Investigation of red blood cell antigens with highly fluorescent and stable semiconductor quantum dots

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Abstract. We report a new methodology for red blood cell antigen expression determination by a simple labeling procedure employing luminescent semiconductor quantum dots. Highly luminescent and stable core shell cadmium sulfide/cadmium hydroxide colloidal particles are obtained, with a predominant size of 9 nm. The core-shell quantum dots are functionalized with glutaraldehyde and conjugated to a monoclonal anti-A antibody to target antigen-A in red blood cell membranes. Erythrocyte samples of blood groups A^+, A_2^+, and O^+ are used for this purpose. Confocal microscopy images show that after 30 min of conjugation time, type A^+ and A_2^+ erythrocytes present bright emission, whereas the O^+ group cells show no emission. Fluorescence intensity maps show different antigen expressions for the distinct erythrocyte types. The results obtained strongly suggest that this simple labeling procedure may be employed as an efficient tool to investigate quantitatively the distribution and expression of antigens in red blood cell membranes. [DOI: 10.1117/1.1993257]

Keywords: biomedical optics; optical materials; quantum dots.

Paper 04214RR received Nov. 8, 2004; revised manuscript received Mar. 23, 2005; accepted for publication Mar. 28, 2005; published online Aug. 1, 2005.

1 Introduction

This work is concerns the use of fluorescent semiconductor quantum dots for biological labeling. Several classes of compounds are conventionally used to generate fluorescence: organic molecules, fluorescent proteins, metal chelators, and chemi- and bio-luminescent agents. All of these fluorophores present one or more of the following disadvantages: lack of brightness, broad emission bands, and high photobleaching rates. Aqueous fluorescent semiconductor quantum dots (QDs) are a new class of markers that present many advantages over conventional fluorophores. QDs are stable, bright particles in the nanometer size range that present high quantum yields, large excitation bands, narrow fluorescence emission bands, very long effective Stokes shifts, high resistance to photobleaching, and can provide excitation of several different emission colors using a single wavelength for excitation.[1] The peculiar characteristics presented by QDs are related to the fact that they are in a quantum confinement regime.[2–4] In the case of semiconductor QDs, one of these special characteristics is the capability of tuning their optical properties, particularly their emission spectra,[5] by controlling the size of the particles. The size tunability of emission bands in addition to single-wavelength excitation make QDs a class of fluorescent markers that can be chemically manipulated according to the desired target molecule in a biological system.

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The first biological applications of QDs were reported in 1998.[6,7] Bruchez et al.[6] and Chan and Nie[7] used CdSe QDs coated with silica and mercaptoacetic acid layers, respectively. Both groups showed specific labeling by covalent coupling of ligands to these surfaces. Subsequently, several authors have reported labeling of whole cells and tissue sections using several different surface modifications of QDs.[8–12] By attaching biomolecules to nanometer-sized bits of semiconductors, sensitive and potentially widely applicable methods for detecting biomolecules and for scrutinizing bimolecular processes have been developed. The QD-labeled molecules remain active for biochemical reactions, and the tagged species produce brightly colored products.[6,7] This methodology takes advantage of the efficient fluorescence and high photostability of the semiconductor QDs. In spite of the great advantage of QDs, their biological applications, however, do not always correspond to simple methodologies that can easily be implemented. As an example, quantum dot preparations reported to data have not been optimized for labeling blood cells. Two articles applying QDs to blood cells published recently reported the use of core-shell zinc sulfide cadmium selenide (CdSe/ZnS) quantum dots for labeling fixed human erythrocytes[13] and T-lymphoma cells.[14] Despite showing a successful conjugation, the methodology utilized for conjugating QDs to red blood cells still requires exceptionally experimental setup steps. In contrast, we developed a very simple methodology for marking living human erythrocytes by using highly fluorescent core-shell cadmium sulfide (CdS)/

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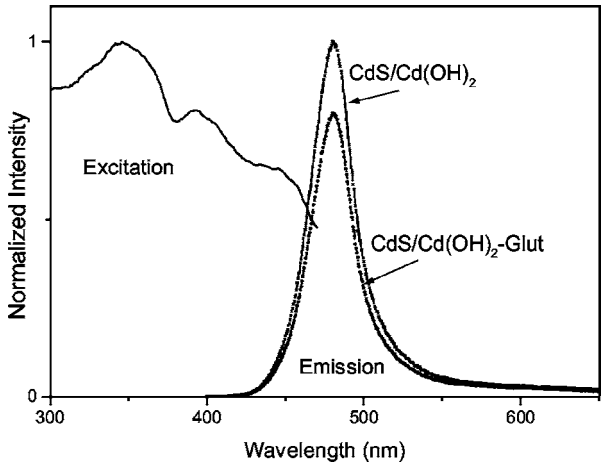

Fig. 1 Excitation and emission spectra for $CdS/Cd(OH)_2$ QDs and functionalized $CdS/Cd(OH)_2$-glutaraldehyde QDs.

cadmium hydroxide $[Cd(OH)_2]$ QDs functionalized with glutaraldehyde. Figure 1 shows the excitation and emission spectra obtained for $CdS/Cd(OH)_2$ QDs before and after functionalization with glutaraldehyde.

2 Material and Methods

9-nm sized core-shell $CdS/Cd(OH)_2$ QDs were synthesized in aqueous medium using the methodology described in a previous work.[15] The pH of the $CdS/Cd(OH)_2$ colloidal suspension was adjusted to 7.8 with chloridric acid 0.1-M diluted solution. The next step consisted of the functionalization of the QDs with a 0.016% glutaraldehyde solution. The resulting pH of the functionalized colloidal suspension was 7.2. The glutaraldehyde acts as an organic functionalizing agent that intermediates the interaction of the QDs with the monoclonal A antibody. After functionalization, the QDs were filtered and incubated with monoclonal anti-A reagent (DiaMed AS, Cressier s/Morat, Switzerland) in isotonic buffer at room temperature for 5 h. The erythrocyte samples used were obtained from blood donors at the Hematology and Tranfusion-Hemotherapy Center of Campinas. Before contact with QDs, the erythrocyte concentrates were diluted in 0.9% saline solution, centrifuged, and separated from the liquid phase. The resulting conjugates QDs/anti-A were incubated with human erythrocytes of blood groups A^+, A_2^+, and O^{+16} for 30 min at 37 °C, 3000 rpm for 2 min, and washed with buffer solution at 37 °C. The optical properties of the as-prepared $CdS/Cd(OH)_2$ and $CdS/Cd(OH)_2$-glut colloidal suspensions were characterized by electronic absorption spectroscopy and emission and excitation spectroscopies using the apparatus described in Ref. 16. The structural properties of the QDs were obtained using electronic transmission microscopy. The samples were prepared by drying a drop of the colloidal suspension on a copper grid coated with carbon and parlodium film. The efficiency of the conjugation QDs/antibody to the erythrocytes was monitored by the confocal microscopy technique. The systems were centrifugated three times at 10,000 rpm for 2 min and washed with buffer solution. The efficiency of the conjugation QDs/antibody to the erythrocytes was monitored by the confocal microscopy technique.

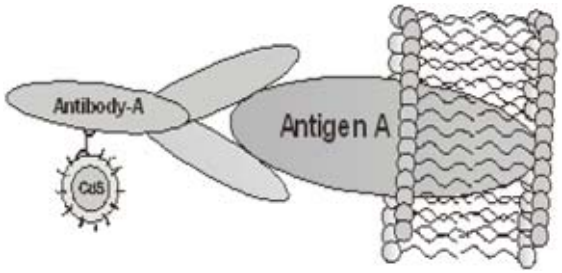

Fig. 2 Schematic diagram proposed for the specific conjugation of the CdS QDs/anti-A on the erythrocyte cell surface.

The cells were examined by the laser scanning confocal microscopy LSM 510 (Carl Zeiss, Jena, Germany) using an apochromatic water immersion, 63× 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 consisted of 1024×1024 pixels. The images were acquired and processed using the software LSM 510 (Carl Zeiss, Incorporated). Laser intensities at the target spot are estimated to be 9 and 1 mW for the argon (488 nm) and He/Ne (543 nm), respectively. For each cell type, the images were reproduced at least three times. To establish a comparative analysis of the luminescence intensity maps, the parameters related to the acquisition of confocal images, such as pinholes, filters, beamsplitters, and photomultiplier gain, were maintained constant.

3 Results and Discussion

The attachment procedure of anti-A coated QDs to the red blood cells needs only a primary incubation step. This is one of the main advantages over other fluorescence assays described in the literature, such as the common immunocythochemical methods. In the present method, we define a specific conjugation site on cell: the antigen-presenting molecules. A proposed schematic diagram for the conjugation of the CdS QDs/anti-A and the erythrocyte cell membrane is depicted in Fig. 2.

The QDs are attached by a cross-linking procedure to the antibody-A molecule. In a second step, the QDs/anti-A is

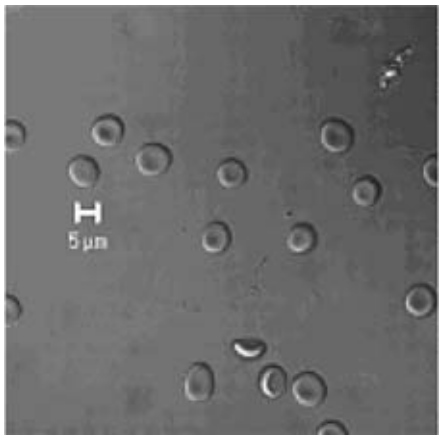

Fig. 3 Interference microscopy image for A^+ erythrocyte conjugated with QDs/anti-A, obtained after more than 2 h of incubation time.

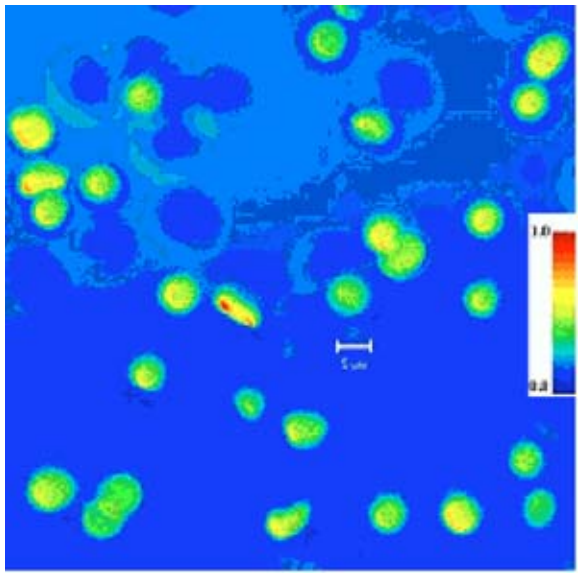

Fig. 4 Fluorescence intensity map obtained for O^+ erythrocyte conjugated with QDs/anti-A. According to the intensity scale, very low levels of QDs/anti-A conjugates can be observed.

placed in contact with the red blood cells at 37 °C, resulting in the formation of the antigen A/anti-A/QDs complex in the cell membrane. The living cells showed no sign of damage after the conjugation procedure and maintained their integrity even after two hours of incubation time, demonstrating the isotonicity of the labeling procedure as well as the low toxicity of the QDs. Figure 3 shows an interference microscopy image of A^+ erythrocytes obtained after more than 2 h of incubation time.

The CdS QDs/anti-A system shows high fluorescence when excited by laser sources, markedly for 488-nm (Ar laser) and 543-nm (He/Ne laser) excitations wavelengths. After the cell conjugation procedure, brightly colored fluorescence microscopy images of the group A^+ erythrocytes are observed, while no luminescence for the O^+ group is observed. Figure 4 shows the fluorescence intensity map for O^+ erythrocytes. As can be observed in the intensity bar, most of the sample shows very low levels of QDs/anti-A conjugates (blue for 0% of luminescence).

The confocal image and fluorescence intensity map for the marked A^+ erythrocytes are presented in Figs. 5(a) and 5(b), respectively. It can be noted that contrary to the fluorescence intensity map obtained for O^+ erythrocytes, the A^+ erythrocytes incubated with QDs/anti-A conjugates show strong levels of luminescence.

Analyzing the intensity map of A^+ marked cells, we observe a homogeneous emission profile throughout the cell surface, suggesting a homogeneous distribution of antigens A on the surface. In contrast, the intensity map analysis of the confocal images shows a distinct intensity pattern for the different A-type erythrocytes studied, suggesting different antigen distribution. The lack of emission of the O^+ erythrocytes is explained by the absence of anti-A binding, indicating an absence of antigen A.

Lack of photostability is one of the known drawbacks during prolonged visualization of tagged material with conventional fluorescence and confocal scanning microscopes. The photobleaching process[17] causes the fluorescent image to become dim, in some cases, after less than one minute of light excitation. To verify the photostability of our new fluorophore, we also tested the QDs under prolonged light excitation prior and after conjugation with the red blood cells. The emission spectrum of the QDs maintained constant intensity for up to one hour of light excitation, and no signs of photodarkening of the blood cell images was observed up to 20 min of constant laser excitation. The observed photostability is due to the QDs's greater energetic stability as inorganic nanocrystals compared to the common organic dyes.[16] This higher stability, and also the observed higher luminescence efficiency, is due to a surface capping layer of higher bandgap energy, which minimizes the surface traps, optimizing the electron-hole recombination process.

In conclusion, we have shown the viability of the use of high luminescent and stable quantum dots as fluorescent la-

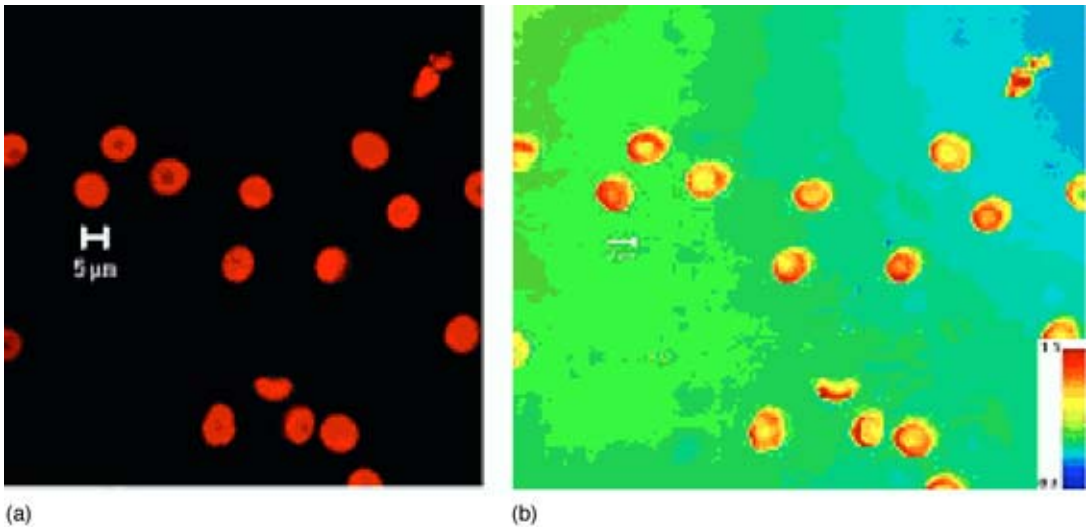

Fig. 5 (a) Confocal image and (b) fluorescence intensity map for the marked A^+ erythrocytes.

bels for human erythrocyte membrane A-antigens by employing a simple and efficient methodology, which can be easily adapted for targeting other antigens in red blood cells as well as for other types of molecules in different kinds of cells. The results presented in this work are hardly obtained via serologic or even molecular techniques. The knowledge of the correct expression of antigens in blood cell membranes represents relevant information to investigate the viability of organ transplantations, and also plays an important role for the development of diagnosis reagents. The specificity of the QDs conjugation in the erythrocyte cell membrane opens up the possibility of using simple QDs-based methodologies as a quantitative tool to investigate the distribution and expression of antigens in red blood cell membranes.

Acknowledgments

This work is partially supported by the following Brazilian agencies: CNPq, IMMC, Renami, and Facepe. This work is also linked to CEPOF (Centro de Pesquisas em Optica e Fotonica from Fapesp). The authors would like to thank the Hematology and Transfusion-Hemotherapy Center, State University of Campinas for the use of the confocal microscopy facilities.

References

1. A. Watson, X. Wu, and M. Bruchez, "Lighting up cells with quantum dots," *BioTechniques* **34**, 296–303 (2003).
2. H. Borchert, D. V. Talapin, C. McGinley, S. Adam, A. Lobo, A. R. B. de Castro, T. Möller, and H. Weller, "High resolution photoemission study of CdSe and CdSe/ZnS core-shell nanocrystals," *J. Chem. Phys.* **271**, 1800–1807 (2003).
3. A. P. Alivisatos, "Semiconductor clusters, nanocrystals, and quantum dots," *Science* **271**, 933–937 (1996).
4. C. Nowak, H. Döllefeld, A. Eychmüller, J. Friedrich, A. Kolmakov, J. O. Löfken, M. Riedler, A. Wark, H. Weller, M. Wolff, and T. Möller, "Innershell absorption spectroscopy on CdS: Free clusters and nanocrystals," *J. Chem. Phys.* **114**, 489–494 (2001).
5. A. Eychmuller, "Structure and photophysics of semiconductor nanocrystals," *J. Phys. Chem.* **59**, 13226–13239 (1996).
6. M. Bruchez, Jr., M. Moronne, P. Gin, S. Weiss, and A. P. Alivisatos, "Semiconductor nanocrystals as fluorescent biological labels," *Science* **281**, 2013–2016 (1998).
7. W. C. W. Chan and S. Nie, "Quantum dots for ultrasensitive biological detection," *Science* **281**, 2016–2018 (1998).
8. B. Ballou, B. C. Lagerholm, L. A. Ernst, M. P. Bruchez, and A. S. Waggoner, "Noninvasive imaging of quantum dots in mice," *Bioconjugate Chem.* **15**, 79–86 (2004).
9. X. Gao and S. Nie, "Molecular profiling of single cells and tissue specimens with quantum dots," *Trends Biotechnol.* **21**, 371–373 (2003).
10. M. E. Äkerman, W. C. W. Chan, P. Laakkonen, S. N. Bhatia, and E. Ruoslahti, "Nanocrystal targeting *in vivo*," *Proc. Natl. Acad. Sci. U.S.A.* **99**, 12617–12621 (2002).
11. P. M. A. de Farias, B. S. Santos, R. L. Longo, R. Ferreira, and C. L. Cesar, "CdS nanoparticles: structural and energetic correlations," *Mater. Chem. Phys.* **89**, 21–27 (2005).
12. L. C. Mattheakis, J. M. Dias, Y. Choi, J. Gong, M. P. Bruchez, J. Liu, and E. Wang, "Optical coding of mammalian cells using semiconductor quantum dot," *Anal. Biochem.* **327**, 200–208 (2004).
13. F. Tokumasu and J. Dvorak, "Development and application of quantum dots for immunocytochemistry of human erythrocytes," *J. Microsc.* **211**, 256–261 (2003).
14. A. Hoshino, K. I. Hanaki, K. Suzuki, and K. Yamamoto, "Applications of T-lymphoma labeled with fluorescent quantum dots to cells tracing markers in mouse body," *Biochem. Biophys. Res. Commun.* **314**, 46–53 (2004).
15. D. V. Petrov, B. S. Santos, G. A. L. Pereira, and C. M. Donegá, "Size and band-gap dependences of the hiperpolarizability of CdxZn1-xS nanocrystals," *J. Phys. Chem. B* **106**, 5325–5334 (2002).
16. D. L. Taylor, A. S. Waggoner, R. F. Murphy, F. Lanni, and R. R. Birge, *Applications of Fluorescent in the Biomedical Sciences*, Alan R. Liss, Inc., New York (1986).
17. G. Daniels, *Human Blood Groups*, Blackwell Science, Oxford (2002).