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Assay of multiplex proteins from cell metabolism based on tunable aptamer and microchip electrophoresis

Xuexia Lin [a,b], Qiushui Chen [b], Wu Liu [b], Linglu Yi [a], Haifang Li [b], Zhihua Wang [a,*], Jin-Ming Lin [b,*,1]

[a] State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing 100029, China
[b] Department of Chemistry, Beijing Key Laboratory of Microanalytical Methods and Instrumentation, Tsinghua University, Beijing 100084, China

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ABSTRACT

A simple and rapid method for multiplex protein assay based on tunable aptamer by microchip electrophoresis has been developed. Different lengths of aptamers can modulate the electrophoretic mobility of proteins, allowing the protein molecules to be effectively separated in hydroxyethyl cellulose buffer with 1.00 mM magnesium ion. A non-specific DNA was exploited as an internal standard to achieve the quantitative assay and to reduce the interference. A fluorescence dye SYBR gold was exploited to improve the sensitivity and to suppress the interference from sample matrix. Under optimum conditions, quantitative assay of PDGF-BB (R^2=0.9986), $VEGF_{165}$ (R^2=0.9909), and thrombin (R^2=0.9947) were achieved with a dynamic range in the 5.00–150.0 nM and RSDs in the 5.87–16.3% range. The recoveries were varied from 83.6% to 113.1%. Finally, the proposed method was successfully applied to analyze cell secretions, and then the concentration of PDGF-BB and $VEGF_{165}$ were detected from 5.15 nM to 2.03 nM, and 3.14 to 2.53 nM, respectively, indicating the established method can be used to analyze cell secretions.

1. Introduction

Protein detection is critically important in basic research and clinical practice, because trace level of proteins can be sufficient to influence the biological functions of cells and trigger pathophysiological processes (D'hondt et al., 2007; Rey and Semenza, 2010; Steeq, 2006). Great efforts have been made to achieve rapid and sensitive analysis of proteins. One difficulty that is still often encountered is the absence of appropriate chromogenic groups on the protein itself, yielding poor absorption in the ultraviolet and visible region, and limiting the selectivity and sensitivity of the analysis. Traditional solid-phase protein detection strategies include enzyme-linked immunity assays, protein microarrays, magnetic-separation assays, lateral-flow assays and biosensors (Gonzalez-Gonzalez et al., 2012; Monecke et al., 2013; Xin et al. 2008; Freeman et al., 2012). These techniques often rely on antibody specificity to the target proteins. However, the cost, stability, and availability of the required antibodies remain problematic. Moreover, these methods may be interfered by cross reactions and non-specific adsorption, often limiting their capacity to perform multiplexed protein analysis. Probably most reliable methods for protein assay in solutions are based on chromatography, which are usually accomplished through the combination of an effective separation technique, such as thin-layer chromatography (Han et al., 2010), high performance liquid chromatography (HPLC) (Chan et al., 2011; Wen et al., 2007), or capillary electrophoresis (De Jong et al., 2012; Haselberg et al., 2011), and a detection step performed by UV/vis, fluorescence or mass spectrometry. However, the above methods are generally tedious and expensive for routine analysis. Given the limitations of these systems, microchip electrophoresis (MCE) is an attractive alternative because it can integrate sample preparation, derivatization, separation, detection and other functional units in an area of a few square centimeters (Ma et al., 2011; Mao et al., 2012). Consequently, MCE can be considered as one of the most successful miniaturized analysis systems for separating biological samples such as cells, genes, amino acids, and proteins (Meagher and Thaitrong, 2012; Ansari et al., 2013; Jin et al., 2013; Ye et al., 2010). For DNA/RNA detection, it can be on-line labeled with fluorescent dye and then optically detected after a separation by MCE (Lin et al., 2013a, 2013b). It is also well known that some DNA/RNA strains can bind to proteins. Therefore, rapid and simple protein

* Corresponding authors.
E-mail address: jmlin@mail.tsinghua.edu.cn (J.-M. Lin).
[1] Tel./fax: +86 10 62792343.

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detection can be achieved by DNA coupling and on-line fluorescence labeling.

Aptamers are single-strand nucleic acids screened from DNA/RNA libraries. This technology has drawn tremendous attention in various fields because it can bind to corresponding targets (such as proteins, cells, and metal ions) with high specificity and affinity (Bao et al., 2011; Chen et al., 2012a, 2012b; Chung et al., 2013). More importantly, unlike other affinity ligands, aptamers could be easily synthesized, and are stable under different experimental conditions including pH varying from 5 to 9 (Hianik et al., 2007; Li et al., 2008). DNA aptamers against thrombin, human vascular endothelial growth factor 165 ($VEGF_{165}$), and platelet-derived growth factor B-chain (PDGF-BB) were selected in this work because they have been thoroughly characterized (Kaur and Yung, 2012; Lin et al., 2014; Xie and Patrick, 2010). The aptamers selected for the analysis of thrombin including a 15-nucleotide aptamer (TBA15) and a 29-nucleotide aptamer (TBA29) (Krauss et al., 2011; Daniel et al., 2013), with dissociation constants (K_d) approximately 100 nM and 22 nM, respectively. For the analysis of $VEGF_{165}$ protein, a 66 mer nucleotide aptamer (Ea66) that binds to heparin-binding domain has been frequently used. An improved version of this aptamer (26 mer DNA aptamer, Ea26) has been also presented, with a K_d of 0.5 nM (Kaur and Yung, 2012; Hasegawa et al., 2008). Finally, a 35 mer (PB35) with a $K_d \approx 0.1$ nM has been used for the analysis of PDGF-BB (Platt et al., 2012). According to recent reports, these aptamers can be isolated from random sequence libraries at concentrations ranging from nanomolar to micromolar. Therefore, it was hypothesized that multiple aptamers could be applied to achieve protein detection by the formation of aptamer–protein complexes, and that these complexes could be separated by MCE for multiple protein analysis. Therefore, the purpose of this work was to develop an aptamer-based MCE for multiple proteins assay. In order to realize the efficient separation of multiple proteins with adequate sensitivity and less sample consumption (about 2 μL), different lengths of aptamers were applied to recognize and tune the electrophoretic mobility of proteins by forming aptamer–protein complexes. As a result, multiple proteins can be separated efficiently (Berezovski and Krylov, 2003; De Jong and Krylov, 2011; Zhang et al., 2008, 2009). Hydroxyethyl cellulose (HEC) was used as sieving matrix to enhance the resolution of DNA–protein complexes separation. SYBR gold, one of the most sensitive reagents for staining nucleic acids (especially suited for double- and single-strand DNA or RNA) was used as chromosphere. Based on this fluorescent dye, the limit of detection (LOD) and selectivity can be greatly improved. In order to demonstrate the capabilities of the system, thrombin, $VEGF_{165}$, and PDGF-BB were used as model analytes. These proteins are related to cancer and have been confirmed implicated in many cell transformation processes, tumor growth and progression.

2. Materials and methods

2.1. Chemicals

Both DNA ladder and SYBR gold solution were purchased from Invitrogen Corporation (Karlsruhe, Germany), and diluted with Tris–HCl buffer (TE buffer, 10.00 mM Tris–HCl, pH 8.0, 1 mM EDTA). HEC was purchased from Sigma (HEC, 250,000 Da, Sigma, USA). 2% HEC (w/v) was prepared with TE buffer (pH 8.0) and 1.00 mM magnesium ion. Recombinant human $VEGF_{165}$ and PDGF-BB were purchased from ProSpec Protein Specialists (East Brunswick, NJ). Thrombin was obtained from Dingguo Biotechnology (Beijing, China). Roswell Park Memorial Institute (RPMI) 1640 medium was from GIBCO (Grand Island, NY). All oligonucleotides were purchased from Sangon Biotech (Shanghai, China), listed in Table 1.

2.2. Cell culture

CaSki cell line and human umbilical vein endothelial cell (HUVEC) line were purchased from Cancer Institute & Hospital Chinese Academy of Medical Science (Beijing, China). CaSki cells and HUVEC cells were cultured in RPMI 1640 media supplemented with 2% fetal bovine serum (FBS), 100 μg/mL penicillin, and 100 μg/mL streptomycin. Only 2% FBS was added to RPMI 1640 media because it can maintain cells viability. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

2.3. Sample preparation

The stock (100.00 μM) as well the working solutions of the aptamer were prepared in 10.00 mM TE buffer (pH = 8.0). Then, the work solution was also diluted with 10.00 mM TE buffer. In order to get the special structures, aptamers were incubated at 85 °C for 5 min and then slowly cooled down to room temperature. Stock solutions of $VEGF_{165}$ and PDGF-BB were 20.00 μg/mL, which were prepared by ultrapure water. Stock solution of thrombin prepared by ultrapure water was 1.00 mg/mL. After that, aptamer was incubated with the corresponding protein at 37 °C for 10 min. For quantitative assay, thrombin, $VEGF_{165}$ and PDGF-BB ranging from 0.50 nM to 300.00 nM were incubated with 500.00 nM aptamer. The incubation buffer contained 10.00 mM Tris–HCl and 1 mM $MgCl_2$ at pH 8.0. To mimic the real sample matrix, various concentrations of three proteins were spiked into RPMI 1640 cell media, and then diluted ten-fold. After that, three kinds of aptamers (PB44, Ea66, TBA57) were added to the diluted solution, and incubated at 37 °C for 10 min before MCE analysis.

Table 1
Proteins and their aptamers used in this study.

Protein name	Aptamer no.	Aptamer size	Aptamer sequence
Thrombin	TBA15	15 mer	GGTTGGTGTGGTTGG
	TBA57	57 mer	GGTTGGTGTGGTTGGCTTGCTCCTCAGCAAGCCAACCACACCATTTTTTTTTTTTTT
	TBA67	67 mer	GGTTGGTGTGGTTGGCTTGCTCCTCAGCAAGCCAACCACACCATTTTTTTTTTTTTTAAGTAGACTGA
$VEGF_{165}$	Ea26	26 mer	CAATTGGGCCCGTCCGTATGGTGGGT
	Ea49	49 mer	CCTCCCCAATTGGGCCCGTCCGTATGGTGGGTGGGGAGGGTGGGGAAGG
	Ea66	66 mer	ATACCAGTCTATTCAATTGGGCCCGTCCGTATGGTGGGTGTGCTGGCCAGATAGTATGTGCAATCA
PDGF-BB	PB35	35 mer	CAGGCTACGGCACGTAGAGCATCACCATGATCCTG
	PB44	44 mer	CAGGCTACGGCACGTAGAGCATCACCATGATCCTGTTTTTTT
	PB80	62 mer	CAGGCTACGGCACGTAGAGCATCACCATGATCCTGTTTTTTTCTCACACGAATTCATCTGAC
Non-specific DNA	NP	49 mer	TGGTCTTGTGTGGCTGTGGCTATGTCTGATCTTA ATCCACGAAGTCACC