USING QD-FRET BASED METHOD TO INVESTIGATE PROTEIN-PROTEIN INTERACTIONS

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Abstract

Förster resonance energy transfer (FRET) is an energy transfer process between a pair of light-sensitive molecules, where the donor fluorophore (initially in its electronic excited state) transfers energy to an acceptor chromophore. In this study, FRET was used to investigate the dimerization of metallothionein isoform MT-1A. The FRET system was developed based on a fluorescent quantum dots (QDs) and cyanine dye 3.5 (Cy3.5) as a versatile tool to probe small distance changes between acceptor and donor fluorophores in nanometer range. Herein, the water-soluble 450-nm emitting QDs as the donor and 590-nm Cy3.5 as the acceptor were covalently conjugated with MT-1 according to the protocol. Previous studies suggest that MTs may form oligomers under certain conditions. Therefore, further studies of this phenomenon, which has been studied here using capillary electrophoresis (CE) coupled with fluorescence detection.

Keywords: Förster resonance energy transfer (FRET), quantum dots (QDs), Oligomerization, Capillary electrophoresis, Metallothionein

1. INTRODUCTION

Optical biosensors often take advantage of fluorescence resonance energy transfer (FRET) which has been used in carefully designed sensing systems for proteins, peptides, nucleic acids and small molecules. FRET is a photophysical mechanism particularly useful to probe the dynamics of the formation of recognition complexes. This phenomenon involves dipole−dipole interactions between a donor−acceptor pair (Figure 1). The process is sensitive to the distance between the FRET partners, and thus provides a versatile spectroscopic tool to monitor events occurring between moieties at intimate contact [1,2]. QDs and organic fluorophores such as cyanine dyes are widely exploited as FRET probes for numerous chemical and biological applications such as protein folding investigation or for sensing and imaging of macromolecular interactions. Quantum dots (QDs) with the exceptional fluorescent properties are excellent FRET donors. In other words, wavelengths optimal for excitation of QDs are far apart from the excitation maxima of acceptors, thereby avoiding their direct excitation [3-5]. Metallothioneins (MTs) are a family of small (6–7 kDa) proteins with cysteine-rich (30%) structure and high affinity for metal ions [6,7]. Due to this reason MTs are involved in the metabolism of heavy metals and they serve as an intracellular reservoir of essential
trace elements. Moreover, due to the high amount of disulphide bonds, MTs can create dimers or higher oligomers, which can be formed either in oxidative (e.g. presence of NO or hydrogen peroxide) or non-oxidative conditions (addition of excess of Cd\textsuperscript{2+}). Investigation of their structural arrangement can help understand the development of oxidative stress and the mechanism of transport of toxic metals. Moreover, the oligomer formation (as well as their structures) may play an important role in a number of neurological disorders such as Alzheimer disease and amyotrophic lateral sclerosis [7].

In the present work, the interactions between metallothioneins covalently conjugated with QDs and Cy 3.5 were investigated using CE coupled with fluorescence detector and fluorescence spectroscopy as well.

2. MATERIALS AND METHODS

2.1. Materials and reagents

MT isoform (MT-1) from rabbit liver was obtained from Enzo Life Science, USA. Sodium borate, zinc acetate, cadmium acetate, sodium phosphate, mercaptosuccinic acid (MSA), isopropanol, N-Ethyl-N’-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS) and commercial Cyanine 3.5 dye (Cy3.5) labelling kit were purchased from Sigma-Aldrich in ACS quality. The stock solution of MT (1 mg/mL) was prepared in ultrapure water and stored in the dark at -20 °C. Ultrapure water purified by Milli-Q system was used for preparation of all solutions.

2.2. Preparation of QDs

Suspension of ZnCd QDs was prepared by mixing stock solutions of the 6 mM zinc acetate, 6 mM cadmium acetate, 20 mM sodium phosphate buffer (PB) pH 7 and 16 mM mercaptosuccinic acid (MSA). The resulting mixture with the typical molar ratio of 1:4:4:6 (Cd\textsuperscript{2+}:MSA:PB:Zn\textsuperscript{2+}) was exposed to UV irradiation for 5 minutes. The ZnCd QDs were precipitated by isopropanol and then isolated by centrifugation. Finally, ZnCd QDs were suspended in sodium phosphate buffer (PB, pH 7.2) and sonicated for 2 minutes before use.

2.3. QDs and cyanine dye bioconjugation

At first, conjugation of ZnCd QDs with 0.1 mM MT-1 was carried out by a method using coupling agents EDC and Sulfo-NHS according to the protocol published elsewhere [8]. Conjugation of Cy3.5 with MT-1 was carried out according to the manufacturer’s protocol.

2.4. MT dimerization

Dimers (or higher oligomers) of MTs were obtained by storing MT-1 (0.1 mM) in 20 mM PB (pH 9.2) in presence of 0.5 mM Cd\textsuperscript{2+} at 8 °C under aerobic conditions for 1 week.

2.5. Capillary electrophoresis

Monitoring of FRET signal between MTs conjugates was performed using a Beckman Coulter CE instrument (P/ACE MDQ, USA) equipped with a light emitting diode (375 nm) as an excitation source and a long pass emission filter (550 nm). Separations were performed in an uncoated fused-silica capillary (Polymicro Technologies, USA) with an internal diameter of 75 µm and external diameter of 375 µm. The total length of the capillary was 48 cm and the effective length to the detection window was 37.5 cm. The capillary was flushed with 0.1 M NaOH for 5 min and with background electrolyte (sodium borate buffer, pH 9) for 15 min prior to the first use. Before each run, the capillary was rinsed for 120 s with background electrolyte (BGE). Sample was injected hydrodynamically by pressure of 1 psi for 4 s. Separation voltage was 20 kV.
2.6. Fluorescence analysis

Samples of MTs conjugates were pipetted (100 µL) in to the well plate and emission spectra were recorded ($\lambda_{\text{Ex}} = 375$ nm and $\lambda_{\text{Em}} = 405 - 800$ nm). Fluorescence signal was acquired by multifunctional microplate reader Tecan Infinite 200 M PRO (TECAN, Switzerland). The samples (100 µL) were placed in UV-transparent 96 well plate with flat bottom by CoStar (Corning, USA). Then excitation or emission spectra were recorded using 2-nm steps and gain 100.

3. RESULTS AND DISCUSSION

Fluorescence-based detection techniques are highly selective and very sensitive with high potential for multiplexing. Moreover, FRET enables the measurements of distances at the molecular level. Thus, this technique was selected for detection of protein-protein interactions. The selection of molecules with optimal optical properties for the FRET pair is crucial. Therefore, firstly, the selection of the FRET probes (donor and acceptor fluorophores) was optimized. Resonance energy transfer is highly dependent on several factors and one of the most important of them is the spectral overlap of the donor emission spectrum and the acceptor absorption spectrum. The combination of cyanine dye (Cy3.5) and quantum dots (QDs) was tested and as can be seen in Figure 2, the optimal combination providing the satisfying spectral overlap was selected. Metallothionein isoform MT-1 was used as a model biomolecule for protein interaction.

![Figure 2](image)

**Figure 2** Spectral overlap between donor (QDs) and acceptor (Cy3.5) which is crucial for the FRET

The capillary electrophoresis method was used to demonstrate the FRET signal which was observed after interactions of MTs. The aliquots of MT-1 protein were fluorescently labelled (each with one member of the FRET pair – Cy3.5 and QDs). In case of MTs interactions (dimerization or oligomerization), the FRET occurred due to the proximity of fluorophores. On the other hand, if there are no interactions between MT-1, the distance of the fluorophores is larger than required and therefore, the FRET signal is not observed. QDs nanoparticles developed in our laboratory were tested as a suitable donor fluorophore for the FRET pair. The emission wavelength could be easily tuned by duration of irradiation.

Conjugates of Cy3.5MT-1, QDsMT-1 and Cy3.5MT-1QDsMT-1 were studied by capillary electrophoresis and their electropherograms are shown in Figure 3. It can be seen clearly that Cy3.5MT-1 provides a weak signal (green trace), which is due to the excitation source 375 nm (Cy3.5 optimal excitation wavelength is 570 nm). The conjugate of QDsMT-1 provides a weak signal as well (red trace), even though the instrumental setting
(emission filter 550 longpass) should disable the detection of the QDs fluorescence (emission maximum of QDs is 450 nm). Probably, these weak signals were caused by broad emission spectra of QDs and Cy3.5 and were considered as background signal. After interaction between the conjugates Cy3.5MT-1 and QDsMT-1 took place, a FRET signal occurred between Cy3.5 and QD and therefore, a FRET signal was obtained (beige trace).

**Figure 3** Electropherogram of QDsMT-1, Cy3.5MT-1 conjugates and their conjugate

In addition, regarding to previous research, metallothioneins can create oligomers under specific conditions (temperature, addition of Cd²⁺ etc.). A solution of MT-1 was stored under aerobic conditions at 8 °C for 1 week with addition of Cd²⁺. By this we confirmed that the MTs oligomerization was increased after Cd²⁺ addition and metal bridges between MTs were formed. Because of metal bridges cause a tighter connection between metallothioneins, stronger FRET signal was observed. This fact was verified by fluorescence spectroscopy (**Figure 4A**) and CE (**Figure 4B**).

**Figure 4** (A) Emission spectra of Cy3.5MT-1QDsMT-1 without and with the addition of Cd(II) and (B) electropherograms of these conjugates

4. **CONCLUSION**

In conclusion, we optimized a method to study protein interactions based on QDs and Cy3.5 FRET pair. Metallothionein isoform MT-1 was used as a model protein. The FRET signal was studied not only by fluorescence spectrometry, but also by CE with fluorescence detection. The main benefit of CE over fluorescence spectrometry is the fact, that in CE only complexes exhibiting stronger interaction are visualized.
In during fluorescence spectroscopic analysis, FRET occurring between fluorophores in close proximity even without any chemical interaction can be detected.

In case of MT oligomerization it should be highlighted that the role of MTs oligomerization in organism is unclear and it is important to note that insufficient literature records are available. However, oligomerization of MTs might play an important role in signal pathways. Therefore, further investigation is needed.

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