also show that QDs-(Con A) were successfully bound to most of the cells.

This information was further verified through quantification of the number of cells that were labeled by QDs-(Con A) by flow cytometry. In fact, cytometric analysis revealed that more than

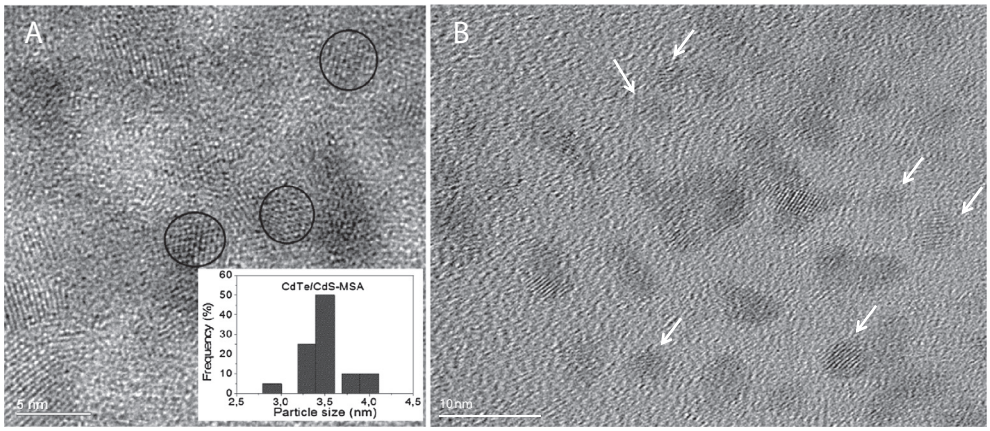

Fig. 2. Representative TEM image of CdTe-MSA QDs. Scale bar for A: 5 nm and for B: 10 nm. The size distribution of the QDs is shown in the insert in A. The encircled regions in A correspond to some individual QDs from which the statistical distribution was done. This was performed by measuring the size of around 40 nanoparticles. The arrows in B point to some individual QDs.

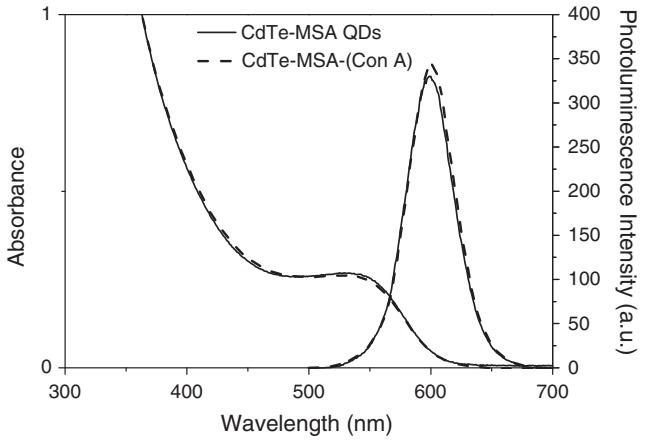

Fig. 3. Absorption and photoluminescence spectra of CdTe-MSA QDs (line) and CdTe-MSA-(Con A) QDs conjugate (dashed line).

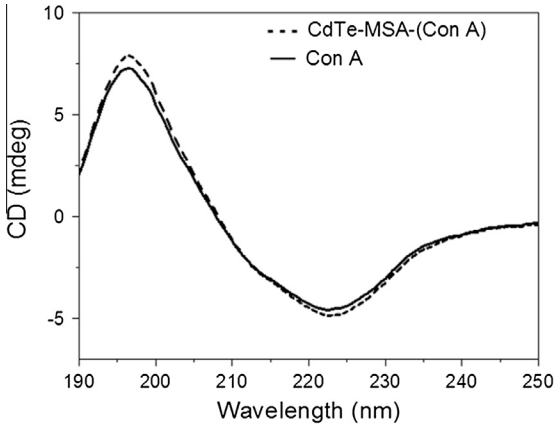

Fig. 4. CD spectra of native Con A (dashed line) and of Con A conjugated to MSA-capped CdTe QDs (line).

Table 1
Hemagglutination activity of the Con A and its conjugate to CdTe-MSA QDs in 2% (v/v) rabbit erythrocyte cell suspension. HU express hemagglutination units.

Systems	Hemagglutination activity
Con A	128
CdTe-MSA QDs	No activity
CdTe-MSA QDs-(Con A)	128

93% of yeast cells were labeled by QDs-(Con A), as shown in Fig. 6. The flow cytometry histogram presents two peaks due to the heterogeneity of morphology in the *C. albicans* population. It can also be observed in the microscopy images of Fig. 5. The heterogeneity of the yeast cell populations is due to the different cell morphologies within the sample (e.g. presence of buddings, germ tube formation, hyphae, etc.). This analysis also confirmed that bare QDs did not label *C. albicans* cells. Therefore, this process confirms that QDs were successfully conjugated to Con A which binds specifically to carbohydrates present in *C. albicans*.

The biofilm was also evaluated in order to examine the presence and distribution of QDs-(Con A) in its arrangement. Particularly, *C. albicans* biofilms are comprised of a complex three-dimensional structure consisting of heterogeneous cell morphologies and several extracellular elements [24]. After *C. albicans* biofilms were incubated with QDs-(Con A), both yeast cells and hyphae displayed a distinct fluorescence emission (Fig. 7), indicating a successfully labeling of different *C. albicans* morphologies embedded in biofilms. We also observed a less intense fluorescence of an amorphous structure around dense cell populations, suggesting that QDs-(Con A) also bound to carbohydrates present in the extracellular matrix. Although *C. albicans* biofilm presents a complex composition and architecture, the QDs-(Con A) conjugate were able to penetrate and diffuse through the whole samples thickness, labeling deeply localized *C. albicans* cells and extracellular matrix. The biofilms incubated with QDs-(inhibited Con A) or bare QDs did not exhibit any detectable fluorescence.

Fluorescent labeling of cells based on binding to carbohydrate is a routine tool used with different purposes. Past studies successfully labeled *C. albicans* cell walls specifically employing AlexaFluor 488-Con A for monitoring phagocytes assays [25] and following biofilm growth [26]. Other studies employed FITC-(Con A) to confirm the presence of binding sites between enteric bacteria and probiotic yeast [27] and to investigate the occurrence of specific receptors in the cell wall in two different lichen phycobionts [28]. Additionally, the QDs-lectin conjugate has been employed as fluorescent probes for diagnostic identification of leukemia cells [29], sensor for the detection of glucose [30] and for tumor recognition [31,8]. Also, the specific recognition of lectin to carbohydrates using QDs-(Con A) conjugate allows a dynamic monitoring of the alteration of cell surface carbohydrate expression in response to drugs [32]. Thioglycolic acid capped-CdTe QDs were

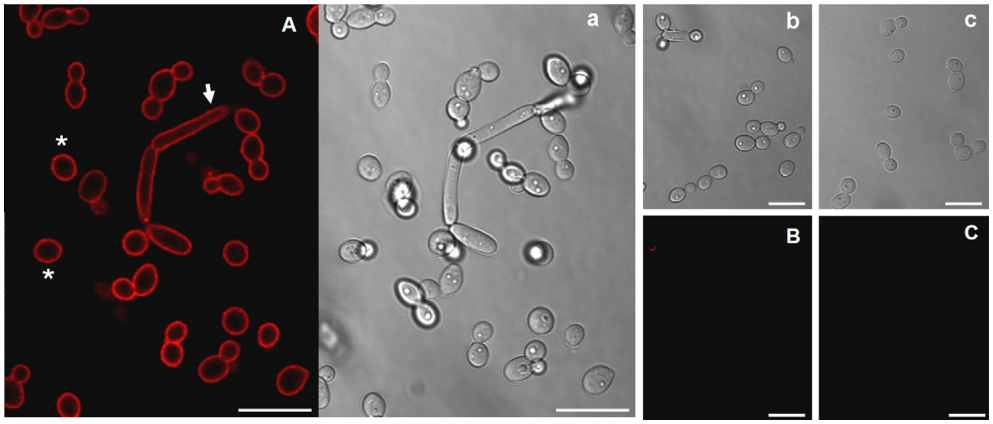

Fig. 5. Fluorescent confocal micrographies of *Candida albicans* cell suspensions labeled with QDs-(Con A) (A, a), bare QDs (B, b), and QDs-(inhibited Con A) (C, c) showing comparable labeling as revealed by phase-contrast (lower cases) and fluorescence (capital letters) confocal microscopy. Both populations of *C. albicans* can be observed in A: hyphae (arrow head) and yeast cells (*). Scale Bar: 10 μm.

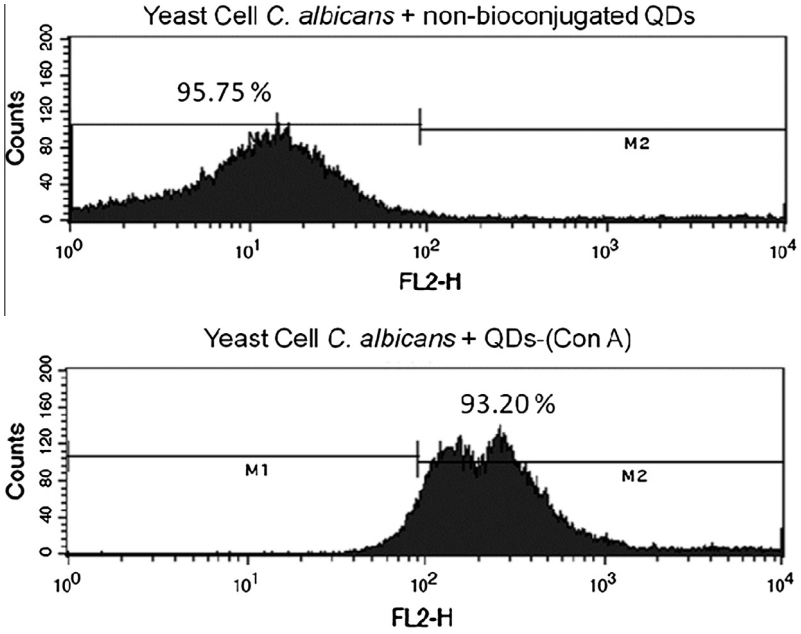

Fig. 6. Histogram statistic of flow cytometry analysis of the cells labeled with bare (non-conjugated) CdTe-MSA QDs and with CdTe-MSA-(Con A).

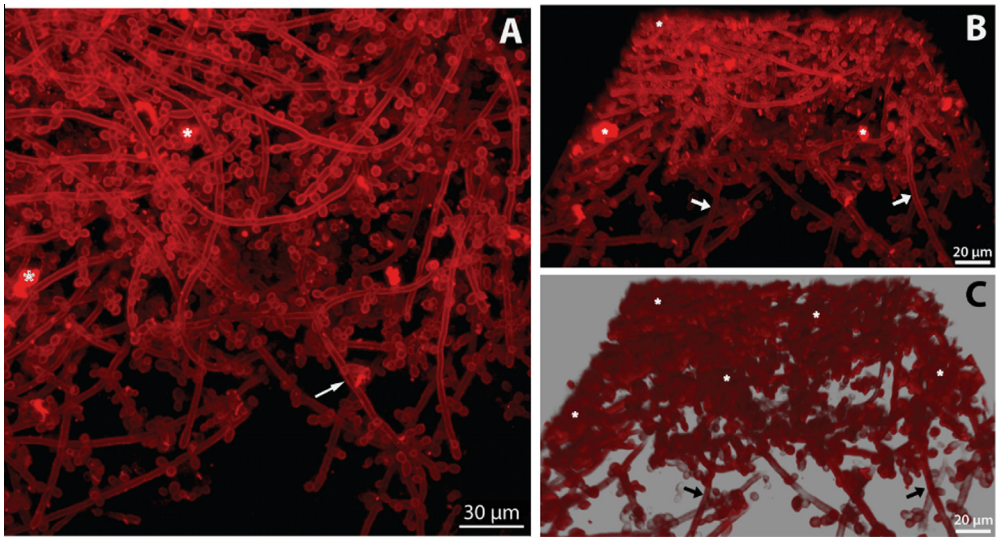

Fig. 7. Confocal laser scanning microscopy images of *C. albicans* biofilm labeled by QDs-(Con A) conjugates. (A) Simultaneous and equivalent efficiency of labeling of hyphae (arrows) surrounded by extracellular matrix (*); (B) cellular surface enhanced 3D reconstruction of the biofilm and (C) a 3D reconstruction of the same field evidencing the smear layer of saccharide rich extracellular matrix (*). Scale bar: 30 μm in A and 20 μm in B and C.

conjugated to sugars (galactose, glucose or mannose) in order to study the interaction of these complexes with yeast cells [33]. It was demonstrated that the conjugates formed by QDs and sugars were recognized by the lectins present on the cells. That was an opposite strategy than the one used in our work.

There are only a few references in the literature that describe the use of QDs to label biofilms. For instance, CdSe/ZnS QDs-capped by hydrophilic 3-mercaptopropionic acid or by hydrophobic aminoacids (leucine or phenylalanine) were used to label the biofilm produced by *Shewanella oneidensis* bacterium [34]. In that work it was found that the final QDs' distribution within the biofilm depends on their surface amphiphilicity and can be controlled by changing the surface ligands. QDs were also applied to label oral biofilms [35]. Conjugated to antibodies, the QDs applied in the mentioned work, showed excellent single-cell resolution in both, *in vitro* and *in vivo* biofilms.

Herein, cell wall and biofilm of *C. albicans* were successfully labeled with MSA-capped CdTe QDs-(Con A) conjugate. Carbohydrates constituents of *C. albicans* cells and biofilm are important for protection, adhesion, cell–cell interconnections and immune responses [36,37]. Given the characteristics and functions of these carbohydrates, QDs-(Con A) conjugate can be a promising tool, for monitoring alterations of cell wall and biofilm formation in response to drugs, fungal physiology and interactions with immune cells. Furthermore, in therapeutic applications, QDs can improve the killing efficacy of photodynamic therapy [38]. Therefore, cell labeling will help new prospects to be explored in biomedical applications as potential light-activated theranostics agents combining fluorescence imaging and optical therapy.

4. Conclusion

We have developed an effective procedure to bioconjugate concanavalin A to CdTe-MSA QDs and have observed a specific labeling of saccharide-rich structures on hyphae and yeast *C. albicans* cells disposed either in suspension or biofilm. Additionally the polysaccharide structure of extracellular matrix formed in biofilms was successfully labeled. Consequently, this work opens new possibilities to monitor glucose- and mannose-rich molecules through fluorescence techniques. The conjugates also represent a potential tool for theranostics techniques by associating infection site detection to improve phototherapy efficiency. Therefore, the conjugates formed by concanavalin A and QDs could be considered as practical probes that can be used for investigations of microbial infections, molecular imaging and saccharide profiling.

Acknowledgements

We are grateful to the following agencies: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) for financial support and student fellowships. We are also grateful to the National Institute of Science in Photonics (INFo) for financial support. Additionally, we would like to thank the Centro de Tecnologias Estratégicas do Nordeste (CETENE) for allowing the use of analytical facilities, Maria Aparecida Barreto Lopes Seabra for TEM images and Luis Claudio Suzuki for confocal images.

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