Biosensors and Bioelectronics 32 (2012) 148–154

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

The homogeneous fluorescence anisotropic sensing of salivary lysozyme using the 6-carboxyfluorescein-labeled DNA aptamer

Mingjian Zou, Yang Chen, Xiao Xu, Hongduan Huang, Feng Liu, Na Li*

Beijing National Laboratory for Molecular Sciences (BNLMS), Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, Institute of Analytical Chemistry, College of Chemistry and Molecular Engineering, Peking University, Beijing, 100871, China

ARTICLE INFO

Article history:
Received 31 August 2011
Received in revised form
26 November 2011
Accepted 28 November 2011
Available online 21 December 2011

Keywords:
Fluorescence anisotropy
6-Carboxyfluorescein (FAM)
Lysozyme
DNA aptamer
Dynamic fluorescence anisotropy
Lifetime

ABSTRACT

A simple and sensitive fluorescence anisotropy method was developed for lysozyme, employing the coupling of fluorophore, 6-carboxyfluorescein (FAM), with lysozyme upon recognition between the target molecule and its DNA aptamer. It was found in this study that the rotational dynamic of the detecting system is crucial to obtain a high anisotropy signal that cannot always be achieved by simply increasing the molecular volume, because molecular volume increase may not be able to efficiently retard the rotational movement of the fluorophore. FAM was selected as the label of the ssDNA aptamer to effectively facilitate the change of the fluorophore from a primarily independent segmental movement to slow global rotation. The time-resolved measurements, including lifetime and dynamic fluorescence anisotropy, were conducted to study the recognition interaction and to better understand the methodology. The proposed method had a wide linear dynamic range of 12.5–300 nM and a high sensitivity with the limit of detection of 4.9 nM (3S/N). This proposed method was successfully applied to assay of human salivary lysozyme. The results based on the standard addition recovery and comparison with enzyme-linked immunosorbent assay (ELISA) demonstrated the feasibility of this method for biological samples. Using coupling between the fluorophore and the analyte can be one of the approaches working toward expanding the application of fluorescence anisotropy based on aptamer–target and antibody–antigen recognitions.

1. Introduction

Fluorescence anisotropy based on recognition reaction is a method that requires no separation of the reagent blank from the test media. Thus, fluorescence anisotropy can be used for homogeneous analysis and has the element for automation with high-throughput capability, providing a powerful tool for drug discovery, biochemical research, clinical diagnoses, and food analyses (Goulko et al., 2009; Gradinaru et al., 2010; Jameson and Ross, 2010; Lakowicz, 2006; Ruta et al., 2009). However, in the determination of macromolecules such as proteins using antibodies for recognition, fluorescence anisotropic is not generally feasible because only small anisotropy changes can be observed (Gokulrangan et al., 2005). In vitro selected DNA- and RNA-based aptamers have been used as recognition elements for numerous applications in biosensor development (Goulko et al., 2009; Hamula et al., 2006; Song et al., 2008). Aptamers are especially advantageous for fluorescence anisotropy determination of proteins because they are generally small compared to the proteins (Gokulrangan et al., 2005); therefore a significant anisotropy change will be observed when an aptamer labeled with a fluorescent dye binds to the target molecule such as a protein. In addition, aptamers are readily available at low cost, have less batch-to-batch variability and reasonable storage characteristics due to their resistance to denaturation and degradation compared to antibodies (Tombelli et al., 2005). Therefore, nucleic acid aptamers provide opportunities for homogeneous assays of proteins (Cai et al., 2002; Chen et al., 1999; Fang et al., 2001; Gokulrangan et al., 2005; Kumke et al., 1995; Li et al., 2007; McCauley et al., 2003; Potyrailo et al., 1998) as well as other entities including small molecules (Perrier et al., 2010; Ruta et al., 2009) and cancer cells (Deng et al., 2010) based fluorescence anisotropy measurements.

Saliva has been increasingly recognized as a biological fluid that can be easily collected in a non-invasive manner for the detection of diagnostic and prognostic biomarkers. Lysozyme is an important defense molecule of the innate immune system and one of the major proteins in saliva with the level varying in the range of 1.4–28 μM (Perera et al., 1997; Virella et al., 1978). Lysozyme is also a clinically important protein, since its level increases in monocytic and monomyelocytic leukemia, tuberculosis, acute bacterial infections, and monocytosis with inflammatory bowel disease (Perera et al., 1997); therefore, the qualitative and quantitative determination of salivary lysozyme has been useful in the diagnosis, treatment and monitoring the progression of such diseases. In addition to

* Corresponding author. Tel.: +86 10 62761187; fax: +86 10 62751708.
E-mail address: lina@pku.edu.cn (N. Li).

0956-5663/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2011.11.052

traditional methods, such as turbidimetric detection (Morsky and Aine, 1983), lysoplate assay (Gupta et al., 1987) and lysorocket electrophoresis (Virella, 1977), immunoassay and enzyme-linked immunosorbent assay (ELISA) have been widely used for lysozyme assay (Gao et al., 1995; Rauch et al., 1995; Sato et al., 2001; Schneider et al., 2010). Since Ellington successfully obtained the RNA lysozyme-binding aptamer (Cox and Ellington, 2001), this RNA (Cho et al., 2006) and its DNA template (Cheng et al., 2007; Huang et al., 2009; Peng et al., 2009; Wang and Yu, 2010; Wang and Liu, 2009) have been used for development of lysozyme aptasensors based on fluorescence intensity (Wang and Yu, 2010; Wang et al., 2009, 2010), voltammetry (Cheng et al., 2007), electrochemiluminescence (Huang et al., 2009), and impedance measurements (Peng et al., 2009). Recently, Jeroen Lammertyn group (Tran et al., 2010) successfully selected new 40-mer DNA aptamers for lysozyme with high affinity ($K_d = 2.8 \pm 0.3$ nM) at pH 8.3 using capillary electrophoresis, which opened possibilities for the development of real DNA aptamer-based lysozyme sensor. Based on the localized surface plasmon resonance light-scattering of gold nanoparticles, we have developed an optical method for lysozyme using this DNA aptamer (Wang et al., 2011). Among the above published biosensing methods, few fully demonstrated the applicability to determination of lysozyme in collected human samples.

In this work, we aimed to combine the advantages of aptamer and homogeneous fluorescence anisotropy assay to develop a method for lysozyme. We for the first time demonstrated the feasibility of the proposed method on human salivary lysozyme in collected human samples, by evaluating standard addition recovery and comparing with ELISA. In this study, we found that the rotational dynamic of the detecting system is crucial to obtain a high anisotropy signal that cannot always be achieved by simply increasing the molecular volume, because molecular volume increase may not contribute effectively to retarding the rotational movement of the fluorophore when the movement of the fluorophore is still segmental dominated. Molecular volume increase can only be effective for anisotropy signal when the rotational movement of the formed complex is integrated with that of the fluorophore, which means the coupling between the fluorophore and the complex. To the best of our knowledge, using the coupling between fluorophore and the formed complex has not been discussed in the literature. Therefore, we carried out systematic studies, including steady-state and dynamic measurements, to explore the mechanism of the significant change of the fluorescence anisotropy signal, i.e., the coupling between 6-carboxyfluorescein (FAM) and lysozyme when FAM-labeled DNA aptamer formed complex with lysozyme. We hope that the study of coupling would provide insight into the methodology of fluorescence anisotropy and is helpful for fluorescence anisotropy method development in the future.

2. Experimental

2.1. Reagents and instrumentation

The aptamer (noted as Apt 80) selected by Jeroen Lammertyn group (Tran et al., 2010) contained a 40-base sequence for specific binding toward lysozyme and two 20-base primers at 5′- and 3′-ends, respectively. The sequence was 5′-AGCAG CACAG AGGTC AGATG GCAGG TAAGC AGGCG GCTCA CAAAA CCATT CGCAT GCGGC CCTAT GCGTG CTACC GTGAA-3′. Apt 40 had only the 40 selected bases, Apt 50 had the first 10 bases of the 5′-end primer located at the same end, and Apt 60 had the original 20 bases of the 5′-end primer. The fluorophores, 6-carboxyfluorescein or 6-carboxy-x-rhodamine (ROX), were attached to the 5′-end of the ssDNA. All the fluorophore-labeled ssDNA were synthesized and HPLC-purified by Sangon Biotech (Shanghai) Co., Ltd.

Recombinant human lysozyme (135,372 units/mg, expressed from rice), γ-globulin and cytochrome c were purchased from Sigma–Aldrich Co. LLC. α-Amylase and lipase were purchased from TCI (shanghai) Development Co., Ltd. Bovine serum albumin (BSA) was purchased from Amresco Inc. Human serum albumin (HSA), glycine (AR) and trizma base (AR) were purchased from Beijing Xinjinke Biotechnology Co., Ltd. ELISA kit for human lysozyme was purchased from Beijing Qisong Biotech. K_2HPO_4 (AR), HCl (AR) and other reagents were purchased from Beijing Chemical Works.

Fluorescence emission spectroscopy and steady-state fluorescence anisotropy were measured using FLS 920 fluorimeter (Edinburgh Instruments Ltd.) with 492 nm excitation. Fluorescence lifetime and dynamic fluorescence anisotropy were measured using a LifeSpec-Red time-correlated single-photon counting fluorimeter (Edinburgh Instruments Ltd.) with a 440-nm laser source and a R7422 PMT detector. Instrument response function was scanned with 30% (wt) colloidal silica suspension in H_2O. ELISA experiment was measured with Genios absorbance reader (Tecan Ltd.).

2.2. General procedure

Unless otherwise stated, FAM was used as the fluorophore for method development, and the binding buffer (25 mM trizma base, 192 mM glycine, 5 mM K_2HPO_4, pH adjusted to 8.3) was prepared as used in aptamer selection by Jeroen Lammertyn group (Tran et al., 2010). Fluorescence measurements were carried out with a controlled water bath at $25 \pm 0.2\,^{\circ}C$. Briefly, the aptamer, buffer and lysozyme solutions were mixed into an individual tube with a final volume of 300 μL, followed by vortex mixing for 10 s; the final concentration of aptamer and the concentrations of lysozyme were indicated in the specific measurement.

2.3. Steady-state fluorescence anisotropic measurements and data fitting

The anisotropy, r, of the test solution was calculated by

$$r = \frac{I_{VV}I_{HH} - I_{VH}I_{HV}}{I_{VV}I_{HH} + 2I_{VH}I_{HV}} \quad (1)$$

where I represents the intensity of the fluorescence signal and the subscripts define the orientation (H for horizontal and V for vertical) of the excitation and emission polarizers, respectively.

Fluorescence anisotropic titration was carried out by varying the concentration of lysozyme with the concentration of DNA aptamer fixed at 10 nM. The titration curve was fitted assuming a two-state binding model (Gokulrangan et al., 2005) at one-to-one binding stoichiometry.

The one-to-one binding model for aptamer (Apt) binding to lysozyme (Lyz) can be written as

$$\text{Apt} + \text{Lyz} \leftrightharpoons \text{Apt–Lyz} \quad (2)$$

where the dissociation constant (K_d) is given by

$$K_d = \frac{[\text{Apt}][\text{Lyz}]}{[\text{Apt–Lyz}]} \quad (3)$$

Thereby the concentration of bound aptamer is given by

$$[\text{Apt}]_b = \frac{r - r_u}{(r_b - r_u)c_{\text{Apt}}} \quad (4)$$

where $[\text{Apt}]_b = [\text{Apt–Lyz}]$, r is the steady-state anisotropy, r_u is the steady-state anisotropy of the aptamer in the absence of Lyz, r_b is the anisotropy at saturation level of Lyz, and c_{Apt} is the total concentration of aptamer.