

NANOPARTICLE-BASED FRET PAIR SELECTION FOR INVESTIGATION OF METALLOTHIONEIN DIMERIZATION

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Abstract

Metalloproteins (MTs) are one of the most diverse classes of proteins, with the intrinsic metal atoms providing a catalytic, regulatory and structural role crucial to protein functioning. When stored under aerobic conditions, MTs might form dimers (as well as higher oligomers) that play an essential role as mediators in oxidoreduction signalling pathways. In this work, we explore the non-oxidative dimerization process characterized by metal (Cd) bridging of MT monomers by Förster resonance energy transfer. The formation of MT dimers/oligomers was investigated by applying capillary electrophoresis and matrix-assisted laser desorption/ionization with mass spectrometric detection.

Keywords: Metallothionein, dimerization, capillary electrophoresis, Förster resonance energy transfer

1. INTRODUCTION

Metallothioneins (MTs) are a family of low molecular weight proteins (6-7 kDa). MTs are involved in the metabolism of heavy metals and they serve as an intracellular reservoir of essential trace elements. In mammals they occur in four isoforms (MT-1 to MT-4) that vary in expression and localization in individual tissues. Each molecule consists of a single polypeptide chain of 61-68 amino acid residues with high cysteine content (30 %). The sulfhydryl group of cysteine express the most notable functional potential. It can form disulphide bonds by which MTs can create oligomers. Oligomers can be formed either under oxidative (e.g. presence of NO or hydrogen peroxide) or non-oxidative conditions (addition of excess of Cd²⁺). Investigation of their structural arrangement can help understand the development of oxidative stress and mechanism of transport of toxic metals. In addition, the oligomer formation may play an important role in a number of neurological disorders such as Alzheimer's or Parkinson's diseases [4,5]. Due to the MT characteristics, such as low molecular weight, unique primary structure with lack of aromatic amino acids, and numerous isoform occurrence analysis of MTs represent a demanding task in terms of satisfactory sensitivity and specificity. In addition to traditional analytical techniques, such as gel electrophoresis and liquid chromatography, capillary electrophoresis (CE) has proven to be a powerful separation method for MT analysis. CE belongs to those few analytical methods enabling the separation of single MT isoforms and their sensitive detection [1-3]. Matrix-assisted laser desorption/ionization with mass spectrometric detection (MALDI MS) represents another effective tool for identification of the MT isoforms and/or MT oligomers.

Förster resonance energy transfer (FRET) is defined as a "long-range" dipole interaction between probes (the donor and the acceptor system) through resonance dipole-dipole coupling occurring in the pico- to nanometer range and thus at a scale relevant to changes in protein conformation [6]. Organic fluorophores such as cyanine dyes and quantum dots (QDs) 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 [7,8].

In the present work, the conjugation of MTs with QDs was investigated using CE coupled with absorbance detector and MALDI MS technique. We propose that the conjugation of protein to QDs could efficiently employed in FRET system which may subsequently serve for efficient investigation of MTs oligomerization.

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) 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.

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 (Cd²⁺:MSA:PB:Zn²⁺) 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 bioconjugation

At first, conjugation of ZnCd QDs with 0.2 mM MT-1 was carried out by a method using coupling agents EDC and Sulfo-NHS according to the protocol published elsewhere [9].

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 different volumes (0.5, 1 and 1.5 μL) 0.5 mM Cd²⁺ at 8 °C under aerobic conditions for 1 week.

2.5. Capillary electrophoresis

Analyses of MTs conjugates were performed using CE 7100 (Agilent technologies, Waldbronn, Germany) equipped with UV/Vis absorbance detector. Fused silica capillary with an internal diameter of 75 μm, total length of 64.5 cm and effective length of 56 cm was used. The capillary was flushed with background electrolyte (BGE) for 15 minutes prior to the first use. The capillary was rinsed for 120 s with background electrolyte before each run to obtain stable measurements. Sample was injected hydrodynamically by pressure of 50 mbar applied for 4 s. Separation voltage was set to 20 kV. Sodium borate buffer (20 mM, pH 9.2) was used as a BGE. The signal was registered at 254 nm.

The method of "short-end injection" [10] was also introduced to be able to visualize the protein dimers/oligomers. Samples were injected at the end of the capillary near to the detector. Effective length of the capillary was 8.5 cm. This was achieved by injecting the sample at the outlet end of the capillary (short-end injection), the capillary was air conditioned to temperature of 25 °C.

2.6. MALDI-MS

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 - 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 $^{\circ}\text{C}$). 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).

3. RESULTS AND DISCUSSION

A solution of metallothionein isoform MT-1 at millimolar concentrations stored under aerobic conditions at 8 $^{\circ}\text{C}$ per 1 week produces a new species characterized here as dimers and/or higher oligomers. Here we confirmed that the environment has a great influence on the oxidation rate. Non-oxidative dimerization caused by Cd^{2+} addition occurred and metal bridges were formed. Samples of MTs with Cd^{2+} were analysed by CE. The obtained electropherograms are shown in **Figure 1**, where distinct changes in the signal intensities are observed. These changes suggest that the Cd^{2+} addition causes significant changes in the MTs structure.

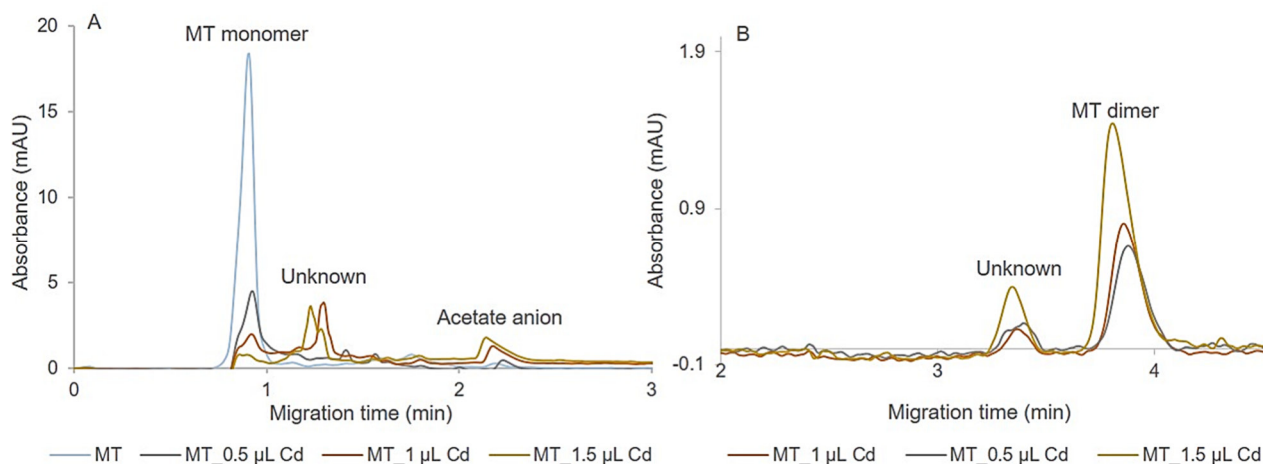


Figure 1 Electropherograms of metallothionein with different volumes (0.5, 1 and 1.5 μL) of 0.05 mM Cd^{2+} , at positive polarity (A) and negative polarity (B) by short-end injection.

The short end injection method was used to demonstrate the dimerization of MT-1. As shown in the **Figure 1A**, with the increased amount of Cd^{2+} , the peak of MT-1 monomer with migration time of 0.9 minute decreased (**Figure 1A**), while the peak of MT-1 dimer with migration time 3.8 minutes increased (**Figure 1B**). Based on these results we hypothesise that the highest volume of Cd^{2+} (1.5 μL 0.05 mM Cd^{2+}) directly affects the formation of metal bridges in the investigated protein MT-1 causing a subsequent dimerization of MTs. MALDI-TOF-MS analysis of the identical samples confirmed the formation of dimers increased after addition of 1.5 μL of 0.05 mM Cd^{2+} to the MT-1 sample and one-week storage at the 8 $^{\circ}\text{C}$ under aerobic conditions (**Figure 2**).

Even though the CE and MALDI-TOF-MS were able to detect and determine the formation of MT dimers/oligomers, these methods do not provide any information about the structure of the formed species. For this reason, we suggest the use of FRET technique for following investigation. At first the FRET pair composed of ZnCd QD and cyanine dye was proposed.

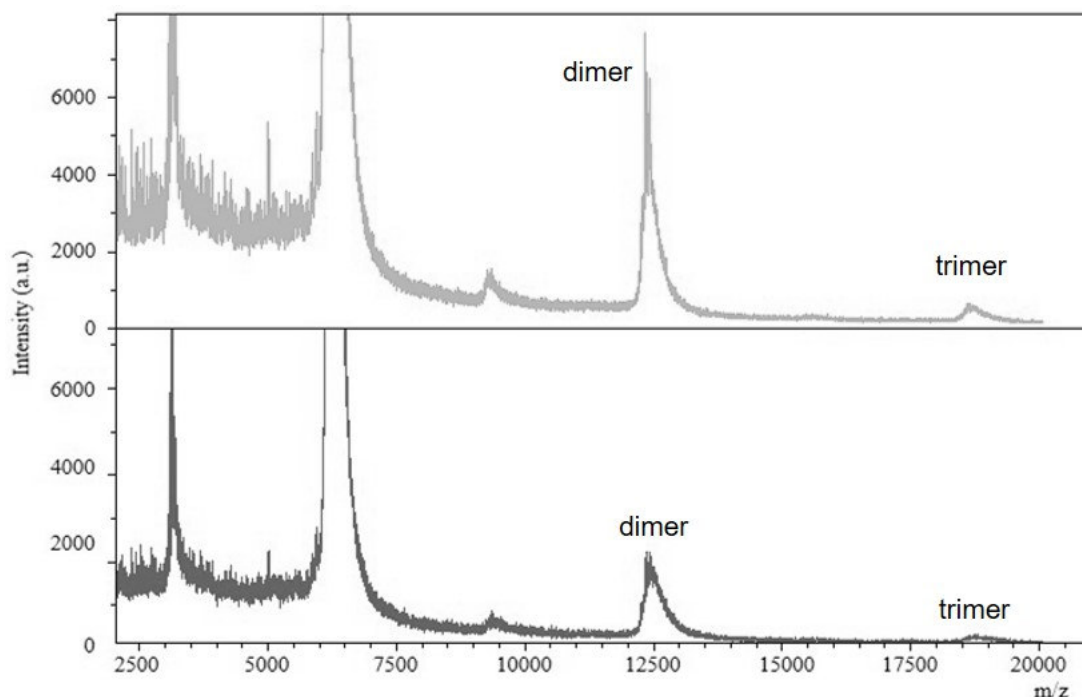


Figure 2 MALDI-TOF-MS spectra of MTs. Sample of MTs without Cd²⁺ addition analysed immediately (bottom) and sample of MTs with Cd²⁺ addition after 1 week-storage (top)

At first, the conjugation of MTs with quantum dots (QDs) was studied in order to evaluate the ability of the FRET system to indicate the structure of MT dimers or oligomers. However, the short-end injection method did not provide sufficient separation resolution. Therefore CE method with the effective length of the capillary of 56 cm was used resulting in successful separation of MT, QDs and their conjugates (**Figure 3**). The conjugation of MTs with organic fluorophores will be subject of further research aiming to study a protein oligomerization by FRET.

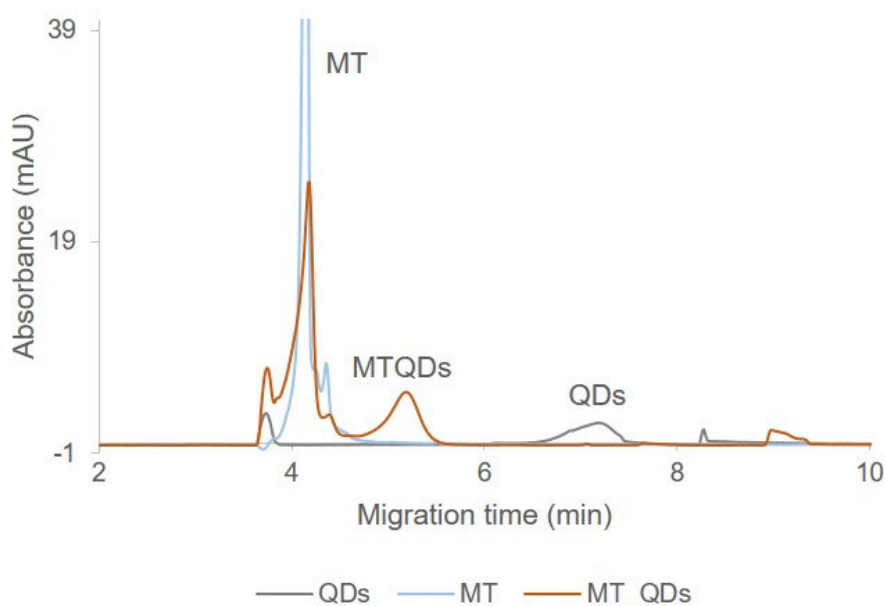


Figure 3 Electropherograms of protein (MT-1) conjugation to quantum dots

CONCLUSION

Metallothionein (MT) may form dimers (as well as higher oligomers) upon storage under aerobic conditions. This dimerization may play an essential role as a mediator in oxidoreduction signalling pathways. In this study, the oligomerization of MT was investigated using capillary electrophoresis coupled to absorbance detection and mass spectrometry. Dimers/higher oligomers of MT were studied by a CE and verified by a MALDI-TOF-MS analysis after Cd²⁺ addition and 1 week-storage. The method of protein conjugation to QDs was employed in order to investigate the FRET system applicability for structural characterization of protein dimerization. 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 and monitor distance changes in nanometer range. This phenomenon is highly dependent on the distance between acceptor and donor fluorophores. In conclusion, analysis based on a combination of CE FRET could be a very useful tool for study of protein oligomerization due to low cost and ease of use.

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