FRET as a powerful tool to study protein dimerization

Kristyna Pavelicova, Lukas Nejdl, Lucie P. Vanickova, Mirek Macka, Marketa Vaculovicova
Department of Chemistry and Biochemistry
Mendel University in Brno
Zemedelska 1, 613 00 Brno
CZECH REPUBLIC

xpavelic@node.mendelu.cz

Abstract: Herein, Förster resonance energy transfer (FRET) was used to investigate the oligomerization of mammalian metallothionein (MT) isoform MT-1. A FRET system was developed based on a highly fluorescent ZnCd quantum dot (QD) and cyanine 3 (Cy3) as a powerful tool to probe small distance changes between acceptor and donor fluorophores in nanometer range. In this study, the water-soluble 450-nm emitting ZnCd QDs as donor and 570-nm Cy3 as acceptor were covalently conjugated with MT-1. Metallothionein MT1 forms dimers (as well as higher oligomers) upon storing under aerobic conditions. These dimers/oligomers were investigated using capillary electrophoresis (CE) coupled with fluorescence detection.

Key Words: FRET, metallothioneins, oligomerization, capillary electrophoresis, quantum dots

INTRODUCTION

Fluorescence resonance energy transfer (FRET) is a non-invasive powerful tool where radiationless energy transfer occurs from an excited donor to an acceptor resulting from dipole-dipole interaction between the donor and the acceptor systems. Only in case the donor and the acceptor moieties are in a close proximity, efficient transfer of energy takes place (Jain 2012, Stanisavljevic et al. 2015). 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. Moreover, it has been demonstrated to have a strong potential for optical data storage, biosensing, development of multicolour fluorescent probes, structural analysis, study of interactions between molecules, and in detection of genes (Saha et al. 2018, Buckhout-White et al. 2014, Li et al. 2010).

In the presented work, a FRET system was used for investigation of metallothionein dimerization. The process was monitored by capillary electrophoresis (CE) coupled with light-emitting diode-induced fluorescence detection.

Metallothioneins (MTs) are a group of small (6–7 kDa) non-enzymatic proteins with cysteine-rich (30%) structure and high affinity to metal ions (Sanz-Nebot et al. 2003, Zangger et al. 2001). Due to the disulphide bonds, MTs can create dimers (and/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^{2+}). Investigation of their structural arrangement can help understand the development of oxidative stress and 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 (Zangger et al. 2001).

MATERIAL AND METHODS

Materials and reagents

MT isoform (MT-1) from rabbit liver was obtained from Enzo Life Science, USA. Commercial Cyanine 3 dye (Cy3) labelling kit, sodium borate, zinc acetate, cadmium acetate, sodium phosphate, mercaptosuccinic acid (MSA), isopropanol, *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS) 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.

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^{2+}:MSA:PB:Zn^{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 subsequently, the solution was sonicated for 2 minutes before use.

QDs and Cy3 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 (Pereira and Lai 2008). Conjugation of Cy3 with MT-1 was carried out according to the manufacturer's protocol.

MT dimerization

Dimers (or higher oligomers) of MTs were obtained by storing QDsMT and Cy3MT mixture (0.1 mM each mixed in 1:1 ratio) in 20 mM PB (pH 9.3) in presence of 0.5 mM Cd^{2+}, at 8 °C under aerobic conditions for 1 week.

Fluorescence spectrometric analysis

Conjugate solutions (Cy3MT, QDsMT and Cy3MTQDsMT) were pipetted (100 µL) into the well plate (UV-transparent 96 well plate with flat bottom by CoStar (Corning, USA)) and emission spectra were recorded (λ_{Ex} = 390 nm and λ_{Em} = 420–750 nm). Fluorescence signal was acquired by multifunctional microplate reader Tecan Infinite 200 M PRO (TECAN, Switzerland). Then excitation or emission spectra were recorded using 2-nm steps and gain 100.

CE analysis

Monitoring of FRET signal between MT conjugates was performed using a Beckman Coulter CE instrument (P/ACE 5500, USA) equipped with a light emitting diode (390 nm) as an excitation source and a band pass emission filter (607 ± 20 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 46.5 cm and the effective length to the detection window was 36 cm. The capillary was flushed with 0.1 M NaOH for 5 min and with background electrolyte (sodium borate buffer, pH 9.3) 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 15 kV.

MALDI-TOF analysis

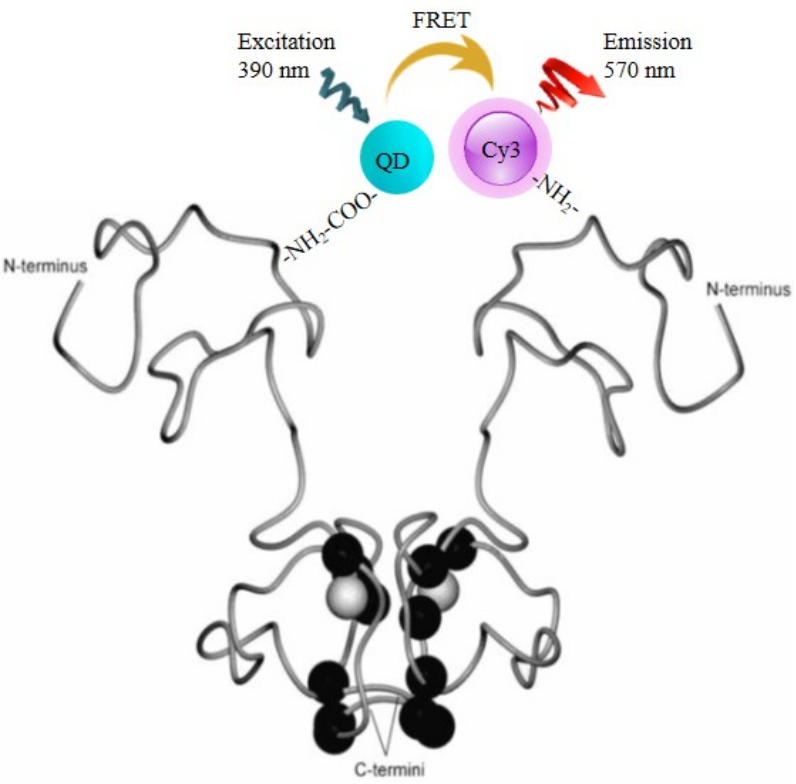
MT-1 was analysed using MALDI-TOF-MS (ultrafleXtreme instrument, Bruker Daltonik GmbH, Bremen Germany) equipped with a laser (operating at wavelength of 355 nm with an accelerating voltage of 25 kV, a maximum energy of 43.2 µJ, and a repetition rate of 2000 Hz) in linear positive ion mode. Organic matrix - namely 2,5-Dihydroxybenzoic acid (DHB) - was chosen. Matrix (1 µL) was applied on the prepared sample of MT-1 with Cd^{2+} (probably dimers or higher oligomers) and dried under atmospheric pressure and ambient temperature (25 °C). The laser frequency was set to 1000 Hz and laser energy was optimized prior to each measurement. External calibration using a protein standard mixture I and II (Bruker Daltonics, Bremen, Germany) was applied in the range of m/z 1–30 kDa. A total of 3000 spectra were summed for each spot using the Random Walk raster pattern, with no evaluation criteria and were analysed with the Flex Analysis software (Version 3.4).

RESULTS AND DISCUSSION

Fluorescence-based detection offers the advantage of low-cost and high resolution and sensitivity. Generally, fluorescence-based detection techniques are very sensitive as well as highly selective

with high potential for multiplexing. Moreover, Förster resonance energy transfer enables the measurements of distances at the molecular level. Therefore, this technique was selected for detection of protein dimerization. The selection of probes for the FRET pair is crucial. Therefore, at first, the selection of the FRET pair (donor and acceptor fluorophores) with optimal optical properties was optimized. The combination of organic dye (Cy3) and quantum dots was tested and the optimal combination providing the highest signal was selected. Mammalian metallothionein isoform MT-1 was used as a model biomolecule for protein dimerization (Figure 1).

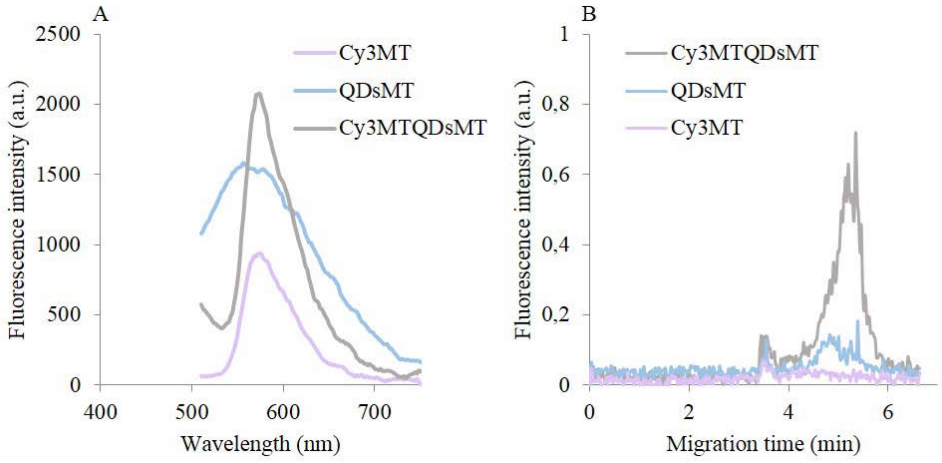
Figure 1 Schematic representation of FRET analysis of non-oxidative dimerization of MTs (labelled by the Cy3 and QD) adapted from (Zangger et al. 2001)



Two aliquots of MT-1 protein were fluorescently labelled (each by one member of the FRET pair – Cy3 and QD). In case of MTs oligomerization, the FRET will occur (ON state) due to the proximity of fluorophores. On the other hand, if oligomerization does not occur, the distance of the fluorophores is larger than required, the FRET signal is not observed (OFF state). QD nanoparticles developed in our laboratory will be tested as suitable donor fluorophore for the FRET pair. These QD nanoparticles are semiconductor nanocrystals prepared by UV irradiation. The emission wavelength can be easily tuned by duration of irradiation. The characterization of fluorescence properties of QDsMT, Cy3MT, and Cy3MTQDsMT conjugates was carried out by fluorescence spectrometry (Figure 2A) and then, the conjugates were studied by CE (Figure 2B).

As seen in Figure 2, the FRET signal was observed for Cy3MTQDsMT conjugate. Therefore, typical emission spectra were acquired (Figure 2A). Subsequently, Cy3MT, QDsMT and Cy3MTQDsMT were studied by capillary electrophoresis and their electropherograms are shown in Figure 2B. It can be seen clearly that Cy3MT provides no observable signal (pink trace), which is due to the excitation source 390 nm (Cy3 optimal excitation wavelength is 550 nm). In contrast, QDsMT provides a weak signal (blue trace), even though the instrumental setting (emission filter 607 bandpass) should disable the detection of the QDs fluorescence (emission maximum of QDs is 450 nm). This weak signal is caused by broad emission spectra of QDs and is considered as background signal. After interaction between the conjugates Cy3MT and QDsMT took place, a FRET signal occurred between Cy3 and QD and therefore, a FRET signal was obtained (grey trace).

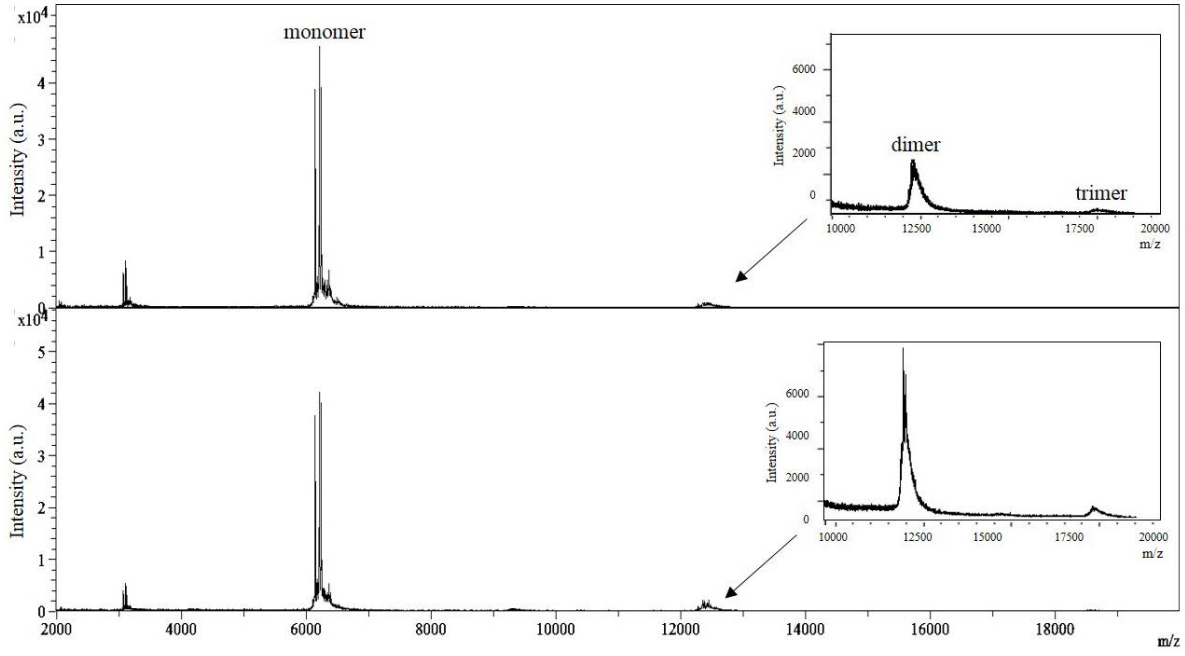
Figure 2 (A) emission spectra of Cy3MT, QDsMT and Cy3MTQDsMT conjugates (A), and electropherograms of these conjugates (B)



In order to verify the dimer (oligomer) formation, MALDI-TOF-MS analysis of MTs after addition of Cd^{2+} was performed. As can be seen in Figure 3, the signal intensity increased significantly in MT sample with Cd^{2+} addition after 1 week-storage at 8 °C under aerobic conditions.

From our results follows that non-oxidative dimerization caused by Cd^{2+} addition occurred and metal bridges were formed. Moreover, we believe that the FRET method may allow to suggest the structure of MT dimers/oligomers. The exact role of MT dimerization is unclear and it is important to note that insufficient literature records are available. Therefore, further investigation is needed. However, dimerization of MT might well play an important role in signal pathways.

Figure 3 MALDI-TOF-MS spectra of MTs. Sample of MTs after Cd^{2+} addition analysed immediately (top) and after 1 week-storage (bottom).



CONCLUSION

In conclusion, we developed a method to study of protein dimerization based on QD and Cy3 FRET pair. MT was used as a model protein. The FRET signal was studied not only by fluorescence spectrometry, but also by CE with fluorescence detection. Analysis based on a combination of capillary electrophoresis with Förster resonance energy transfer could be a very useful tool for many investigations due to low cost and ease of use.

ACKNOWLEDGEMENTS
The research was financially supported by IGA grant, no. IP_025/2019.

REFERENCES

Buckhout-White, S. et al. 2014. Assembling programmable FRET-based photonic networks using designer DNA scaffolds. Nature Communications, 5: 5615.

Jain, K.K. 2012. The handbook of nanomedicine. New York: Humana Press.

Li, Y.Q. et al. 2010. High-sensitivity quantum dot-based fluorescence resonance energy transfer bioanalysis by capillary electrophoresis. Biosensors and Bioelectronics, 25(6): 1283–1289.

Pereira, M., Lai, E.P.C. 2008. Capillary Electrophoresis for the Characterization of Quantum Dots After Non-Selective or Selective Bioconjugation with Antibodies for Immunoassay. Journal of Nanobiotechnology, 6: 10.

Saha, S. et al. 2018. Evidence of homo-FRET in quantum dot–dye heterostructured assembly. Physical Chemistry Chemical Physics, 20(14): 9523–9535.

Sanz-Nebot, V. et al. 2003. Characterization of metallothionein isoforms from rabbit liver by liquid chromatography coupled to electrospray mass spectrometry. Journal of Chromatography B, 796(2): 379–393.

Stanisavljevic, M. et al. 2015. Quantum dots fluorescence resonance energy transfer-based nanosensors and their application. Biosensors and Bioelectronics, 74: 562–574.

Zangger, K. et al. 2001. Oxidative dimerization in metallothionein is a result of intermolecular disulphide bonds between cysteines in the alpha-domain. Biochemical Journal, 359: 353–360.