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Molecular Differentiation of *Leishmania* Protozoarium using CdS Quantum Dots as Biolabels

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ABSTRACT

In this work we applied core-shell CdS/$Cd(OH)_2$ quantum dots (QDs) as fluorescent labels in the *Leishmania amazonensis* protozoarium. The nanocrystals (8-9 nm) are obtained via colloidal synthesis in aqueous medium, with final pH=7 using sodium polyphosphate as the stabilizing agent. The surface of the particles is passivated with a cadmium hydroxide shell and the particle surface is functionalized with glutaraldehyde. The functionalized and non-functionalized particles were conjugated to *Leishmania* organisms in the promastigote form. The marked live organisms were visualized using confocal microscopy. The systems exhibit a differentiation of the emission color for the functionalized and non-functionalized particles suggesting different chemical interactions with the promastigote moieties. Two photon emision spectra (λ_{exc}=795nm) were obtained for the promastigotes labeled with the functionalized QDs showing a significant spectral change compared to the original QDs suspension. These spectral changes are discussed in terms of the possible energy deactivation processes.

Keywords: quantum dots, *Leishmania amazonensis*, CdS/$Cd(OH)_2$, fluorescent biolabel.

INTRODUCTION

Fluorescent semiconductor nanoparticles or Quantum Dots (QDs) represent a new class of fluorescent biolabels, which possess many advantages over conventional fluorophores. Semiconductor nanoparticles in the quantum confinement regime may be prepared in order to present (i) high quantum yields, (ii) large excitation bands, (iii) narrow fluorescence emission bands, (iv) very long effective Stokes shifts, (v) high resistance to photobleaching, (vi) the capability of tuning their optical properties, particularly their emission spectra[1] by controlling the size of the particles and (vii) the possibility of excitation of several different emission colors using a single excitation wavelength. All these features combined with the highly active chemical surface of the particles, make QDs a class of fluorescent markers that can be chemically manipulated according to the desired target molecule in a biological system[1,2].

Since the first well succeeded experiments reported in the literature using CdSe/ZnS core-shell nanocrystals as fluorescent biolabels [3] there has been a great increase of work in this area. There are still some features to unravel regarding the preparation and the application of QDs in biological systems. The general synthetic methods presented in the literature describe the controlled growth of semiconductor particles up to the nanometer size range employing known colloidal methods. In order to avoid the exciton

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deactivation through radiationless processes, improving its photoluminescence capability, a second semiconductor layer (with a higher bandgap energy) is grown on the suface of the particles.

This process is refered to as passivation. This procedure also protects the particles from oxidaton, prevents leeching of the surface atoms into the surrounding solution. Although the presence of this passivation layer there are some reports of energy transfer processes occuring within the QDs and the biomolecules attached to its surface [4]. Mamedova et al [5] for example reported an increase of the emission efficiency in the CdTe/albumin bioconjugate system due to intermolecular energy transfer processes (antenna effect). This feature opens up the possibility of fluorescence resonance energy transfer studies (FRET). Recently some of us have employed highly luminescent CdS nanoparticles passivated with a cadmium hydroxide layer ($CdS/Cd(OH)_2$) in order to investigate red blood cell membrane antigen expression [6,7]. After the conjugation procedure the original emission maximum shifted to lower energies, resulting in dual luminescence. This spectral change, explained by energy transfer processes in the shiff-base linkage of the glutaraldehyde and the amine groups presented in the Anti-A protein, suggests that the $Cd(OH)_2$ coating layer although improving the particles photoluminescence, it still allows different pathways of energy leakage. So, in order to increase our understanding on the spectral changes of $CdS/Cd(OH)_2$ QDs conjugated to a biological system, we use in this work $CdS/Cd(OH)_2$ core-shell nanoparticles stabilized with polyphosphate anions $(PO_3^-)_n$ and functionalized with glutaraldehyde molecules ($O=CH-(CH_2)_3-HC=O$) to label *leishmania amazonensis* parasites in a flagellar form called promastigote. This parasite is responsible for a diffuse type of leishmaniosis causing injuries to mucous membranes of the human body and other mammals. The leishmaniosis is an endemic disease that affects millions of people around the world especially in tropical and sub-tropical countries [12].

EXPERIMENTAL METHODOLOGY

Preparation and passivation of the QDS

The $CdS/Cd(OH)_2$ nanocrystals (8-9 nm) are obtained via colloidal synthesis in aqueous medium, with sodium polyphosphate as the stabilizing agent, using the methodology described in a previous work[8]. Their surface is passivated with a cadmium hydroxide shell by increasing the pH suspension up to 10 and adding a solution of $Cd(ClO_4)_2$.

Functionalization of the QDs

To conjugate the particles to the parasites the pH of the suspension was decreased down to 7.6 with HCl 0,1M. In a second step, the particles are functionalized with a 0.016% glutaraldehyde solution at room temperature. The resulting pH of the functionalized colloidal suspension was 7.2. The functionalized particles will be from here on denominated $CdS/Cd(OH)_2$/Glut.

Conjugation of the QDS to the promastigotes

In a typical conjugation experiment, 300μL of a 10^{13}/mL nanocrystal suspension (functionalized and non-functionalized) was added to 100μL of the promastigote suspension in RPMI culture medium (Sigma) containing also 10% of phetal bovine inactivate serum, containing approximately 10^4 live parasites. The system was incubated at 26^o C for 18h and then washed with saline solution (0,9% NaCl) and centrifuged prior to the microscopic characterization.

Characterization of the QDS and the bioconjugates

Emission and excitation spectroscopies were performed by using an ISS K2 Multifrequency Phase Fluorimeter.

The marked live organisms were examined by the laser scanning confocal microscopy LSM 510 (Carl Zeiss, Jena, Germany) using an apochromatic water immersion, 63x with numerical aperture of 1.2, objective lens. Two wavelengths were used to promote excitation of the marked samples: 488 and 543 nm. The recorded image consists of 1024x1024 pixels. The images were acquired and processed using the software LSM 510

(Carl Zeiss Inc.). Laser intensities at the target spot are estimated to be 9 mW and 1 mW for the Argon (488 nm) and He/Ne (543 nm) respectively.
Two photon excited spectra of the CdS/$Cd(OH)_2$/Glut conjugated *leishmania* parasites were performed using an optical tweezers system. The micro-spectroscopy and Optical Tweezers system used both, cw Nd:YAG or cw Ti:Sapphire lasers for trapping. The laser beams were focused through a inverted microscope. A super notch filter was used not only as a filter for the backscattered light but also as a mirror to reflect the femtosecond and the cw Ti:Sapphire laser beams to the microscope. We used a short pass color filter to transmit the visible and cut the IR for the TPE luminescence. The spectra were acquired with an excitation wavelength of 785 nm and the final spectrum registered the sum of at least 1000 scans.

RESULTS AND DISCUSSION

Figure 1 shows a schematic representation of the CdS/$Cd(OH)_2$ and the CdS/$Cd(OH)_2$/Glut nanoparticles (d=8-9nm) used in this investigation. The polyphosphate anions show a polimerization degree of nine phosphate units (which results an average size of 2 nm) [9]. In the CdS/$Cd(OH)_2$/Glut nanoparticles we suggest that the bonding of the $Cd(OH)_2$ surface with the bi-dentate functionalizer induces hemiacetal formation [10].

The emission spectra of the CdS/$Cd(OH)_2$ and of the CdS/$Cd(OH)_2$/Glut nanocrystals are shown in Figure 2(a). The emission maximum of the original and the functionalized suspension used in the conjugation experiments is shown to be at 489 nm. The decrease in luminescence of the CdS/$Cd(OH)_2$/Glut suspension is due to the dilution performed during the functionalization procedure.

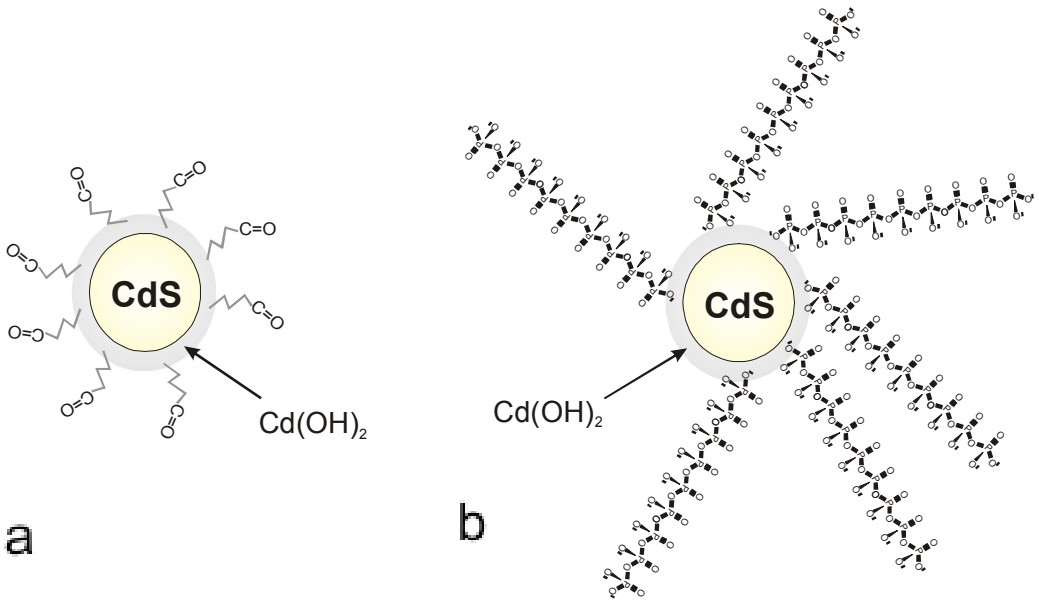

Figure 1. Schematic drawing of (a) CdS/$Cd(OH)_2$/Glut and (b) CdS/$Cd(OH)_2$ core-shell nanoparticles used in the conjugation of promastigote parasites.

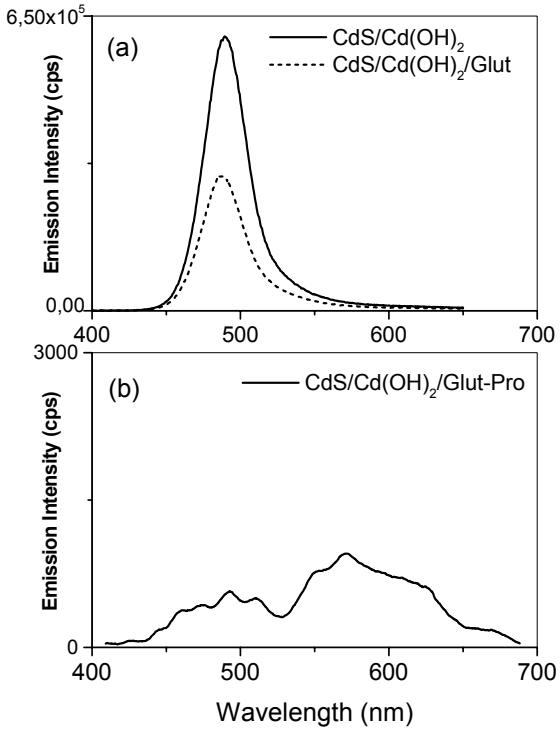

Figure 2. (a) emission spectra of $CdS/Cd(OH)_2$ (line) and $CdS/Cd(OH)_2$/Glut (dash). (b) Two photon excited luminescence of a promastigote labeled with $CdS/Cd(OH)_2$/Glut.

The emission spectra of the $CdS/Cd(OH)_2$ and of the $CdS/Cd(OH)_2$/Glut nanocrystals are shown in Figure 2(a). The emission maximum of the original and the functionalized suspension used in the conjugation experiments is shown to be at 489 nm. The decrease in luminescence of the $CdS/Cd(OH)_2$/Glut suspension is due to the dilution performed during the functionalization procedure.

Figure 2(b) shows the two photon excitation spectrum (Exc: 785nm) for a promastigote conjugated to $CdS/Cd(OH)_2$/Glut nanoparticles. The spectrum shows a new band profile, with two main bands, the first in the green region resembling the original emission of the QDs and the second extending up to the red region with a maximum around 570 nm.

The living parasites showed no sign of damage after the conjugation procedure and maintained their integrity even after 18 hours of incubation time, demonstrating the isotonicity of the labeling procedure as well as the low toxicity of the functionalized and non-functionalized QDs.

The confocal microscopic analysis of the marked promastigotes show two main features: (1) both sets of promastigotes show dual emission; excited at 488 and 543 nm the samples show green and red emission respectively and (2) a different labeling pattern for the functionalized and non-functionalized nanoparticles is observed.

For the $CdS/Cd(OH)_2$/Glut–promastigote the color emission shown in Figures 3(a) and 4(a) (red) and 3(b) and 4(b) (green) although being pseudo-colors correspond to the spectral region acquired by the instrument (550-700 nm and 500-535 nm respectively). This two emission regions agree well with the two-photon excited spectrum shown in Figure 2b for the same system. Excitation at 543 or 488 nm resulted in the same fluorescent image showing dual luminescence homogeneously distributed on the whole parasite. The

confocal microscopic image series shows an uniform marking not diferentiating any characteristic moiety of the parasite.

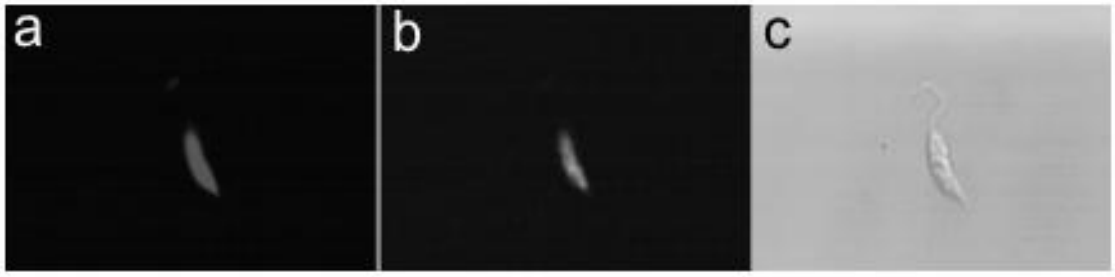

Figure 3. Confocal microscopy images of a *leishmania* amazonensis promastigote conjugated to CdS/$Cd(OH)_2$/Glut nanoparticles excited with (a) 543 nm and (b) 488 nm. The image shown in (c) is the differential interference contrast image of the promastigote.

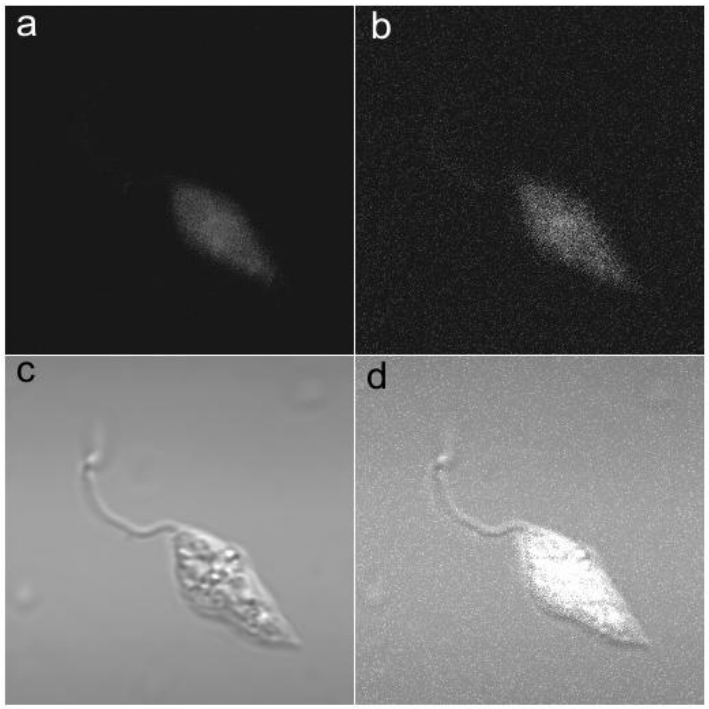

Figure 4. Confocal microscopy image of *leishmania amazonensis* promastigote conjugated to CdS/$Cd(OH)_2$/Glut nanoparticles excited with (a) 543 nm and (b) 488 nm. The image shown in (c) is the differential interference contrast image of the promastigote and (d) is the resulting superposed images.

On the other hand the promastigotes conjugated to CdS/$Cd(OH)_2$ nanocrystals show the emission pattern described in Figure 5(a-c). Figure 5(a) shows the microscopic confocal image under excitation at 543 nm while Figure 5(b) shows the image of the same system under excitation at 488 nm. Under 543 nm excitation wavelenght the emission profile ressembles the homogeneous pattern observed in promastigotes marked with the funcionalized CdS/$Cd(OH)_2$ QDs.

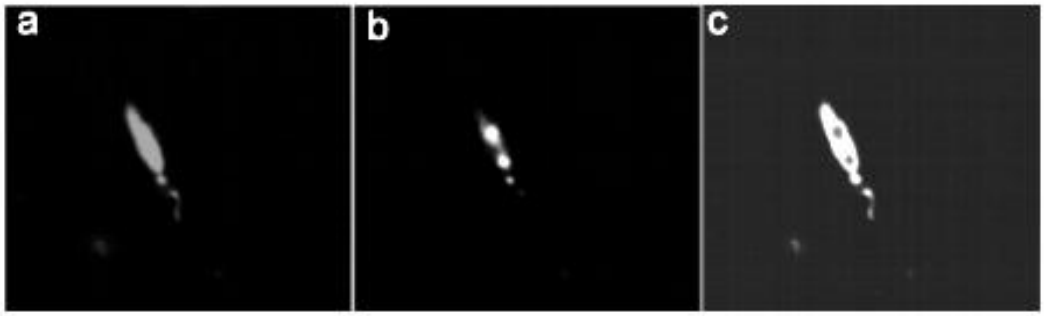

Figure 5. Confocal microscopic images of the leishmania amazonensis promastigote labelled with $CdS/Cd(OH)_2$ QDs excited at (a) 543 nm and (b) 488 nm. The image shown in (c) is the overlapping image of (a) and (b).

In order to further confirm the extracellular and intracellular labelling of the promastigotes we used confocal microscopy to visualize its depth emission profile. For the functionalized-QD conjugates, the depth profile maintained the homogenous pattern observed in Figures 3 and 4. The confocal microscopic depth profile of the $CdS/Cd(OH)_2$ labelled parasites is shown in the series of images depicted in Figure 6. We observed that the emission profile maintains the observed pattern shown in Figure 5.

The emission pattern observed under 488 nm excitation (Figure 5b) evidenciates the labelling of different internal structures present in the promastigote. The fact that we observe two different emission range (green under 488 nm excitation and red under 543 nm excitation) suggests that these cellular regions have distinct molecular environment promoting different deactivation pathways. To explain this emission behavior we have to examine the possible binding sites for the functionalized and non-functionalized nanoparticles in the parasite.

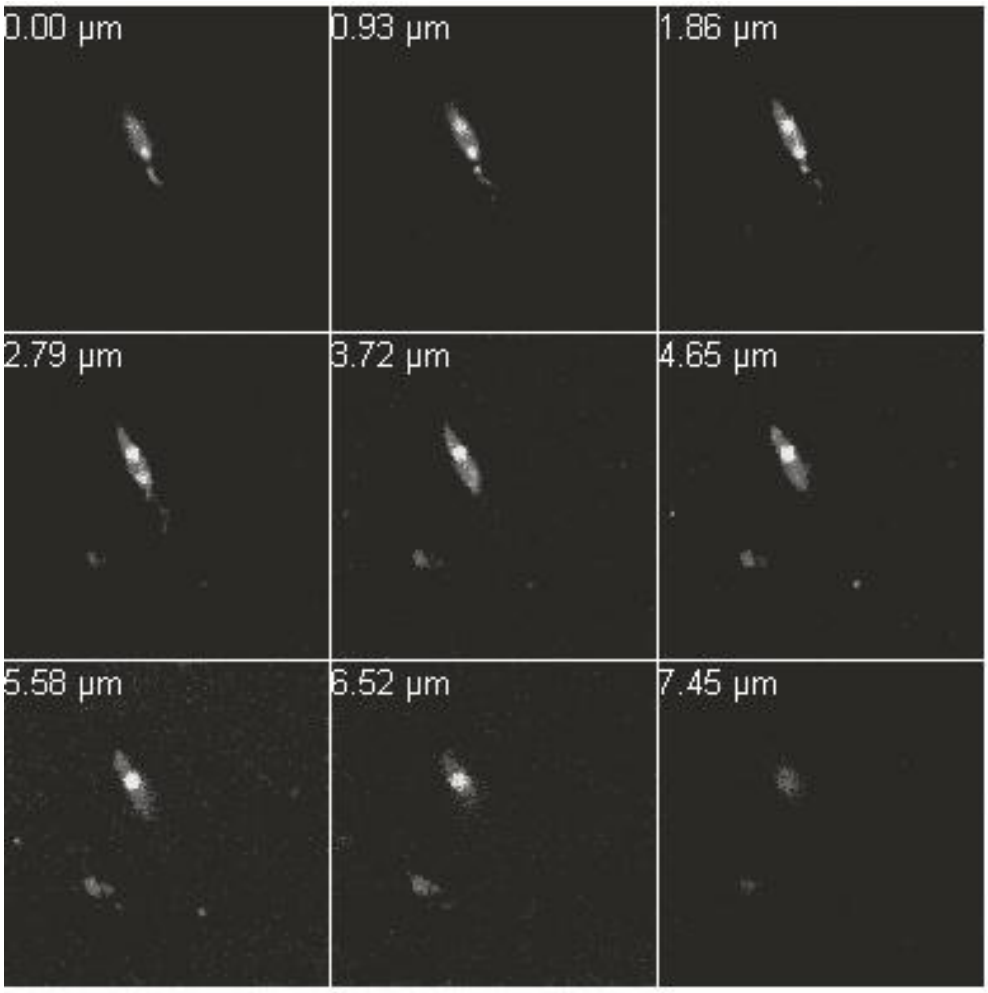

Figure 6. Confocal microscopic depth profile of the $CdS/Cd(OH)_2$ labelled parasites.

Figure 7 shows a schematic representation of a leishmania promastigote. It possesses a boby of about 10-15 µm long and a nucleus, a kinetoplast and a flagellar pocket as its three relevant internal parts.

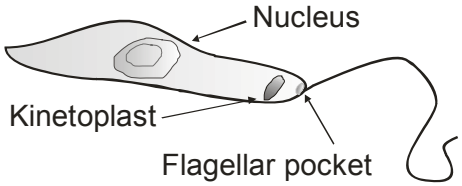

Figure 7. A schematic representation of a leishmania promastigote parasite, showing three different internal structures, the nucleus, the kinetoplast and the flagellar pocket.

The nucleus, kinetoplast and the flagellar pocket have a high concentration of nuclear molecules, such as DNA, contrasting to the citoplasmatic region which has a more proteic nature. As seen in a previous work on the binding of CdS/$Cd(OH)_2$/Glut QDs to erythrocyte membranes [7] the binding to the Anti-A protein caused a broadening of the original QDs emission band. In that case, it was suggested that the change of the emission band profile of the QDs after their binding to the Anti-A molecule moiety occurred through deactivation either by surface traps of the functionalized QDs or by energy transfer processes to near-resonant electronic states related to the biomolecule. This last hypothesis is favored in the promastigotes labelled with CdS/$Cd(OH)_2$/Glut considering that the glutaraldehyde molecules presented in the particles surface interact with the amine groups of the molecules presented in the parasite through very effective Shiff base reactions [11]. Since this binding is also inespecific regarding the protein target we would expect an uniform labelling pattern throughout the citoplasmatic region.

Conversely, the non-functionalized particles possess a phosphate rich surface (Figure 1b) which represents a very attractive energy source for the DNA producing and consuming structures inside the parasite, mainly the nucleus, the kinetoplast and the flagellar pocket. Speccially this last one which controls the flagellar motion. Still we observe two emission regions in the CdS/$Cd(OH)_2$/promastigote. Taking into account the favored chemical interactions between the QDs and the parasite biochemical moieties we suggest that (i) the non-functionalized QDs are being allowed to incorporate the DNA rich internal structures and (ii) the observed emission differentiation is altering the original energy pathway of the isolated crystals is due to distinct energy deactivation pathways occurring between the QDs and the molecules to which they are directly attached in the promastigote. This strongly suggests that the passivation surface of these particles is allowing the leakage of the exciton recombination energy of the CdS nanocrystals. How this deactivation processes occurs is a function of the interacting biomolecule(s) attached either via the polyphosphate anions or the glutaraldehyde molecule and will be published elsewhere.

CONCLUSION

We have labelled *Leishmania amazonensis* live parasites with phosphate and aldehyde rich surface CdS/$Cd(OH)_2$ luminescent quantum dots. We investigated their chemical interactions with the parasites intracellular structures by analyzing its emission pattern using confocal microscopy. The images show that the phosphate rich QDs interact with specific DNA rich structures of the promastigote showing a distinct fluorescence pattern when compared to the aldehyde rich QDs. The observed emission ranges indicate different fluorescent deactivation pathways. These findings are supported by spectroscopic data (absorption, excitation and emission spectra and lifetime measurements that will be published elsewhere). The preliminary results are very promising and suggest the use of CdS/$Cd(OH)_2$ nanocrystals as versatile probes of cellular processes in living cells.

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