Real-Time Detection of DNA Hybridization Assay by Using Evanescent Field Microscopy

Do-Kyun Kim, Yong-Sung Choi, Yuji Murakami, Eiichi Tamiya and Young-Soo Kwon

Abstract - The determination of DNA hybridization reaction can apply the molecular biology research, clinic diagnostics, bioengineering, environment monitoring, food science and other application area. So, the improvement of DNA detection system is very important for the determination of this hybridization reaction. In this study, we report the characterization of the probe and target oligonucleotide hybridization reaction using the evanescent field microscopy. First, we have fabricated DNA chip microarray. The particles which were immobilized oligonucleotides were arranged by the random fluidic self-assembly on the pattern chips, using hydrophobic interaction. Second, we have detected DNA hybridization reaction using evanescent field microscopy. The 5`-biotinylated probe oligonucleotides were immobilized on the surface of DNA chip microarray and the hybridization reaction with the Rhodamine conjugated target oligonucleotide was excited fluorescence generated on the evanescent field microscopy. In the foundation of this result, we could be employed as the basis of a probe olidonucleotide, capable of detecting the target oligonucleotide and monitoring it in a large analyte concentration range and various mismatching condition.

Key Words - DNA hybridization, Evanescent field microscopy, DNA chip microarray, Real-time detection

1. Introduction

The bacterium *Escherichia coli* O157:H7 is a worldwide threat to public health and has been implicated in many outbreaks of haemorrhagic colitis, some of which included fatalities caused by haemolytic uraemic syndrome [1, 2]. Close to 75,000 cases of O157:H7 infection are now estimated to occur annually in the United States [3]. In Japan, outbreaks and sporadic cases have occurred frequently since May 1996. According to a report from the Food Sanitation Division of the Ministry of Health and Welfare, reported cases of verotoxin-producing *Escherichia coli* O157:H7 infection numbered 9451; including 1808 hospitalizations and 12 deaths in 1996 alone [4].

Verotoxin is also termed Shiga-like toxin, and two immunologically distinct types, known as type I and type II, exist. These have homologies of 56% in the amino acid sequences and 58% structural similarities in the nucleotide sequence. A total of 3021 strains of verotoxin-producing *Escherichia coli* O157:H7, comprising 28 different serotypes, were isolated in 1996. Of these, 2352 strains (77.9%) produced both verotoxin I and verotoxin II, while 359 strains (11.9%) produced verotoxin II only, and 307 strains (10.2%) produced verotoxin I. It has been reported that the verotoxin II production verotoxin-producing *Escherichia coli* O157:H7 was related to hemolytic uremic syndrome [5].

Therefore, we considered that the investigation of detection of the verotoxin II gene should precede that of the less toxic verotoxin I gene. In this study, we will measure a method, which involves a hybridization reaction based on the evanescent field microscopy using fluorescence conjugated oligonucleotides, for the rapid detection of the verotoxin II gene and, hence, for the detection of about 90% of the total amount of verotoxin-producing *Escherichia coli* O157:H7.

The hybridization reaction of the probe and target oligonucleotide in solution lead to change of mass, electrical charge or optical properties to be detected by microgravimetric, potentionmetric, amperometric or optical transducers [6]. So, We have detected DNA hybridization reaction by used evanescent field microscopy with optical properties. The properties of evanescent field microscopy which used in our research are as following.: 1) Real-time analysis of biomaterials. 2) Development of biomaterials chip with high density, many channel and high-throughput. 3) Detection of biomaterials of a high · low molecular weigh and low concentration. Therefore, the purpose of our research is the development of the high function, density system that can analyze various biomaterials with real-time function.

In this research, 5-biotinylated oligonucleotide

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probes were immobilized on the surface of particles as template for DNA chip microarrays via biotin-avidin bridge, and the hybridization reaction with the Rhodamne conjugated target sequences (be involved mismatching sequences) were studied *in vitro* by evanescent field microscopy excited fluorescence generated on the surface of particles. With the evanescent field excitation and real-time detection method used, we suggest that a very sharp discrimination of bulk fluorescence against surface excitation in combination with high excitation intensities can be achieved. And it can identify a single base mismatch in the hybridized duplex under experimental conditions. Hybridization performance of surface immobilized oligonucleotides to various concentrations of target oligonucleotides in solution were studied.

2. Experimental

2.1 Materials

All oligonucleotide sequences of the verotoxin II subunit A gene in *E. coli* O157:H7 strain used in this study were synthesized and HPLC purified by Nisshinbo Co. in Japan. Biotin phosphoramidite and fluorescein phosphoramidite (Rhodamine) were used in the synthesis of 5`-biotinylated oligonucleotides and 5`fluorescein conjugated oligonucleotides, respectively. The sequences of these oligonucleotides were given in Table 1.

Table 1 Oligonucleotide sequences

Oligonucleotide	Base sequence
Probe (PB-1)	5` Bio-TGCAGAGTGGTATAACTG 3`
Complementary (CR-1)	5` CAGTTATACCACTCTGCA 3`
Mismatching 1 (MR-1)	5` GGTTTCCATGACAACGGA 3`
Mismatching 2 (MR-2)	5` CAGTTATAGGACTCTGCA 3`
Mismatching 3 (MR-3)	5` CAGTTATACCACTCTGGG 3`

Where, Complementary (CR-1) and Mismatching 1 (MR-1) oligonucleotides are a part of the sequence encoding verotoxin II subunit A from *Escherichia coli* O157:H7. And solid lines are indicated mismatching sequence. The probe and target oligonucleotides were supplied as a dry powder and were reconstituted to 100 μM with TE buffer solution (10mM Tris, 1mM EDTA, pH 7.4) and stored at -20℃ until need for use. And, the Concentration of oligonucleotides were adjusted from 1pM to 1 μM by the aqueous buffer solution (10mM Tris-HCl, 0.2M NaCl, pH 7.9).

2.2 Fabrication of particles

One side of a cover glass (0.13～0.16mm, 18mm× 18mm, Takahashi Giken, Glass Co.) was made hydrophobic by cyclized perfluoro polymer (CPFP : 9.0wt-%, 0.5 μm) treatment. Then the cover glass was baked at 115℃ for 4hr. Cr (0.5～1.0Å/s, 50Å)/Au (5.0～10.0Å/s, 450Å) layers were evaporated on the other side. The particles were cut from cover glass using dicing machine (A-WD-10A, Tokyo Seimitsu), were 300 μm in length.

2.3 DNA immobilization

The clean bare Au electrode side of the particles was soaked into an aqueous solution (3mL) of 3,3`-dithiodipropionic acid (1mM) at room temperature for 30 min. Before drying, the carboxylic acid on the Au electrode was reacted with N-hydroxysuccinimide in the presence of water-soluble carbo-diimide[1-ethyl-3-(3-dimethylamino propyl)carbodiimide] in the aqueous solution for 30min. The Au electrode having the activated carboxyl groups was immersed in the aqueous buffer solution (10mM Tris-HCl, 0.2M NaCl, pH7.9) of avidin (100 μg in 1mL) for 1hr. the Avidin was not able to be removed from the electrode after rinsing with aqueous solution several times. The avidin-bound Au electrode was immersed into the aqueous solution (1mL) of ethanol-amine (1M) for 30min to deactivate the carboxyl group as β -hydroxyethylamide. The avidin-bound electrode was immersed into 1mL of the aqueous buffer solution (10mM Tris-HCl, 0.2M NaCl, pH7.9) of biotinylated oligonucleotide at 25℃ for 1hr, the electrode was picked up to control the immobilization amount. The biotinylated oligonucleotide was calculated to bind to one of the four binding sites of the avidin molecule. The immobilized amount of biotin-DNA was controlled by the immersion time in oligonucleotide solution.

2.4 Fabrication of pattern chip

One side of a cover glass (1.2～1.5mm, 20mm× 30mm, Takahashi Giken, Glass Co.) was made hydrophobic by CPFP treatment. The cover glass was baked at 115℃ for 4hr. Cr/Au layers were evaporated onto the CPFP. The negative photoresist (SU-8) was applied on the cover glass by a spin coater (1st : 500rpm/10sec, slope : 10sec, 2nd : 4000rpm/20sec, slope : 5sec, 3th : 5000rpm/5sec, slop : 5sec ; 1H-DXII, MIKASA) and baked at 100℃ for 30min. The cover glass was exposed to UV light (MJB3 UV400, Karl Suss) through a photo-mask on the resist film for 20sec and baked at 100℃ for 30min. It was then immersed in developer (SU-8(50)) for 30min,

rinsed with Millipore water twice, and then blown dry by N_2 gas.

The Au layer was etched in Au etchant (KI : I2: H2O = 4 : 1 : 40) for 80sec, rinsed with Millipore water 2 times, and blown dry by N_2 gas. The Cr layer was etched in Cr etchant (NaOH : K3[Fe(CN)6] : H2O = 2 : 5 : 20) for 40sec, rinsed with Millipore water twice, and then blown dry by N_2 gas. Finally, the patterned chip was divided by hydrophilic and hydrophobic area, respectively.

2.5 Fabrication of DNA chip microarray

The particles were arranged by the random fluidic self-assembly method on the patterned chip, using a hydrophobic interaction. One side of particles was treated as hydrophobic, and probe oligonucleotide was immobilized to the other side. The particles were arranged onto the hydrophobic sites of the patterned chip randomly in a suspension. The particles were arranged stably because a thick walls were fabricated. Finally, DNA chip microarray will be obtained if oligonucleotides are immobilized on the particles.

2.6 Evanescent field microscopy

The apparatus used for real-time fluorescence intensity measurements is the evanescent field microscopy. Its main parts are light source (YAG laser, 532nm), a standard plasmon resonator support (a trapezoid glass prism), Sample (DNA chip microarray and probe oligonucleotide), detector of light intensity and CCD camera. The YAG laser have 532nm emission line and expanded, collimated and reflected by dichroic mirrors. Input laser power was maintained at 10mW for all measurement. The expanded fluorescence image is focused by the o.45-inch diameter, f/10× lens. Image detection performed, by using CCD camera interfaced to PC. So, we could confirm the fluorescence image of sample on DNA chip microarray by real-time after spreading solution in which there was target oligonucleotide.

2.7 Hybridization assay measurement

The DNA chip microarray with immobilized probe oligonucleotide was fixed on a trapezoid glass prism of the evanescent field microscopy. For the hybridization reaction measurement, fluorescein conjugated target oligonucleotides of 400 μl that prepared in the aqueous buffer solution at a various contcenration and mismatching condition were spread onto the surface of DNA chip microarray that was immobilized probe oligonucleotide. The *in vitro* fluorescence signal was recorded in real-time every 1min for up to 60min. The hybridization reaction temperature was set at room temperature (23℃). For all the hybridization reaction, a new DNA chip microarray prepared from the same batch was used for each measurement. It should be noted that the optimal hybridization condition are different for different reaction method [7].

3. Results and Discussion

Real-time *in vitro* hybridization analysis offers the opportunity to obtain biological information, such as the specificity and kinetics of binding of biomolecules. These will give an insight into the relationship between the molecular structures and function.

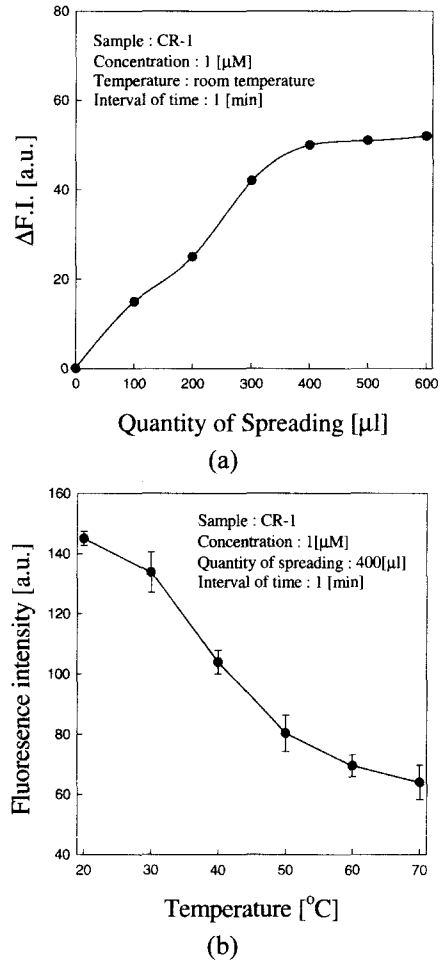


Fig. 1 (a) The time courses of fluorescence intensity to oligonucleotide hybridization at room temerature in the presence of different quantity f spreading
(b) dependence of hybridization on the reaction temperature

The time courses of fluorescence intensity to oligonucleotide hybridization at room temperature in the presence of different quantity of spreading are

shown in Fig. 1 (a). Here, the ⊿FI is indicated the shift of fluorescence intensity. the ⊿FI was given by subtracting the measured fluorescence intensity after spreading with the measured fluorescence intensity before spreading. In this figure, the ⊿FI was increased with quantity of spreading and saturated after about 400 μl. Given these considerations, the optimized quantity of spreading was determined to be 400 μl. So, We used the quantity of spreading of 400 μl in all further experiments. The dependence of hybridization on the reaction temperature is shown in Fig. 1 (b). The idea was to perform hybridization at a temperature below the selective temperature and then to gradually raise the hybridization temperature until melting temperature (T_m) of complementary oligonucleotides (CR-1, T_m = 62.2℃). In this figure, the ⊿FI was decreased with raise of Temperature and saturated after about 70℃. This is due to the decrease in bulk refractive index of the aqueous buffer solution as the temperature increases. In separate experiments, when the temperature was raised even further, the curve eventually went completely plateau (it means that the image of hybridization reaction go completely dark). An interesting area of further study for this technique would be to investigate the surface melting behavior of oligonucleotide hybrids, to see what difference between surface behavior and solution behavior are exhibited. In doing so, it would be important to make the distinction true melting experiments, in which probe oligonucleotide above the surface of DNA chip microarray exists in equilibrium with target oligonucleotide in solution bound to the surface. So, We used the room temperature (about 23℃) in all further experiments.

The Fig. 2 and 3 show the comparison of hybridization reaction to concentration of complementary oligonucleotide (CR-1). As shown in this figure, the fluorescence response that was measured by evanescent field microscopy was not detected gradually with decreasing of molar. it is considered that when the probe oligonucleotide was hybridized with target oligonucleotide until molar of 1nM, It can detect the fluorescence intensity by evanescent field microscopy. However, when the concentration of target oligonucleotide was essentially unchanged less than 0.1nM throughout the experiment.

The Fig. 2 also illustrates the experimental complication that shows the presence of optical interference fringes giving the image a corrugated appearance. For reason not completely understood, some cover glass or patterned chip-prism assemblies resulted in interference. Vapor deposition of gold film directly onto the prism itself might eliminate the problem, but was not done because of the convenience of working with disposable DNA chip microarray sample.

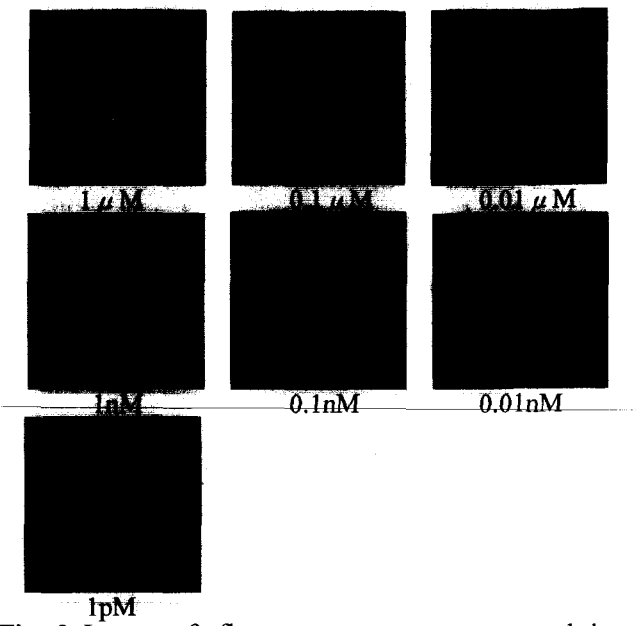
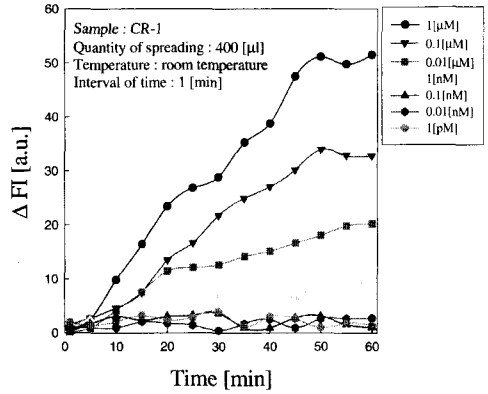
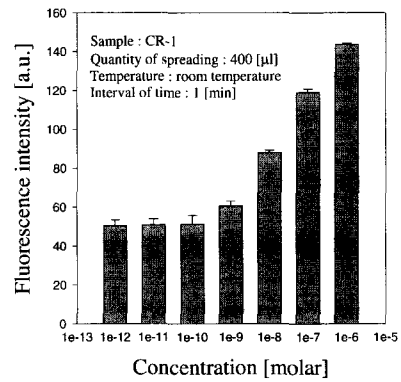


Fig. 2 Image of fluorescence response to real-time hybridization reaction as to concentration of target oligonucleotide (CR-1)



(a)



(b)

Fig. 3 comparison of hybridization reaction to concentration of complementary oligonucleotide (CR-1)
(a) ⊿F.I. versus time as to concentration of target oligonucleotide (CR-1)
(b) Fluorescence response versus concentration of target oligonucleotide (CR-1)

The differences in the fluorescence intensity between complementary oligonucleotide and mismatching oligonucleotide as to each mismatching condition are given in the Fig. 4. The oligonucleotides with many mis-matching sequence was observed only very low hybridization. For oligonucleotide with a complementary sequence hybridization becomes more stable and the fluorescence intensity seems to be similar to the mismatching sequence. The MR-2 and MR-3 have each mismatching sequence of central and terminal. With this set the role of point mutations was to be investigated. As results, a terminal mismatching sequence is shown more high Fluorescence intensity than central mis-matching sequence. The fluorescence response increased with the concentration of oligonucleotide in solution from 1nM. It is not relation mismatching condition generally.

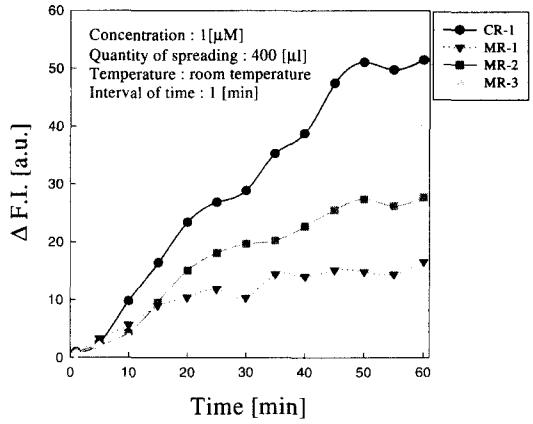


Fig. 4 comparison of hybridization reaction as to each mismatching condition
(a) ⊿F.I. versus time as to mismatching condition of target oligonucleotide
(b) Fluorescence response versus concentration of complemantary and each mismatching condition of target oligonucleotide

4. Conclusion

For the confirmation of development possibility of high function sensitivity system that can analyze various biomaterials with real-time function, we have measured as following. ; First, for the fabrication of DNA chip microarrary, the particles which were immobilized probe oligonucleotide was arranged by the random fluidic self-assembly on the pattern chips, using hydrophobic interaction. Second, Detection of DNA hybridization assay in accordance with a various condition using evanescent field microscopy. In the foundation this results, the image detection technique of evanescent field microscopy was shown to be able to distinguish between probe and target oligonucleotide regions on the surface at a various concentration and mismatching condition.

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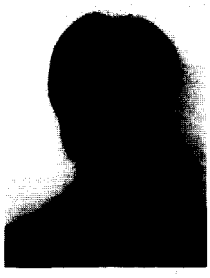

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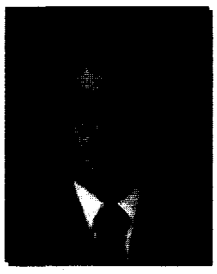

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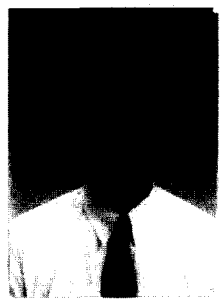

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