Fluorescence Plate Reader for Quantum Dot-Protein Bioconjugation Analysis

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We present here a new and alternative method that uses a Fluorescence Plate Reader in a different approach, not to study protein–protein interactions, but to evaluate the efficiency of the protein bioconjugation to quantum dots (QDs). The method is based on the QDs’ native fluorescence and was successfully tested by employing two different QDs-proteins conjugation methodologies, one by promoting covalent binding and other by inducing adsorption processes. For testing, we used bioconjugates between carboxyl coated CdTe QDs and bovine serum albumin, concanavalin A lectin and anti-A antibody. Flow cytometry and fluorescence spectroscopy studies corroborated the results found by the Fluorescence Plate Reader assay. This kind of analysis is important because poor bioconjugation efficiency leads to unsuccessful applications of the fluorescent bioconjugates. We believe that our method presents the possibility of performing semi-quantitative and simultaneous analysis of different samples with accuracy taking the advantage of the high sensitivity of optical based measurements.

Keywords: Quantum Dot, CdTe, Bioconjugation, Fluorescence, Biomolecule.

1. INTRODUCTION
Quantum dots (QDs) are colloidal semiconductor nanocrystals which show unique physicochemical properties due to their quantum confinement regime in three dimensions. Some of the main optical features observed for these systems are: wide excitation bands, narrow emission spectra, size-controlled emission at different regions of the visible-light spectra and great photostability. These properties offer significant advantages for QDs when compared with conventional organic dyes, which are commonly used as fluorescent probes.¹–³ Additionally, the fluorescence of organic dyes tends to quench rapidly, so they are not suitable for long term analysis.⁴

QDs are nanocrystals made up of elements from the II–VI (e.g., CdSe, CdTe, CdS, and ZnSe), III–V (e.g., InP and InAs) or IV–VI (e.g., PbSe) group. As their surface atoms render a greater part of their structure, compared to bulk crystals, they show intrinsic electronic defects that have to be overcome. This is usually accomplished by chemically coating the nanocrystal core with monolayers of a semiconductor that usually has a higher bandgap, a process called passivation. These core–shell nanostructured systems can be synthesized using different chemical approaches. For biological applications they are prepared using colloidal chemistry either in organic or in aqueous media. When compared to organometallic routes, water-based QDs’s preparation method is less toxic, applies low-cost precursors and is intrinsically biocompatible to applications in biological fields.⁵

Due to the improvement of the colloidal synthesis methodologies, especially the aqueous based, in the last 15 years, QDs have been successfully used as fluorescent
probes in biological and medical field. In this time lapse we observed an exponential growth of the use of this new class of fluorophores in bioimaging (fixed cell, \textit{ex vivo} live cell and \textit{in vivo} animal targeting), bioanalytical assays, in immunofluorescence assays, as potential photosynthesisers for photodynamic therapy and as probes to trace the pharmacological routes of some drugs.\textsuperscript{1–3}

In almost all of these applications, the coating of QDs with antibodies and other proteins plays an important role, because they form inorganic-biological hybrid nanoparticles that combine characteristics of both materials, which are the fluorescence properties of QDs with the biochemical functions of the proteins. This process called bioconjugation is an important step for promising biological applications and needs to be characterized independently of the conjugation approaches employed, such as covalent binding or adsorption. The characterization and evaluation of the QDs bioconjugation is still a current critical problem for successfully using these new optical probes.

Many research groups have been reporting different methods to characterize QDs bioconjugates, Sapsford et al. (2011)\textsuperscript{4} in a recent review article compiled bioconjugation characterization approaches for various nanomaterials and classified them as separation, scattering, microscopy and spectroscopy techniques. Among the most cited methods are the gel and capillary electrophoresis,\textsuperscript{6} the dynamic light scattering (DLS)\textsuperscript{7} and the fluorescence correlation spectroscopy (FCS).\textsuperscript{8} However, these methods currently used still present one or other disadvantages: laborious methodologies, lack of standardization for the interpretation of the results or the use of expensive equipments. Electrophoresis, for example, is a laborious technique and, despite to be able to analyze and compare bioconjugation for various samples at same time, it can be applied only for qualitative analyses of charged specimens. Moreover, if the conjugated molecule has low or medium weight, the electrophoresis is no more appropriate.\textsuperscript{9,10} DLS can qualitatively answer if the bioconjugation happened, for one sample at a time, based on the average sizes, but has poor resolution and can only resolve particle populations (within the same sample) if they differ in size by at least a factor of 3.\textsuperscript{3} FCS technique is similar to DLS. However, although FCS is more sensitive than DLS for discriminating similar sizes, it needs a confocal microscopy, and then it is an expensive technique. Moreover, the FCS can answer if the bioconjugation happened, but it cannot give much information about the efficiency. Therefore, new and complementary methods are always welcome to evaluate bioconjugation process.

We present here an alternative optical method to confirm QDs bioconjugation by using a Fluorescence Plate Reader. This analytical approach is based on the native QDs’ fluorescence detection of samples containing the bioconjugates and the absence of signal in all other samples named controls. The Fluorescence Plate Reader is routinely applied in immunoassays to detect antigen-antibody interactions as already described in the literature.\textsuperscript{11} This detection is possible because one of the proteins (antigen or antibody) was previously conjugated to a fluorescent marker, which can also be a quantum dot.\textsuperscript{12} In this work, we propose the use of the Fluorescence Plate Reader not to study protein–protein interaction, but instead, for a different approach: to evaluate the efficiency of the protein bioconjugation to QDs. This analysis of bioconjugation is important because poor efficiency leads to unsuccessful applications of the fluorescent bioconjugates.

Among the methods used to evaluate bioconjugation, electrophoresis is, by far, the most used approach. When compared to the most applied methods, especially with electrophoresis, our approach has the advantages to be able to evaluate and discriminate semi-quantitatively a great number of different types of bioconjugates simultaneously (for example, there are microplates with 96 wells). Moreover, it is a simple, practical, fast and sensitive method. Besides, it is not limited by size or charge of the bioconjugates. Therefore, why do not use this procedure as a complementary approach to detect and evaluate the QDs-protein bioconjugation? In order to demonstrate this alternative method, we tested CdTe QDs bioconjugated to different proteins: bovine serum albumin (BSA), anti-A antibody and concanavalin A lectin (ConA). We employed two different conjugation methodologies, one by using covalent binding\textsuperscript{10} and other by adsorption, a phenomenon mainly mediated by electrostatic and hydrophobic/hydrophilic interactions.\textsuperscript{13}

2. EXPERIMENTAL PROCEDURES

2.1. General Experimental Details

All reagents for the QDs synthesis were purchased from Sigma Aldrich and used as received. Microplate-based fluorescence measurements were performed using a WALLAC 1420 microplate reader with the software Victor\textsuperscript{2} (PerkinElmer). Black 96-well Optiplate F HB microplates were purchased from PerkinElmer. The biomolecules used were BSA (Sigma Aldrich), ConA (Sigma Aldrich) and anti-A antibody (DiaMed—Minas Gerais—Brazil). Absorption spectra of bare and conjugated QDs were collected on a DU 7500 UV/VIS spectrophotometer (Beckman) or in a HR 4000 spectrophotometer (Ocean Optics) in the range of 200 to 600 nm. Emission spectra of bare and conjugated QDs (excited at 365 nm) were recorded using a PC1, (a photon-counting fluorescence spectrometer from ISS with a xenon lamp of 300 W as excitation source), employing excitation and emission slits of 1 mm and voltage = 15 V.

2.2. CdTe QDs Synthesis

Aqueous colloidal dispersion of CdTe core/shell QDs were synthesized by adapting a previously reported method.\textsuperscript{14} Briefly, QDs were prepared by the addition
of Te\(^{2-}\) in a Cd(ClO\(_4\))/Cd solution of pH > 10 in the presence of TGA (thioglycolic acid), MPA (3-mercaptopropionic acid) or MSA (mercaptosuccinic acid) (Sigma Aldrich) as stabilizing agents in a 2:1:4.2/4.6/2.4 ratio of Cd:Te:TGA/MPA/MSA respectively. The Te\(^{2-}\) aqueous solution was prepared by using metallic tellurium (Sigma Aldrich) and NaBH\(_4\) (Sigma Aldrich), at a high pH and under argon or nitrogen saturated atmosphere. The reaction proceeded, in argon or nitrogen, under constant stirring and heating at 80 °C during 1 hour for TGA and 7 and 15 hours at 100 °C for MPA and MSA, respectively.

2.3. Plate Reader Bioconjugation Detection Method

The method here described proposes to analyze and verify the QDs-protein bioconjugation based on the fluorescence intensities of samples placed in a Fluorescence Plate Reader. It is known that proteins have affinity for polystyrene, which is one of the most used plastic in microplates employed for protein immobilization in immunoassays.\(^{15-17}\) Bare QD samples do not efficiently link to the polymer because they do not have enough specific groups required for a strong interaction. Therefore, after washing the microplate with a buffer solution such as PBS (phosphate buffered saline), the QDs are removed and no fluorescence signal is detected. The biomolecules alone do not present considerable fluorescence in the same wavelength region of the QDs employed. So, in the case of a well succeeded QD-protein bioconjugation, the Fluorescence Plate Reader will give a fluorescence signal indicating that QDs are bound to the proteins attached to the well. Based on these features, the adsorption or covalent conjugation between carboxylate QDs and amine groups of biomolecules such as peptides, proteins and DNA\(^1\) can be confirmed by comparing the conjugates and controls signals intensities values, according to Figures 1 and 2 respectively.

Each sample set is consisted on three/four components: two/three controls and the QD-biomolecule conjugates. The first control corresponded to bare colloidal QDs. The second control consisted on protein molecules alone and the third control (only used for conjugation by covalent binding) corresponded to all reagents used in the bioconjugation process, except the QDs.

If the signal intensity of the QDs-biomolecules sample was at least 100% higher than the average controls values, this was considered a good indication of a satisfactory conjugation. On the other hand, if the signal intensity was of the same order of the controls, the conjugation was considered unsuccessful. These methodologies are summarized in Figure 1 (adsorption) and Figure 2 (covalent bond) and the correspondent results are presented in Tables I and II respectively.

2.4. Conjugation by Covalent Binding

TGA-coated CdTe QDs were conjugated with BSA protein using EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and Sulfo-NHS (N-hydroxysulfo succinimide) as coupling reagents.\(^9,10\) Firstly, 20 nmol of CdTe was added to the activation buffer at 0.1 M MES (2-[morpholino]ethanesulfonic acid) at pH 6.0. After 15 minutes, 1 mL of EDC (2.08 mM) and 1 mL of Sulfo-NHS (5.06 mM) in ultrapure water were added to the system. Then, 1.4 \(\mu\)L of 2-mercaptoethanol (20 mM) were used to quench the EDC. After that, we added 1 mL
of BSA (1 mg/mL) in a hydroalcoholic solution (80:20). All this procedure was performed in inert atmosphere up to 4 hours at room temperature.

2.5. Conjugation by Adsorption
The MPA-coated or MSA-coated CdTe conjugations, by adsorption, with anti-A, BSA and ConA were performed by adjusting the QDs pH to 8.0 and placing the molecules in contact with QDs for 2 hours at room temperature without stirring. The QD:anti-A proportion was 50:1 (v/v). For ConA (0.2 mg/mL), the QD:ConA proportion was 7:1 (v/v) and QD:BSA (10 mg/mL) was 60:1 (v/v). Con A is a lectin that targets sugars such as mannose and glucose.

2.6. Procedure of Bioconjugates Immobilization and Analysis
A solution of PBS, at 7.4 pH, was employed as wash buffer. All samples were measured in triplicate. The control samples (described as QDs in water or in PBS (1×) and biomolecules in PBS (1×), both at the same concentration used in the conjugation) and conjugated samples were incubated in the microplate wells (200 μL in each well) for 2 hours in an incubator (water bath, humid chamber) at 37 °C. After this time, the wells were washed 3 times with PBS. Then, the microplate was placed in the Plate Reader and the signals of each well (with controls and QDs-biomolecules samples) were analyzed under F480 (480 nm/15.5 nm) or F485 (485 nm/7 nm) excitation filters, F535 (535 nm/12.5 nm) or F595 (595 nm/30 nm) emission filters. The acquisition time was 1 s, the lamp was set to 20,000 or 60,000 cW and normal slits were used for excitation and emission.

2.7. Procedure for Confirmation of the Bioconjugation with Other Methods
Analysis of emission spectra was used to confirm the covalent conjugation results of BSA obtained by Fluorescence Microplate Reader. Flow cytometry assay was applied to evaluate the adsorption conjugation results for anti-A antibody obtained by Fluorescence Microplate Reader. For this, red blood cells (RBCs) were labeled by the QDs-(anti-A) conjugates and analyzed in a FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA). The software used for data processing was Cell Pro (Cell QuestTM Software, Becton Dickinson immuno-cytometry system, San Jose, CA, USA). Around 10,000 events per second were acquired. The fluorescence in cells was excited at 488 nm and measured with FL1 filter (530 nm/15 nm) or FL2 filter (585 nm/21 nm). RBCs type A and type O collected in EDTA (Ethylenediamine tetraacetic acid) tubes were used to prepare a 5% diluted
respectively. The average diameters were 2.7 nm, 2.6 nm and 3.3 nm. They do not present A antigens in their membranes.

The fluorescence microplate reader for the detection of free and bioconjugated QDs systems by adsorption interaction.

<table>
<thead>
<tr>
<th>System</th>
<th>Fluorescence intensity (a.u.)</th>
<th>Relative fluorescence intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1 (QDs-MPA)</td>
<td>705 ± 3</td>
<td></td>
</tr>
<tr>
<td>Control 2 (anti-A)</td>
<td>837 ± 6</td>
<td></td>
</tr>
<tr>
<td>Average control</td>
<td>771 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>QDs-MPA-(anti-A)</td>
<td>2037 ± 6.6</td>
<td>164% ± 7.7%</td>
</tr>
<tr>
<td>Control 1 (QDs-MSA)</td>
<td>200 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>Control 2 (anti-A)</td>
<td>218 ± 6</td>
<td></td>
</tr>
<tr>
<td>Average control</td>
<td>209 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>QDs-MSA-(anti-A)</td>
<td>886 ± 9</td>
<td>325% ± 10%</td>
</tr>
<tr>
<td>Control 1 (QDs-MSA)</td>
<td>312 ± 5</td>
<td></td>
</tr>
<tr>
<td>Control 2 (ConA)</td>
<td>435 ± 3</td>
<td></td>
</tr>
<tr>
<td>Average control</td>
<td>374 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>QDs-MSA-ConA</td>
<td>1078 ± 7</td>
<td>188% ± 7.4%</td>
</tr>
<tr>
<td>Control 1 (QDs-MSA)</td>
<td>429 ± 6.2</td>
<td></td>
</tr>
<tr>
<td>Control 2 (BSA)</td>
<td>383 ± 10</td>
<td></td>
</tr>
<tr>
<td>Average control</td>
<td>406 ± 6</td>
<td></td>
</tr>
<tr>
<td>QDs-MSA-BSA</td>
<td>884 ± 6.6</td>
<td>117% ± 7.4%</td>
</tr>
</tbody>
</table>

Notes: *Measurements performed with F480 excitation filter and F535 or F595 emission filters, acquisition time of 1 s, 20,000 and normal slits for excitation and emission. QDs employed were CdTe-MPA and CdTe-MSA.

Suspension of cells using saline solution. After that, RBCs were incubated with QD-(anti-A) conjugates for one hour at 37 °C using a proportion of 3:1 v/v (QDs conjugate cells). RBCs type O were the negative control, since they do not present A antigens in their membranes.

3. RESULTS AND DISCUSSION

Initially, all QDs employed in the experiments were characterized by absorption and emission spectra which are shown in Figures 3 and 4. According to optical characterizations, CdTe-TGA, CdTe-MPA and CdTe-MSA nanoparticles maxima emissions and the FWHM (full width half maximum) were 560 nm (FWHM = 35 nm), 550 nm (FWHM = 53 nm), 604 nm (FWHM = 50 nm), while the average diameters were 2.7 nm, 2.6 nm and 3.3 nm respectively.18,19 The QDs concentrations used were 1 μM for CdTe-TGA, 13 μM for CdTe-MPA and 6 μM for CdTe-MSA.20

The signal intensities for the QDs conjugated to proteins, the control 1 (QDs alone), control 2 (proteins alone) and control 3 (see Experimental Procedure Section) for each experiment performed on the Fluorescence Microplate Reader are shown in Tables I and II. The Figures 1 and 2 can be used to an appropriate interpretation of these tables.

The relative fluorescence intensity, which evaluates how higher was the bioconjugates signals when compared to controls, was calculated by Eq. (1).

RelativeFL (%) = \frac{BioconjugatedFL - ControlFL}{ControlFL} \times 100 \%

where BioconjugatedFL is the fluorescence intensity of the QDs-biomolecules sample and ControlFL is the average controls signal detected.

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where BioconjugatedFL is the fluorescence intensity of the QDs-biomolecules sample and ControlFL is the average controls signal detected.

Table I. Fluorescence intensity measurements obtained, in triplicate, in the fluorescence microplate reader for the detection of free and bioconjugated QDs systems by adsorption interaction.

Table II. Fluorescence microplate reader results of fluorescence intensity from triplicate detection of free and bioconjugated QDs systems by covalent binding.

Notes: *Acquisition performed with the F485 nm excitation filter and F535 emission filter, acquisition time of 1 s, 20,000 and normal slits for excitation and emission. QDs employed were TGA capped CdTe.
By comparing the analysis for QDs-BSA obtained by different time periods (2 and 4 hours) of covalent binding reaction, the results show relative fluorescence intensities of 91% and 406% respectively. Therefore, an increase in the conjugation reaction time is clearly followed by an increase in the relative intensity value, indicating that this method is semi-quantitative and can help to monitor and to improve the bioconjugation process. However, considering that the fluorescence intensity is given in arbitrary units only relative comparisons can be made. In our experiments, the highest intensity values were obtained after 4 hours of bioconjugation reaction time, indicating that the bioconjugation was more effective after 4 hours of reaction, but after this period of time it is important to test the functionality of the biomolecule. A good reaction time has to promote an efficient conjugation between QDs and proteins and also preserve the biomolecule activity. Moreover, it is important to highlight that QDs can be efficiently bioconjugated to proteins, however this procedure can cause changes in the biomolecule properties. In this way a good result obtained in a bioconjugation analysis is an indication, but is not a complete guaranty, of a successfully biological application.

In Figure 3, we present emission spectra for CdTe-TGA alone and CdTe-TGA-BSA bioconjugate (after 4 hours of reaction). The blue shift of the maximum emission peak from 560 to 540 nm (excitation at \( \lambda = 365 \) nm) confirms the bioconjugation. Both CdTe-TGA bare QDs and CdTe-TGA-BSA QDs presented symmetric and narrow emission spectra. BSA shows no fluorescence in the wavelength range employed. The BSA fluorescence is related to tryptophan residues which provide major contribution to the intrinsic fluorescence of this protein near 340 nm.21, 22 Also, it was observed a small decay in the fluorescence emission spectra intensity for bioconjugated QDs which may be explained by the effect of the surrounding environment pH. Previous study23 reported the effect of various factors on QDs fluorescence, including pH, after the conjugation procedure with BSA. Their results showed that the maximum relative fluorescence intensity (RFI) occurred at pH 6.83, while for lower pH the values of the RFI decreases. The authors described that this decay is also probably caused by depassivation, where the protonation of the surface-binding thiolates results in the deconstruction of the Cd\(^{2+}\)-TGA complexes. This prior study corroborates with our results because we employed pH 6.0 in the covalent bioconjugation process (with EDC plus Sulfo-NHS) and obtained a small decrease in the intensity of the emission spectra when compared to the bare QDs.

As it can be observed in Table I, a value of 325% for the relative intensity demonstrated a more effective conjugation for QDs-MSA-(anti-A), while for QDs-MPA-(anti-A) it was observed a value of 164%. The flow cytometry analysis corroborated the results obtained using the Plate Reader. Figure 5 shows the labeling of approximately 60% of A erythrocytes by QDs-MSA-(anti-A), in contrast to around 30% for QDs-MPA-(anti-A). Erythrocytes type O did not present fluorescence, confirming the specific labeling (Fig. 5(2)). These results indicate that, when the properties of proteins are preserved, a greater signal obtained by our method can be related to a more efficient bioconjugation process. In fact, we believe that the flow cytometry and the Fluorescence Plate Reader approach are complementary. The analysis with the Plate Reader indicates if the bioconjugation of proteins to QDs was efficient, or not, and the flow cytometry results can be used as a contra proof to validate this methodology of evaluation of the bioconjugation. As higher is the signal observed in the Plate Reader, the more efficient is the bioconjugation procedure and a higher number of labeled cells is expected to be detected by flow cytometer.

The flow cytometry results were performed more than five times and were reproducible. The analysis showed that we could label specifically only A erythrocytes. Although these results were reproducible, 100% of the cells were not labeled due to the conjugation process used. The adsorption is driven by electrostatic forces and may not be totally effective because it does not promote a connection among all antibodies to all QDs of the sample, so some QDs are not covered by antibodies and bare QDs do not label RBCs. The labeling of cells incubated with MSA-QDs was...
more efficient than the labeling of cells incubated with MPA-QDs, probably because the MSA agent presents two free carboxyl groups promoting a better interaction with biomolecules, while MPA has only one carboxyl group.

4. CONCLUSION

The alternative Plate Reader bioconjugation method here proposed was successfully tested and confirmed by flow cytometry and spectroscopic analysis. This bioconjugation detection method consists in a fast semi-quantitative analysis showing sensitivity and reproducibility. By using this method it is possible to analyze multiple types of samples (QDs associated to different proteins or QDs associated to molecules by different conditions such as pH and stoichiometric proportion) at the same time. It is an alternative bioconjugation detection method for researchers especially working in the optical field. Moreover, small amount of reagents and samples are required, taking advantage of the native fluorescence of QDs. Therefore, this method could be used as a complementary approach to analyze QD-protein bioconjugation using microplates wells with Fluorescence Plate Reader. Additionally, we limited this optical method in this work to QD-protein bioconjugate detection, but the same idea shows a great potential to be extrapolated for other fluorophores and for other kind of biomolecules, including uncharged biomolecules as well as uncharged pegylated QDs, which are hard to detect by standard non optical methods.

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References and Notes


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