Semiconductor nanocrystals and fluorescence microscopy in biological labeling

Patricia M. A. Farias*, Beate S. Santos, Adriana Fontes and Carlos L. Cesar

1Department Biophysics and Radiobiology, Universidade Federal de Pernambuco, Cidade Universitária, Recife, PE, Brazil, CEP: 50670-901
2Department of Pharmaceutical Sciences, Universidade Federal de Pernambuco, Cidade Universitária, Recife, PE, Brazil, CEP: 50670-901
3Department of Quantum Electronics, Instituto de Física Gleb Wataghin, Universidade Estadual de Campinas, Campinas, SP, Brazil, CEP: 13083-970.
4Research Group on Nanostructures and Biological Interfaces

This work presents and discuss some features concerning the use of fluorescence microscopy for the analysis of living biological samples labeled with fluorescent colloidal semiconductor nanocrystals in the quantum confinement regimen (quantum dots). The nanostructured systems utilized in this work are shown to be very efficient tools for biological labeling and may be used in experimental procedures in order to obtain precise diagnostic of some pathologies, such as a wide variety of cancer types.

Keywords fluorescence microscopy; semiconductor quantum dots; biolabeling; bionanotechnology.

1. Introduction

Fluorescence provides an important tool for the investigation of basic physical properties of biological structures. The high sensitivity of fluorescence, combined with the advances in measurement techniques, allows the detection of ultra small quantities of specific species present in biological systems. The fluorescence process one of the known luminescence phenomena in which susceptible molecules or aggregates emit light from electronically excited states created by either a physical (for example, absorption of light), mechanical (friction), or chemical mechanism. Generation of luminescence through excitation by ultraviolet or visible light photons is a phenomenon termed photoluminescence, which is formally divided into two categories, fluorescence and phosphorescence, depending upon the electronic configuration of the excited state and the emission pathway. Fluorescence is a property presented by some atoms, molecules and nanocrystals, that allow them to absorb light at a particular wavelength and to subsequently emit light of longer wavelength after a brief interval, termed the fluorescence lifetime.

The analysis of biological samples, such as cells and tissues by fluorescence microscopy, is rapidly expanding its application today, both in the medical and biological sciences, a fact which has triggered the development of more sophisticated microscopes and numerous fluorescence accessories. Modern fluorescence microscopy techniques combine the power of high performance optical components with computerized control of the instrument and digital image acquisition to achieve a level of sophistication that far exceeds that of simple observation by the human eye. By using fluorescence microscopy, the precise location of intracellular components labeled with specific fluorophores can be monitored, as well as their associated diffusion coefficients, transport characteristics, and interactions with other biomolecules. In addition, the dramatic response in fluorescence to localized environmental variables enables the investigation of pH, viscosity, refractive index, ionic concentrations, membrane potential, and solvent polarity in living cells and tissues.

There is a great number of compounds used to generate fluorescence, such as organic molecules, fluorescent proteins, metal chelators, chemi- and bioluminescent agents. All of these fluorophores present one or more the following disadvantages: lack of brightness, broad emission bands and high

* Corresponding author: e-mail: pmaf@ufpe.br, Phone: +55-81-21268535 Fax: +55-81-21268560
photobleaching rates. In the last decade a new class of fluorescent materials, constituted by semiconductor nanocrystals in the quantum confinement regimen, known as quantum dots (QDs) is being tested as biolabels. Fluorescent semiconductor quantum dots have been used mainly for bioimaging, using convencional or confocal fluorescence microscopy to study cellular biology and also for optical diagnostics. However, they can also be used in any experiment that is conventionally performed with fluorescent dyes, such as: flow citometry, fluorescence resonance energy transfer (FRET) and fluorescence lifetime measurements (FLIM), as well as in two-photon/multiphoton microscopy [1,2].

Quantum dots are nanometric inorganic particles, which present special characteristics. The special features presented by the quantum dots, are concerned with the fact that they are in quantum confinement regimen [3–5]. In the case of semiconductor quantum dots, one of these special characteristics is the capability of tuning their optical properties, particularly their emission spectra [6] by controlling the size of the particles. The first biological applications of quantum dots were reported in 1998 [7, 8]. Bruchez et al. [7] and Chan et al. [8] used CdSe QDs coated with silica and mercaptoacetic acid layers, respectively, and 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 [9–13]. By attaching biomolecules to nanometer-sized bits of semiconductors, a sensitive and potentially widely applicable method for detecting biomolecules and for scrutinizing biomolecular processes was developed [14-16]. The quantum dot-labeled molecules remain active for biochemical reactions and the tagged species produce brightly colored products [7, 8]. This methodology takes advantage of the efficient fluorescence and high photostability of the semiconductor quantum dots, representing a new class of biological dyes. Hydrophilic quantum dots in water medium and at physiological pH conditions, have the potential to expand conventional protocols used for cancer diagnostic, which needs previous tissue/cell fixation, and extend it to investigate living cellular and tissular neoplastic mechanisms in real time. Quantum dot applications in the investigation pathological processes, such as neoplastic ones, which may give rise to a wide variety of cancer, constitute a topic of crescent interest, in which many questions still remain unanswered. In the pursuit of sensitive and quantitative methods to detect and diagnose cancer, nanotechnology has been identified as a field of great promise.

2. Methodology

Biological samples, such as microorganisms (Saccharomyces cerevisiae, Leishmania amazonensis and Tripanosoma cruzi) as well as red blood cells and healthy and neoplastic glial, breast and cervical cells were incubated with colloidal aqueous suspensions in which the disperse phase consists of core-shell fluorescent Cadmium Sulfide/Cadmium hydroxide (CdS/Cd(OH)2) 6 nm quantum dots. The cells were labeled in their respective culture medium, by dropping stoichiometric volumes of the quantum dots colloidal suspensions, in order to promote the interaction between the inorganic quantum dots and the biological samples. For each kind of analyzed sample, a different incubation time is required. Highly fluorescent labeled cells are then, ready for measurements and to be analyzed by fluorescence microscopy techniques. In the next section, some of the results obtained for the labeling of red blood cells and for cancer cells will be presented and discussed. For further reading see References [15-17].

The fluorescent labels, CdS/Cd(OH)2 quantum dots, were obtained by reacting Cd2+ and S2- ions in aqueous solution. Due to the intrinsic thermodynamical instability of colloidal suspensions, a stabilizing agent (in this case sodium polyphosphate) was added to the reacting system. Subsequent surface passivation with Cd(OH)2 was carried out to improve luminescence. At a pH of 7.2 the QDs were functionalized with a 0.01% glutaraldehyde solution (QD-glut), as described in reference [14]. The glutaraldehyde is an organic cross-linking functionalizing agent that intermediates the interaction of the QDs with the cells. All samples were in culture medium and the incubation with the QDs was performed at room temperature (25°C). Tissue and cell staining were evaluated by the fluorescence patterns obtained by laser scanning confocal microscopy (Leica TCS SP2 AOLS and LSM 510 Carl Zeiss confocal microscopes), as well as by conventional fluorescence microscopy (Carl Zeiss, Jena, Germany),
In order to compare the results, constant acquisition parameters, such as such as pinhole, filters, beam splitters and photomultiplier gain and off-set, were used. The images were further processed using the software Leica Lite and LSM 510 (Carl Zeiss Inc). Laser scanning confocal microscopy measurements were performed, using 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 images were taken using dual-channel scanning and consisted of 1024x1024 pixels. For each cell type the images were reproduced at least three times to assure a comparative analysis of the luminescence intensity maps.

For an optimal performance in biological imaging (and competitive to the commercial organic fluorophores), semiconductor quantum dots are being developed in order to optimize their luminescent, surface and chemical stability properties. These conditions result in a very complex multilayered chemical assembly where the nanocrystal core determines its emission color, the passivation shell determines its brightness and photostability and the organic capping layer determines its stability and functionality.

A schematic representation of this “hybrid bio/organic/inorganic nanostructured assembly” is described in Figure 1.

![Fig.1: A diagrammatic representation of a passivated, functionalized and bioconjugated quantum dot.](image)

### 3. Results and discussion

Structural and optical characterization of the QDs were performed by X-Ray Diffraction, Electronic Transmission Microscopy and Electronic Absorption, Emission and Excitation Spectra. A representative Transmission Electronic Microscopy image of the core-shell CdS/Cd(OH)\(_2\) quantum dots functionalized with glutaraldehyde is shown in Fig. 2, in which the scale bar corresponds to 40 nm. The averaged size of the QDs is about 9 nm. Fig. 3 illustrates the excitation and emission spectra for as prepared core-shell CdS/Cd(OH)\(_2\) quantum dots, in which may be observed a broad excitation band and a narrow Gaussian emission band of about 50 nm (FWHM).
Fig. 2: Transmission Electron Microscopy image of the CdS/Cd(OH)$_2$ core shell quantum dots. Scale bar: 40 nm.

Fig. 3: Excitation and emission spectra of as prepared CdS/Cd(OH)$_2$ quantum dots.

Different labeling strategies were developed for the labeling of different biological samples, as presented bellow.

3.1 Labeling red blood cells with fluorescent CdS Quantum Dots for precise determination of A-antigen expression

Core-shell CdS/Cd(OH)$_2$ quantum dots obtained in aqueous medium were successfully used as efficient fluorescent labels for living human red blood cells. The aim of this investigation was to precisely determine the Antigen-A expression in subgroups of group A erythrocytes.
Luminescent CdS/Cd(OH)₂ nanoparticles were functionalized via one-pot cross linking glutaraldehyde procedure. These functionalized nanoparticles were conjugated to monoclonal antibody Anti-A for 5 hours. Living human red blood cells, before the contact with QDs, 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⁺, and O⁺ for 30 minutes at 37°C. Prior visualization, the samples were centrifugated for 2 minutes (3000 rpm) in saline buffer solution and washed. A schematic diagram for the conjugation of the QDs/anti-A as well as for their interaction with red cell membrane antigen is depicted in Fig. 4. Functionalization and conjugation steps were performed in an aqueous medium and physiological pH (7.3 – 7.4).

The cells conjugated with the quantum dots were characterized by confocal laser scanning microscopy as well as by conventional fluorescence microscopy. The conjugates QDs/anti-A intensely marked group A erythrocytes, showing different intensities of luminescence for A⁺ group investigated, and did not show any luminescence for group O erythrocytes [16]. Fig. 5 shows respectively a fluorescence confocal image obtained for QDs/anti-A marking A⁺ erythrocytes and for QDs/anti-A marking O⁺. The lack of emission of the type O⁺ erythrocytes is explained by the absence of Anti-A binding, indicating the absence of antigen A.

![Fig. 4: Schematic diagram for the specific conjugation QDs/anti-A - QDs/Anti-A interaction with red cell membrane antigen-A.](image)

![Fig. 5: Fluorescence image obtained for QDs/anti-A marked living A⁺ (left) and O⁺ (right) erythrocytes.](image)
3.2 Labeling neoplastic cells with fluorescent semiconductor quantum dots

Hydrophobic quantum dots at physiological pH conditions, have the potential to expand conventional protocols used for cancer diagnostic, which needs previous tissue/cell fixation, and extend it to investigate living cellular and tissular neoplastic mechanisms in real time.

Here it is presented and discussed some results concerning the application of water soluble colloidal semiconductor quantum dots for the purpose of cancer diagnostic in living cells. The fluorescence was used as a primary tool in order to explore and differentiate the labeling of the samples. Tissues and cells conjugated with QDs were analyzed by the laser scanning confocal microscopy and conventional fluorescence microscopy. In order to confirm the presence of QDs inside the cells, some of the conjugated systems were also characterized by transmission electronic microscopy (TEM). This kind of measurement complements the fluorescence analysis, as they show that the QDs were internalized by the cell. The images obtained show that the nanocrystals accumulate near the nuclear envoltorium. Fig. 6 shows a representative TEM image of core-shell CdS/Cd(OH)$_2$ quantum dots functionalized with glutaraldehyde (QD/ Glut) and conjugated in vitro with living human glioblastoma cells, for 3 minutes. The cells showed no signs of damage after the conjugation procedure and maintained their integrity even after five days of incubation time, demonstrating the low toxicity of the QDs for in vitro studies. Fig. 7 shows fluorescence image (left) and the corresponding fluorescence intensity map (right), obtained by the incubation of fluorescent CdS/Cd(OH)$_2$ quantum dots with fresh sample of ductal filling carcinoma, a very aggressive form of breast cancer. In the fluorescence intensity map shown in Fig. 7, the blue regions correspond to the absence of fluorescence, which means no marked regions, while the red regions correspond to high level of fluorescence, which means a strong interaction between the quantum dots and the neoplastic cells. The interaction mechanisms between quantum dots and healthy and neoplastic cells will not be focused here. For a better understanding, see reference [17].

Fig. 6 - Transmission Electron Micrography image of Glioblastoma labeled cells: highest QDs concentration at the nuclear envoltorium.
Breast cancer tissue samples were incubated with the functionalized QDs-glut, and as can be noticed from Fig. 7, the neoplastic cells filling up the mammary duct clearly show highest QD concentrations compared with normal breast tissues samples.

4. Concluding Remarks

In summary, fluorescence microscopy techniques consist in a very important class of methods for analyzing and monitoring cellular and tissular mechanisms in living cells and tissues. The use of colloidal fluorescent semiconductor quantum dots, as highly luminescent stains, expands the use of fluorescence microscopy for beyond the use in fixed cells and tissues, which may represent a potential tool for fast and precise diagnostic of different kinds of cancer and other pathologies.

Acknowledgements The support by CNPq, Facep, Fapesp, Renami, Cepof and IMMC Brazilian agencies and networks as well as by Philips Corp., is gratefully acknowledged.

5. References

