Electrochemical Detection of Vascular Endothelial Growth Factor by an Aptamer-Based Bound/Free Separation System

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ABSTRACT

Vascular endothelial growth factor (VEGF) is expected to find application as a prognostic biomarker in cancer diagnosis. In this study, we measured VEGF using a simple bound/free separation system that utilizes an aptamer, whose capacity for hybridization with capture DNA immobilized on beads changes in the presence or absence of target molecules. Two systems were constructed using a VEGF aptamer selected by in vitro screening and a VEGF aptamer improved by a dimerization strategy. We can detect VEGF based on electrochemistry using both aptamers. We previously reported the detection of two proteins in addition to VEGF—IgE and thrombin—and the success of VEGF detection with this system suggests that this is a versatile system for the detection of various molecules.

Keywords: Aptamer, Vascular Endothelial Growth Factor, Electrochemical Sensor, Conformational Change

1. Introduction

Vascular endothelial growth factor (VEGF) is associated with angiogenesis, which is necessary for tumor growth, progression, and metastasis.1,2 It has been reported that VEGF levels within tumor tissue are elevated in solid tumors.3 As an increased VEGF expression was found to correlate with bad prognoses in several cancers,4 VEGF is expected to find application as a biomarker for cancer diagnosis. Although many VEGF detection systems have already reported and been used clinically, most of them are required large and expensive devices. Cheap and miniaturized biosensor that can detect VEGF with high sensitivity and rapidly would be attractive tools for diagnostics, especially for point of care testing.

VEGFs are homodimeric glycoproteins and have 12 isoforms that are produced by the alternative splicing of VEGF mRNA. VEGF165 is the most abundantly expressed isoform and plays a vital role in angiogenesis. We have also reported DNA aptamers that bind VEGF165 and VEGF121.6,7 Aptamers are commonly selected by a form of in vitro selection named SELEX (Systematic Evolution of Ligands by Exponential enrichment).8,9 Although SELEX is a powerful method for obtaining aptamers, the selected aptamers require sequential optimization to improve their affinities. Many researchers have reported an improvement in the affinity of aptamer by dimerization, which expands the interaction area between the aptamer and target molecules,10,11 and we also reported dimeric VEGF aptamers, which improve the affinity of selected aptamers.7,12

Aptamers can be used as a biosensing element instead of antibodies because aptamers, which can be easily synthesized and are more stable than antibodies, also recognize molecules with a high affinity and specificity. In addition, aptamers have an advantage over antibodies in biosensing in that a structural change in aptamers can be designed using straightforward hybridization rules. Using this advantage, we constructed an aptamer-based homogeneous sensing system called an Aptameric Enzyme Subunit.13

We have already reported a simple bound/free (B/F) separation system based on the structural change of an aptamer for protein detection, which consists of an aptamer and its part of the complementary strand.14 The sensing system is shown in Fig. 1. The structure of the aptamers is stabilized by binding to target molecules. We designed the system utilizing the difference in the hybridization ratio of the aptamer with the complementary DNA of the aptamer in the presence and absence of target molecules by designing complementary DNA that hybridize with the aptamer in the absence of target protein, but does not hybridize with it in the presence of target protein. We previously reported thrombin detection and IgE detection using this system.14

Thrombin aptamer and IgE aptamer have been characterized in depth by many researchers. In this study, to demonstrate the versatility of this strategy, we constructed a simple B/F separation system using aptamers that we selected against VEGF165. We also constructed a simple B/F separation system using a dimeric aptamer.

2. Materials and Methods

2.1 Materials

Recombinant human VEGF165 purified from the insect cell line Sf21 was purchased from R&D Systems in the form of carrier-free lyophilized powders. Immobilized NeutrAvidin was purchased from Thermo Scientific. PQQGDH from Acinetobacter calcoaceticus was purified as described previously.15 All oligonucleotides used in this study were purchased from Invitrogen. The sequences of the oligonucleotides are listed in Table 1.

2.2 Electrophoretic mobility shift assay (EMSA)

The aptamers were heat-denatured at 95°C for 3 min and then cooled to 25°C over a period of 30 min. The aptamer solution was then mixed with VEGF and incubated for 30 min at room temperature. FITC-labeled complementary DNA (100 nM) (M = mol dm⁻³) was added and incubated for 15 min at room temperature. The mixture was electrophoresed in a 12% polyacrylamide gel in a TBE buffer (45 mM tris, 35 mM H₂BO₃, 2 mM EDTA) at room temperature. FITC fluorescence imaging was then carried out using a Typhoon 8600 (GE Healthcare).

2.3 Preparation of PQQGDH-labeled aptamers

Avidin-PQQGDH conjugate was prepared using glutaraldehyde crosslinking, as has been described elsewhere, to label the biotin-
The activity of the PQQGDH-avidin conjugate was measured using 1 mM 2,6-dichlorophenol-indophenol (DCIP), 6 mM 1-methoxy-5-methylphenazine methsulfate (m-PMS), 60 mM glucose, and we defined 1 unit of GDH activity as the amount of enzyme required to reduce 1.0 µmol DCIP per min in the presence of glucose. To prepare the PQQGDH-labeled VEGF aptamer, 1 nmol PQQGDH-avidin conjugate and 0.5 nmol biotin-labeled VEGF aptamer were mixed in 500 µL of reaction buffer [10 mM Tris-HCl (pH 7.0), 100 mM NaCl] and incubated for 30 min at 4°C.

2.4 Preparation of capture DNA (CaDNA) immobilized on NeutrAvidin beads
To reduce nonspecific binding of proteins using beads, we used NeutrAvidin beads that have a near-neutral isoelectric point. NeutrAvidin beads (10 µL) were washed with a binding buffer [100 mM phosphate (pH 7.2), 150 mM NaCl], and a biotinylated complementary strand (0.5 nmol) was added to the 0.5-ml solution of NeutrAvidin beads and incubated for 1 h. The NeutrAvidin beads were then collected by centrifugation and washed with the binding buffer. The beads were next blocked using biotin and NIP551 (GE Healthcare) to reduce nonspecific binding of target proteins to the beads. First, the NeutrAvidin beads were incubated with 4 mM biotin for 15 min. After washing with the binding buffer, the NeutrAvidin beads were incubated with a reaction buffer [50 mM Tris-HCl (pH 8.0), 5 mM KCl, 100 mM NaCl] that contained 1% NIP551 for 1 h, and then washed with the reaction buffer. All steps were carried out at room temperature.

2.5 Electrochemical detection
PQQGDH-labeled VEGF aptamers (0.1 U) and various concentrations of VEGF or bovine serum albumin (BSA) were mixed with 100 µL of reaction buffer and incubated for 30 min at room temperature. The mixture was then added to 10 µL of CaDNA immobilized beads and incubated for 15 min at room temperature. After centrifugation (5000 g, 1 min), 20 µL of supernatant was added to 20 µL of glucose solution [10 mM MOPS, 1 mM CaCl₂, 1 mM PQQ, 6 mM m-PMS, 60 mM glucose], and 30 µL mixture was placed on the electrode area of a DEP chip (Bio Device Technology Co., Ltd.). The response current was measured at 25°C at a potential of 0.1 V vs. Ag/AgCl.

Table 1. Sequences of oligonucleotides in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<td>Veap</td>
<td>ATACCGTCTATTTCAATTTGGGCCCGTCCGTATGGTGCGGTGTGCTGGCCAGATAGTATGTGCAATCA</td>
</tr>
<tr>
<td>Veap dimer</td>
<td>ATACCGTCTATTTCAATTTGGGCCCGTCCGTATGGTGCGGTGTGCTGGCCAGATACCAGTCTATTCAA</td>
</tr>
<tr>
<td>ΔVeap dimer</td>
<td>TTTGGGCCCGTCCGTATGGTGCGGTGTGCTGGCCAG</td>
</tr>
<tr>
<td>CaDNA1</td>
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<td>CaDNA2</td>
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<td>CaDNA-D2#3</td>
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Figure 2. Native PAGE analysis. a) Evaluation of hybridization between each CaDNA and VEap. FITC-labeled CaDNA (100 nM), VEap (100 nM), and VEGF (500 nM) were electrophoresed on a 12% polyacrylamide gel. Lanes marked a, FITC-labeled CaDNA, lanes marked b, FITC-labeled CaDNA and VEap, lanes marked c, FITC-labeled CaDNA, VEap, and VEGF, a lane marked d, FITC-labeled VEap, a lane marked e, FITC-labeled VEap and VEGF. b) Effects of VEGF concentration of hybridization. Lane 1, FITC-labeled VEap, a lane marked e, FITC-labeled VEap and VEGF, lanes marked b, FITC-labeled CaDNA and VEap, lanes marked c, FITC-labeled CaDNA only, lane 2, FITC-labeled CaDNA and VEap, lanes 3–7 FITC-labeled CaDNA, VEap, and VEGF (31, 63, 125, 250, or 500 nM, respectively).

3. Results and Discussion

3.1 Design of CaDNA for VEGF aptamer

For VEGF detection using an aptamer-based B/F separation system, we designed CaDNA that hybridizes with VEGF aptamer without VEGF but not with VEGF. We used 66-mer VEGF aptamer (VEap), the secondary structure of which is predicted as three stem loops. As previous mutational analysis indicated, the predicted second stem loop structure of VEap is important for VEGF recognition. Therefore we hypothesize that binding VEGF to VEap stabilizes this predicted stem loop structure, and VEap that binds to VEGF does not hybridize with the complementary DNA of this predicted stem loop structure. Next, we designed seven CaDNA candidates that are complementary to the sequence around the predicted second stem loop structure, and we calculated melting temperature (Tm) values, which represent stability of structure, of similar to that of the predicted second stem loop structure.

3.2 Evaluation of CaDNA for VEGF aptamer

We evaluated the effect of VEGF binding on the hybridization of VEap to the designed CaDNA by means of an electrophoretic mobility shift assay (EMSA) using FITC-CaDNA [Fig. 2(a)]. The band for CaDNA2 and 4 showed an upward shift, whereas the other CaDNA did not shift upward in the presence of VEap. These results indicate that the Tm values of CaDNA1, 3, 5, 6, and 7 were too low to break the VEap intramolecular structure. In the presence of both VEGF and VEap, the fluorescence intensity of the bottom band of CaDNA4 and CaDNA2 increased. Since the change ratio of the result of CaDNA4 was higher than that of CaDNA2, we used CaDNA4 for subsequent experiments.

We also evaluated the dependence of the hybridization ratio of VEap to CaDNA4 on VEGF concentration [Fig. 2(b)]. 100-nM VEGF and 0–500 nM VEGF were incubated with 100 nM FITC-CaDNA4 for the EMSA. A fluorescence intensity analysis of the lowest band of CaDNA4, which does not hybridize to VEap, increased in intensity along with an increase in VEGF concentration [Fig. 2(c)]. This result indicates that the hybridization ratio of VEap to CaDNA4 is dependent upon the VEGF concentration.

3.3 VEGF detection using PQQGDH-labeled VEGF aptamer and CaDNA4

Biotinylated CaDNA4 was immobilized on NeutrAvidin beads and biotinylated VEap was labeled with avidin-PQQGDH. 10-µL CaDNA4 immobilized beads were incubated with various concentrations of VEGF and 0.1 U PQQGDH-labeled VEap. We measured PQQGDH activity in the supernatant fluid that contained the VEGF-PQQGDH-labeled VEap complex, which was not trapped by the CaDNA4 immobilized beads. In the absence of VEGF, the background current was approximately 200 nA. Increases in the electric current were observed along with increases in the concentrations of VEGF in the sample solutions, but a little increase was observed with BSA. The magnitude of the electric current depended on the VEGF concentration within 10 nM to 1 µM (Fig. 3). These results indicate that VEGF can be measured using the PQQGDH-labeled VEap and CaDNA4 immobilized beads by means of a single centrifugation step. However, we observed slight increase of electric current in the presence of BSA with concentration dependency. Since we observed little increase of electric current for thrombin detection system, it might indicate that electric current increase in the presence of BSA causes nonspecific binding to the aptamer. Therefore we need to improve binding specificity of this aptamer.

3.4 Design of CaDNA for dimeric VEGF aptamer

For highly sensitive detection of VEGF, we designed CaDNA to hybridize with an improved aptamer: ΔVEap dimer. ΔVEap dimer is composed of two of the same truncated mutants of VEap, and each ΔVEap is connected by a double-stranded palindromic sequence. Since VEGF is a homodimeric protein, each domain of ΔVEap dimer will bind to each domain of a VEGF monomer simultaneously.

First, we determined whether CaDNA4 hybridizes to ΔVEap dimer in the absence of VEGF, but not in the presence of VEGF using EMSA. There was little change in the fluorescence intensity of Figure 3. Calibration graph for VEGF using simple B/F separation. PQQGDH-labeled VEap (0.1 U) and CaDNA4 (10 µL) were used. The current was measured at 25°C with a potential of 0.1 V vs. Ag/AgCl in 40 µL of 10 mM MOPS buffer (pH 7.0) that contained 60 mM glucose, 6 mM m-PMS, and 20 µL of supernatant (mean ± SD; n = 3). The background current was approximately 200 nA, and the increased currents from the background current are shown on the y-axis.
the bottom band of CaDNA4 in the presence of 4–100 nM VEGF, while fluorescence intensity increased in the presence of 500 nM VEGF (data not shown). The fluorescence intensity of the bottom band of only CaDNA4 was higher than that of CaDNA4 with VEap dimer. It would indicate that CaDNA4 can hybridize with VEap dimer regardless of binding of an VEap domain to a VEGF molecule because the other domain can hybridize CaDNA4. Therefore, CaDNA would be required to hybridize both stem loop structures in each VEap, so as to prevent CaDNA from hybridizing with VEap dimer whose only one domain binds to a VEGF. Next, we designed six CaDNA candidates that we categorized into two groups (Table 1, Fig. 4). The first group has a complementary sequence that can hybridize with both second-stem loop sequences in each VEap, and each complementary sequence is connected with a thymines linker. The second group has a complementary sequence that can hybridize not only second-stem loop sequences but also central stem sequences that comprise a palindromic sequence. We designed three CaDNA candidates for each group, which have various thymines linkers or various length complementary sequences for second-stem loop sequences.

3.5 Evaluation of CaDNA for dimeric VEGF aptamer

CaDNA-D1#3, 2#2, and 2#3 can hybridize to a VEap dimer, and in the presence of VEGF and VEap dimer, the fluorescence intensity of their bottom band was increased [Fig. 5(a)]. Then we evaluated the dependence of the hybridization ratio of the VEap dimer to each CaDNA on VEGF concentration. The fluorescence intensity analysis of the band of CaDNA-D2#3, which does not hybridize to the VEap dimer, increased in intensity along with an increase in VEGF concentration from lower concentration than the result obtained using VEap and CaDNA4 [Figs. 5(b) and 5(c)].
Although the method used in this study can remove aptamers that do not bind to target molecules, it cannot remove molecules that affect noise. Therefore this system requires pretreatment if a sample includes molecules that affect noise.

However, for capturable aptamer, we have to design an aptamer that can hybridize with CdNA when it binds to a target molecule, but does not hybridize with CdNA when it does not bind to a target molecule. Therefore, the design of a capturable aptamer requires additional DNA complementary to CdNA and careful design of CdNA, the complementary DNA of which does not affect the properties of the aptamer. On the other hand, the structural changes in the aptamers in this study are based on their stability rather than their conformation. We have to consider only the difference in stability between the bound and unbound state of the aptamer, and this does not require the addition of DNA to the aptamer and a structural prediction of the aptamer for CdNA design. Therefore, its design is easier than that for a capturable aptamer. We expect this system will be applied to various fields by selecting an appropriate system for each target molecule.

4. Conclusion

VEGF measurement was successfully conducted using an aptamer-based B/F separation system. We designed CdNA for the monomer aptamer selected by SELEX, and the dimeric aptamer improved its affinity for VEGF, and we could detect VEGF using both aptamers. Although dimeric aptimer has more complicated structure than monomeric aptamer, we succeeded in construction of CdNA for dimeric aptamer. Since dimerization is attractive approach for improvement of affinity, this system would achieve highly sensitive detection system using dimeric aptamer. We have reported several strategies for aptameric-sensing systems based on their structural changes. We can measure various target molecules in various fields by selecting an appropriate system for each target molecule.

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References