Novel Protein Detection System Using DNA as Constituent Material

● Shozo Fujita ● Kenji Arinaga ● Tsuyoshi Fujihara ● Michihiko Aki
● Tomoyasu Kichise

A quick and simple means of measuring proteins is in much demand in the medical and healthcare fields for identifying markers for cancer and other diseases and in the food industry for detecting toxins associated with food poisoning. Fujitsu Laboratories has been developing novel protein-detection technologies using deoxyribonucleic acid (DNA), which is relatively easy to handle, as a constituent material and has succeeded in developing a modified DNA aptamer technology in which modified amino-acid side chains along the DNA strands (modified DNA aptamers) are used for detecting proteins. A protein detection technology (switchSENSE) in which the artificially induced movement of DNA molecules is used as an index of protein presence has also been developed through collaboration with Technische Universitat Munchen. These two technologies using DNA as a constituent material can be easily combined due to the double-helix formation of DNA, making it easy to develop a system for detecting new target proteins. Additionally, through collaboration with Nagoya University, Fujitsu Laboratories has shown that protein toxins associated with food poisoning can be quickly detected using this protein detection system. Fujitsu Laboratories is making efforts to commercialize these technologies in the form of front-end sensors for a human-centric information system to improve the quality of life and create a safe and secure society.

1. Introduction

Protein immunoassays are used to detect pathogenicity factors. In the field of medicine, these factors may be cancer marker proteins, and in the food industry, they may be protein toxins associated with food poisoning or prions associated with mad cow disease (bovine spongiform encephalopathy). Animals produce antibody proteins that protect them against infection and other threats. However, animals cannot produce antibodies against antigens resembling naturally occurring proteins. Creating sufficiently effective antibodies against such antigens that are applicable to immunoassay is thus difficult.

The sandwich analysis method known as the enzyme-linked immunosorbent assay (ELISA) is widely used to analyze non-labeled proteins. This method uses two types of antibodies with respect to the proteins targeted for detection: a primary antibody to immobilize the target and a secondary antibody to label the immobilized target. This method is called "sandwich analysis" because the object targeted for detection is positioned between two types of antibodies. One drawback of this method is the long time required to establish the quantitative binding reaction for creating an antigen-antibody complex.

Given these technical problems, we set out to develop innovative technologies that would enable creation of methods to replace the currently used methods (antibody and sandwich analysis) for performing immunoassays. In this article, we describe two technologies comprising a protein detection system: modified DNA aptamer

technology and switchSENSE technology.

2. Development approach

Functional components for protein detection consist of a probe molecule that recognizes a protein and binds to it and a signal converter molecule that optically visualizes the binding state (**Figure 1**).

To establish such a system, we used deoxyribonucleic acid (DNA) as a constituent material instead of antibody protein because proteins tend to become denatured and lose their functionality. We chose DNA for our molecular system because of its stability as evidenced by the fact that the great majority of living organisms use DNA to store their genetic information. Moreover, we can use a wide range of technologies like chemical synthesis, cloning, amplification, and sequencing already developed for DNA handling if DNA is used as a constituent material.

3. Probe molecule (Modified DNA aptamer technology)

We focused on the use of aptamers to develop a probe molecule system because aptamers are a nucleic acid having a material-recognition function similar to that of antibodies. Ribonucleic acid (RNA) aptamer technology was established by research activities in the 1980s and 1990s. RNA aptamers are generated by identifying, from a mixture of randomly synthesized RNA molecules, a unique RNA sequence that has high affinity to the target molecule. RNA aptamers are currently used in therapeutic medicine for eye disease, *i.e.*, age-related macular degeneration (AMD).

However, RNA is hard to use because it decomposes very rapidly. There have thus been many attempts to develop stable DNA aptamers. Up to now, though, no one has been successful in obtaining DNA aptamers with good enough characteristics.

In response to this problem, we began development of DNA aptamers displaying a variety of modified amino-acid side chains along the DNA strands (referred to below as “modified DNA aptamers”), as shown in **Figure 2**. The idea behind introducing amino-acid side chains is to form many intermolecular interactions between an aptamer and a target protein using a variety of chemical species and thus to achieve high affinity interaction of aptamers.

3.1 Modification of DNA aptamers

The process we used to modify DNA aptamers is shown in **Figure 3**. First, many modified nucleotide monomers having different amino-acid side chains, denoted by “Ri” in the figure, are synthesized separately and then used as the building blocks of modified DNA aptamers.

The modified nucleotide monomers are mixed together and used to randomly elongate DNA strands. This creates a random library of modified DNA aptamers, i.e., a mixture of DNA aptamers with amino-acid side chains arranged in a random manner along a single strand of DNA. The diversity of this mixture depends on the types of modified nucleotides used and the length of the DNA strand. It is relatively easy to obtain 10^{15} variations of the molecular structure, which exceeds the diversity found in the animal

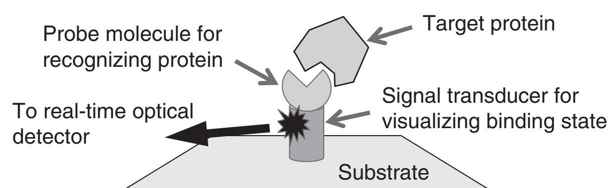


Figure 1
Functional components for protein detection.

Figure 2
Concept of modified DNA aptamer.

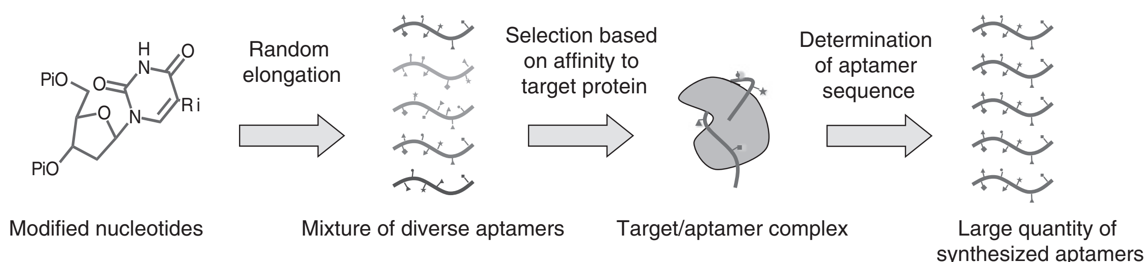


Figure 3
Process used to modify DNA aptamers.

immune system.

The target protein is then dipped into this random library. Those aptamers with which it interacts are recovered from the library as they have the ability to bind strongly to the target. Since hundreds of modified DNA aptamers are recovered, we utilize standard biotechnology methods to isolate each one and to determine its sequence. The DNA sequence information thereby obtained is used to synthesize each of the modified DNA aptamer molecules separately. By analyzing the binding characteristics of these aptamers, we can identify the most effective aptamer for the target protein.

However, the amino-acid side chains are lost during this modification process because the enzymes used in standard biotechnology methods do not recognize such side chains on DNA sequences. This means that we cannot get information about the side chains randomly incorporated into DNA even if we determine the DNA sequence of the aptamer. To overcome this problem and recover the side-chain information, we developed the "block coding" method illustrated in **Figure 4**. In this method, a code table is created beforehand to associate side-chain information with two-character combinations, or dimer molecules, of DNA sequences. When a random library of modified DNA aptamers is synthesized from modified dimers corresponding to the code table, we can recover the side-chain information from the DNA sequences because there is a correlation between the code table and the determined DNA sequence.[1)]

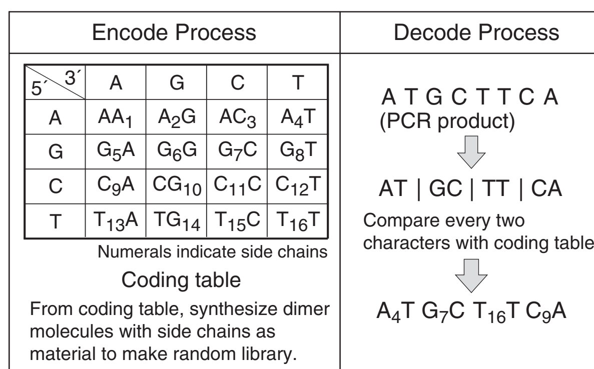


Figure 4
Block coding methodology.

Table 1
Affinities of modified DNA aptamers

Target Molecule	K_D
Green fluorescent protein (GFP)	1 nM
Enterotoxin from *Staphylococcus aureus*	2 nM
A protein circulating in blood	10 nM
Cancer marker candidate	< 1 nM
Disease marker candidate	< 0.6 nM

K_D: Dissociation constant

3.2 Characteristics of modified DNA aptamers

We have so far developed modified DNA aptamers for five types of target molecules using the modification process described above. The affinity of each modified DNA aptamer for its target molecule is shown in **Table 1**. In all cases, the dissociation constant, which is an index of affinity, is no larger than 10 nM, which satisfies

the characteristics required of antibodies used in diagnostic applications. These results demonstrate the effectiveness of increasing the affinity of DNA aptamers by introducing a variety of side chains.

4. Binding visualization (switchSENSE technology)

We have started to investigate ways to utilize the mechanical effects of the balance between thermal fluctuation and induced forces. In terms of mechanical properties, a protein is a sphere-like molecule, and one with a mass of 50 000 daltons has a diameter of about 6 nm. In contrast, a double-helix DNA molecule, which is shaped like a long rod, has a diameter of about 2 nm. Therefore, we considered it useful to monitor the observable changes in the induced movement of a DNA molecule during its interaction with a protein molecule. We thus established a technology for optical detection of artificially induced DNA molecular motion using fluorescent-labeled DNA molecules immobilized on a gold electrode. Research using this technology was carried out through a decade of collaboration with Technische Universitat Munchen, Walter Schottky Institut (TUM-WSI).

In brief, we began by observing fluorescent-labeled DNA strands that had been tethered at one end to a gold electrode. We discovered that the emitted fluorescence fluctuates with the voltage applied to the electrode. This fluctuation was first discovered by the TUM-WSI team,[2] and it became evident that this phenomenon originates from the change in DNA molecular conformation on gold. Our analysis of the parameters affecting this fluctuation revealed that factors such as the amplitude of the external electric field, the length of the DNA molecules, and the density of immobilized DNA molecules on the gold electrode[3] play important roles. As shown in **Figure 5**, interaction between a negatively charged DNA molecule and an ion layer rearranged by an external electric field causes the molecule to move. This motion changes the distance between the gold electrode and the fluorescent dye at the other end of the DNA strand, so this motion changes the quenching intensity of the fluorescence. On the basis of this phenomenon, we developed a completely new detection technology called switchSENSE that can be used to detect the state of DNA molecular motion by optically monitoring the fluorescence quenching phenomenon.

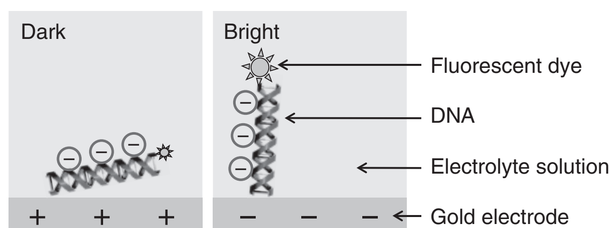


Figure 5
Illustration of switchSENSE technology.

The bright and dark cycle of the fluorescence intensity traces the phase change of the external electric field. When a probe molecule like an antibody or aptamer immobilized at the top of a DNA molecule recognizes its target and binds to it, the cycle is disturbed. This is because the diameter of the target protein molecule is three times that of the DNA molecule, and a protein molecule resists the motion of DNA due to the viscosity of the sample solution. This means that we can identify the status of the interaction between the probe and the target by comparing the intensity of the fluorescence before and after the interaction. This method can also be used to monitor molecular interaction in real time.

5. Combination of functional elements

To construct a protein sensor, we simply combine the two elemental components described above (the modified DNA aptamer technology and the switchSENSE technology). Since both components use DNA as a constituent material, they are easily combined by hybridizing the

complementary single-strand DNA added to each component in advance. We applied this process to the detection of enterotoxin contamination of food. Enterotoxin is a protein toxin associated with food poisoning and is produced by the staphylococcus aureus bacterium. A sensor to detect enterotoxin was developed in collaboration with the Innovative Research Center for Preventive Medical Engineering (PME), Nagoya University.[4)]

First, a modified DNA aptamer that targets enterotoxin was selected from a random library, and its sequence was identified [**Figure 6 (a)**]. Simultaneously, a DNA molecule containing a fluorescent dye (Cy3) at one end and a thiol group at the other was synthesized. This molecule was chemically immobilized on a gold surface through its thiol group [**Figure 6 (b)**]. Then the modified DNA aptamer was synthesized. It had an added complementary DNA sequence that targets immobilized DNA. When it was added to the solution, it hybridized with the immobilized DNA molecule [**Figure 6 (c)**]. The result was a sensor that changes its fluorescence in the presence of enterotoxin [**Figure 6 (d)**].

On attaching a microfluidic channel to this sensor and then streaming 10 μL of 0.8 nM aqueous solution (equivalent to 8 fmol) of enterotoxin as the target molecule, we were able to monitor in real time the decrease in fluorescence intensity caused by the binding of the target molecule to the modified DNA aptamer and to observe that the binding reaction entered a state of equilibrium in about 10 min (**Figure 7**). The protein detection time with this system is about 1/100 compared with that of conventional sandwich technology. Its application to the inspection of food products before shipping

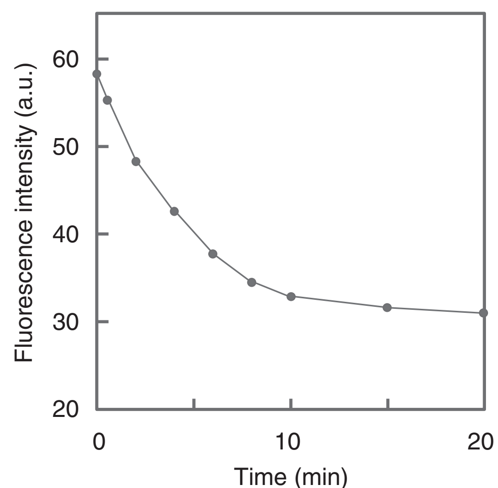
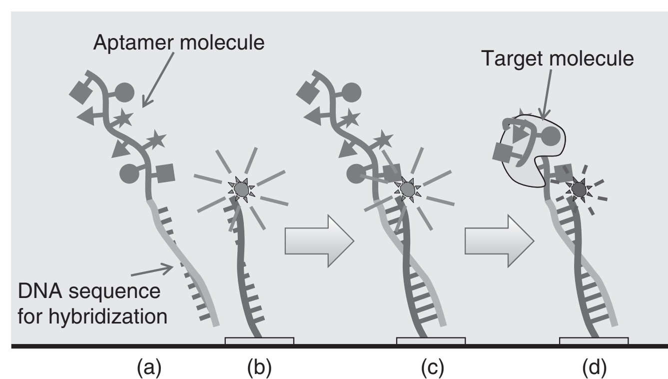


Figure 7
Fluorescence change caused by enterotoxin binding.



(a) Synthesis of modified DNA aptamers having DNA sequence for assembly
(b) Fixing of DNA with fluorescent dye and complementary sequence to gold electrode
(c) Assembly by formation of complementary double helix structure
(d) Detection of fluorescence change caused by binding of target molecule to modified DNA aptamer

Figure 6
Detector integration by self-hybridization of DNA.

would dramatically shorten inspection time and thereby contribute to their shipment in a safer and fresher condition.

6. Future developments

We plan to expand practical usage of the two elemental components described above in several ways.

For the modified DNA aptamer technology, we have already started producing aptamers having high affinity to target molecules provided by customers. This should lead to the commercialization of new diagnostic products using probe molecules that interact strongly with target proteins for which effective antibodies are barely generated.

For the switchSENSE technology, we aim to commercialize inter-molecular interaction analysis equipment in a team effort with TUM-WSI, which has been our research partner for over ten years. This equipment will enable not only high-sensitivity and real-time detection of disease marker proteins but also evaluation of molecular conformation changes.

7. Conclusion

This paper described the development background and the component technologies of a novel protein detection system achieved using DNA as a constituent material. As a substance that is easy to synthesize and analyze while being stable, DNA is an ideal material in the development of highly functional molecules created by the self-assembly of various functional components. Looking forward, we plan to expand our activities in turning our research results into practical products.

The development of modified DNA aptamers was supported by the Bio-IT Integrated Device Development Project (fiscal years 2002–2005) of the New Energy and Industrial Technology Development Organization (NEDO), and the joint research with Nagoya University was supported by the Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program (fiscal years 2006–2009) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT).

The authors would like to extend their deep appreciation to Professor Gerhard Abstreiter and Doctor Ulrich Rant and the entire research team at TUM-WSI for their invaluable cooperation in the development of the switchSENSE technology and to all concerned at the Innovative Research Center for Preventive Medical Engineering, Nagoya University, for their gracious help in the research of the enterotoxin detector.

References

1) Fujitsu: Functional molecules and their manufacturing method. T. Fujihara, S. Takeishi, S. Fujita. Patent No. 3978187, 2007-6-29. (in Japanese).
2) U. Rant et al.: Dynamic Electrical Switching of DNA Layers on a Metal Surface. *Nano Lett.*, Vol. 4, No. 12, pp. 2441–2445 (2004).
3) U. Rant et al.: Structural Properties of Oligonucleotide Monolayers on Gold Surfaces Probed by Fluorescence Investigations. *Langmuir*, Vol. 20, No. 23, pp. 10086–10092 (2004).
4) M. Aki et al.: High-speed, High-sensitivity Unlabeled Protein Detection Method—Application to Detection of Toxic Protein—. 58th Annual Meeting of the Japan Society for Analytical Chemistry (JSAC), Sapporo, 2009. (in Japanese).

Shozo Fujita
Fujitsu Asia Pte Ltd.
Dr. Fujita is engaged in the practical application of DNA aptamers.

Michihiko Aki
Fujitsu Laboratories Ltd.
Dr. Aki is engaged in the research and development of protein detection technology.

Kenji Arinaga
Fujitsu Laboratories Ltd.
Dr. Arinaga is engaged in the research and development of protein detection technology.

Tomoyasu Kichise
Fujitsu Asia Pte Ltd
Dr. Kichise is engaged in the research and development of DNA aptamers.

Tsuyoshi Fujihara
Fujitsu Asia Pte Ltd.
Dr. Fujihara is engaged in the research and development of DNA aptamers.