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ORIGINAL ARTICLE

DNA capturing machinery through spore-displayed proteins

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Abstract

Aims: The purpose of this study was to develop a general method for the facile development of a new DNA biosensor which utilizes streptavidin-displayed spores as a molecular machinery.
Methods and Results: Fluorescence spectroscopy was used as a monitoring tool for the streptavidin displayed on the surface of *Bacillus thuringiensis* spores and as a diagnosis method for DNA detection. As a proof-of-concept, four pathogenic bacteria including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella pneumonia* were used for the detection of pathogenic species. In addition, a set of mutant variants of Wilson's disease were also used for the detection of single nucleotide polymorphism (SNP) in this system.
Conclusions: This strategy, utilizing streptavidin-displayed spores, is capable of capturing DNA targets for the detection of pathogenic bacteria and for mutation analysis in Wilson's disease.
Significance and Impact of the Study: This approach could be useful as a simple platform for developing sensitive spore-based biosensors for any desired DNA targets in diagnostic applications.

Introduction

The microbial cell surface display has been employed for a wide range of biotechnological and industrial applications, including vaccine development (Liljeqvist *et al.* 1997), peptide screening (Boder and Wittrup 1997), antibody production (Dubel *et al.* 1993), environmental bio-adsorbent (Richins *et al.* 1997), whole cell catalyst (Lee *et al.* 2003) and biosensor development (Richins *et al.* 1997). For decades, a set of efficient display systems have been developed through the C-terminal, N-terminal and sandwich fusion on the surface of various micro-organisms, such as bacteria, yeast and phage (Xu and Lee 1999; Lee *et al.* 2003). Indeed, several anchoring motifs including *Escherichia coli* OmpC (Xu and Lee 1999), PhoE (Agterberg *et al.* 1990), FimH (Pallesen *et al.* 1995) and PapA (Steidler *et al.* 1993) were identified and characterized. However, the size of foreign proteins or peptides to be displayed on the surface is a critical factor (Lee *et al.* 2003). Thus, new cell surface display machinery that allows robust expression and display of target proteins is needed.

Current methods for the detection of pathogenic bacteria and single nucleotide polymorphism (SNP) by using enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), capillary electrophoresis and nanoparticles rely on antibodies or capture probes as the biomolecular recognition element and therefore require multistep processing (Yoo *et al.* 2010; Dwivedi and Jaykus 2011). For example, antibody-based immunoassays have some limitations including their high cost and long time to develop new antibodies for emerging targets. In addition, the antibody generally lacks the stability to

detect the targets under harsh conditions (Lee *et al.* 2003).

By contrast, the spore surface display technique has several advantages in the biosensor development (Ricca and Cutting 2003): (i) it is highly resistant to harsh environments such as ultraviolet and ionizing radiation, toxic chemicals and heat (Leuschner and Lillford 1999), (ii) proteins or peptides can be displayed easily and produced cost effectively (Lee *et al.* 2003), (iii) surface-displayed proteins or peptides are more amenable than antibodies from the point of view of biomolecular engineering and notably, (iv) the stability of the spore-anchored proteins on the surface of bacterial spore is a useful approach that could overcome some drawbacks of other systems for the biosensor applications (Ricca and Cutting 2003).

In this study, we developed a novel cell surface display machinery system that could detect DNA targets for the detection of pathogenic bacteria and SNP in Wilson's disease, as a proof-of-concept.

Materials and methods

Bacterial strains and plasmids

The *inhA* gene (Park *et al.* 2009) containing its promoter was amplified by PCR from the chromosome of *Bacillus thuringiensis* subspecies *kurstaki* HD1 using the following two primers: 5′-ATAAGAATGCGGCCGCATGTAATTCC-TCCCTAATTAT-3′ and 5′-GCTCTAGAACGATATAAAC-GAAC-3′.

The amplified fragments were digested with het restriction enzymes *Not*I and *Xba*I (sites underlined) and cloned into a *Bacillus* plasmid pS to make pSD-SA. Then, DNA fragments encoding core streptavidin (Argarana *et al.* 1986) were obtained by PCR amplification of genomic DNA from *Streptomyces avidinii* (ATCC 27419). The amplified streptavidin was cloned into pSD1 to make pSD-SA. The plasmid was transformed into *B. thuringiensis* 4Q7 by electroporation (Bio-Rad, Hercules, CA, USA). Other general molecular biological experiments were carried out following standard procedures (Sambrook and Russell 2001). The bacterial strains and plasmids used in this study are listed in Table 1. Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Ipswich, MA, USA) and used as recommended by the manufacturer.

Purification of spores

We cultured *B. thuringiensis cells* harbouring an expression vector for displaying proteins in CDSM media (Nicholson and Setlow 1990) at 37°C with 590 ***g*** for 48–60 h. Pellets were obtained from 100 ml of culture by centrifugation (2350 ***g***), and the pellet was suspended in 1 ml of 20% urografin (Sigma, St Louis, MO, USA), as described previously (Park *et al.* 2009, 2011). This suspension was gently layered over 10 ml of 50% urografin in a 15-ml centrifuge tube and then centrifuged at 4°C for 10 min at 15 870 ***g***. The pellet contained only free spores.

DNA hybridization

The amplified products (15 μl each) were directly added to hybridization solution (6X SSPE containing 1 mol l^{-1}

Table 1 Bacterial strains and plasmids used in this study

Strains	Relevant characteristics	Reference or source
Strains		
Escherichia coli JM109	*F′traD36 proA+B+ laclq Δ(lacZ) M15/Δ(lac-proAB) glnV44 e14- gyrA96 recA1 relA1 endA1 thi hsdR17*	New England Biolabs*
Streptomyces avidinii	Soil bacterium for streptavidin cloning	ATCC†27419
Bacillus thuringiensis subsp. *israelensis*	4Q7	BGSC‡4Q7
B. thuringiensis subsp. *kurstaki*	HD1, wild-type isolate	BGSC‡4D1
Plasmids		
pTrc99A	4·2 kb, *bla*, *trc* promoter	Pharmacia§
pS1	3·1 kb, *cat*, multicloing site	(Park *et al.* 2009)
pSD1	7·7 kb, *cat*, *inhA*, *trc* promoter	(Park *et al.* 2009)
pSD-SA	8·8 kb, *scFv* against HBV surface antigen::core streptavidin, pSD1 derivative	(Park *et al.* 2011)

*New England Biolabs, Ipswich, MA, USA.
†American Type Culture Collection, Manassas, VA, USA.
‡*Bacillus* Genetic Stock Center, Columbus, OH, USA.
§Pharmacia Biotech, Uppsala, Sweden.

NaCl, 60 mmol l^{-1} $NaH_2PO_4 \cdot H_2O$, 6 mmol l^{-1} EDTA and 20% formamide, pH 7·4). During hybridization on the surface of spores, the reaction chamber was shaken for 30 min at 30°C. After successful hybridization, all samples were washed three times with phosphate-buffered saline (PBS, pH 7·4).

Detection of pathogenic bacteria

To detect several pathogenic bacteria, we used the probe sequence as follows: 5′-GAAGTGCCGAGCATG-3′. The PCR was performed using universal primers with chromosomal DNA from clinically predominant bacteria, *Pseudomonas aeruginosa*, *Acinetobacter baoumannii*, *E. coli* and *Klebsiella pneumonia*, and the products were purified. Primer pairs were 5′-TTGTACACACCGCCCGC-3′ and 5′-TTCGCCTTTCCCTCACGGTACT-3′ for the first region (16S rDNA 1542-23S rDNA 477), 5′-AGTACCGTGAGGGAAAGGCGAA-3′ and 5′-TGGCTGCTTCTAAGCCAACATCCT-3′ for the second region (23S rDNA 477–1073), and 5′-AGGATGTTGGCTTAGAACAGCCA-3′ and 5′-CCCGACAAGGAATTTCGCTACCTTA-3′ for the third region (23S rDNA 1054–1926), as shown in Tables 2 and 3. The downstream primers that were used to amplify the strand complementary to the probes, modified with biotin at 3′-terminus were labelled with FITC for the detection. PCR amplifications with three primer sets covering the target genes were carried out over 10 cycles, each consisting of an initial denaturation at 94°C for 5 min and a second denaturation at 94°C for 50 s, annealing at 56°C for 50 s and extension at 72°C for 50 s, and then 30 cycles each consisting of a third denaturation at 94°C for 50 s, annealing at 56°C for 50 s and extension at 72°C for 50 s. The resulting PCR products were fractionated by agarose gel electrophoresis and finally purified. The detection of pathogenic bacteria was confirmed by a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Detection of SNP

For the detection of SNP to Wilson's disease, all of oligonucleotide probes (Table 4) were modified with biotin at the 3′-terminus. Genomic DNA from patients with Wilson's disease and nonpatients were kindly provided by the School of Medicine, Ajou University (Suwon, Korea).

Table 2 Primer sequences for 23S and 16S rRNA gene bacterial PCR in this study

Primer	Sequence (5′ to 3′)	Location (*Escherichia coli*)*	Use	Reference
1585F	TTGTACACACCGCCCGTC	1542–1559	16S PCR	This work
23BR	TTCGCCTTTCCCTCACGGTACT	457–478	23S PCR	(Dhillon *et al.* 1999)
23BF	AGTACCGTGAGGGAAAGGCGAA	457–478	23S PCR	(Dhillon *et al.* 1999)
MS37R	TGGCTGCTTCTAAGCCAACATCCT	1054–1077	23S PCR	(Dijkshoorn *et al.* 1993)
MS37	AGGATGTTGGCTTAGAAGCAGCCA	1054–1077	23S PCR	(Dijkshoorn *et al.* 1993)
MS38R	CCCGACAAGGAATTTCGCTACCTTA	1950–1926	23S PCR	(Dijkshoorn *et al.* 1993)

**Escherichia coli* 16S rRNA or 23S rRNA.

Table 3 Probe sequences for the detection of several pathogenic bacteria

Species	Source	Probe	GenBank accession no.	Sequence 5′to 3′
Pseudomonas aeruginosa	ATCC15522	Pae03	Y00432	GAAGTGCCGAGCATG
Acinetobacter baumannii	KCTC2771	Acti004	Direct sequencing	TGATGGAACTTGCTT
Escherichia coli	ATCC25922	Eco003	AB089505	CTGAAGCGACAAATG
Klebsiella pneumoniae	ATCC700603	Kpn002	X87284	GCTGAGACCAGTCGA

Table 4 Probe sequences for the detection of Wilson's disease (ATP7B)

No.	Name	Probe sequence	Modification	Length (mer)
1*	R778R	CTG GGC **CGG** TGG CTG	3′-Biotin	15
2†	R778P	CTG GGC C<u>C</u>G TGG CTG	3′-Biotin	15
3†	R778L	CTG GGC C<u>T</u>G TGG CTG	3′-Biotin	15
4†	R778Q	CTG GGC C<u>A</u>G TGG CTG	3′-Biotin	15

*Bold nucleotides indicate normal DNAs.

†Underlined nucleotides indicate single-mismatch point, having in patients with Wilson's disease.

The PCR amplification of each mutation region was performed in 50 μl reaction mixture containing 10X Taq buffer, 0·2 mmol l^{-1} dNTPs, two units of Taq polymerase (Takara Shuzo, Shiga, Japan), 100 ng of genomic DNA, 5 pmol l^{-1} upstream and 25 pmol l^{-1} downstream primers. Primer pairs were 5′-GCCCTGTGACATTCTTCGA-3′ and 5′-GCTGCTGTTACCTTTGCCA-3′ for mutation detection. PCR amplification was carried out for 40 cycles: 1 min at 95°C, 1 min at 57°C and 30 s at 72°C. To prevent the formation of self-dimers, each primer was designed to have A or AA at the 3′-end. The *ATP7B* coding region, including a Korean mutation from clinical samples, was amplified by PCR yielding an amplicon of 97 base pairs in size. The detection of SNP was observed with a SpectraMax M2 microplate reader.

Results

Display of streptavidin on the surface of spores

To display streptavidin on the spore surface, a DNA fragment encoding core streptavidin that included four biotin binding regions was obtained by PCR from genomic DNA in *S. avidinii* ATCC 27419 and cloned to make pSD-SA. The plasmids were transformed to *B. thuringiensis* 4Q7 to display streptavidin on the surface of the spores. As shown in Fig. 1(a), the streptavidins displayed on the surface of the spores were reacted with biotinylated peptides conjugated with FITC and specifically interacted with biotinylated peptides, suggesting that streptavidin was externally exposed and displayed on the spore surface. This may be due to the multivalent interaction of streptavidin displayed on the spore surface to the targets. The control cells that did not have streptavidin did not react to corresponding targets. Thus, most of the streptavidins were successfully displayed on the surface of the *Bacillus* spores. It is interesting to note that molecular recognition events between streptavidin and biotinylated targets could be detected on the *Bacillus* spore surface.

Detection of pathogenic bacteria

To demonstrate further insight into biosensor development, we used streptavidin-displayed spores for the detection of several pathogenic bacteria including *Ps. aeruginosa*, *Ac. baumannii*, *E. coli* and *Kl. pneumonia*. The primer sets and probe sequences used in this study are shown in Tables 2 and 3. The detection of pathogenic bacteria was carried out by means of a fluorescence multiwell reader. As expected, several pathogenic bacteria could be detected on streptavidin-displayed spores by fluorescence assay, as shown in Figs 1(b) and 2. We saw that the spores in without streptavidin showed a poor mean fluorescence intensity (thin lines in Fig. 2). However, the fluorescence intensity of the streptavidin-displaying spores was seen to have an approx. 2–4-fold increase in mean fluorescence over background (solid lines in Fig. 2). More interestingly, this approach is available within 30 min after DNA hybridization for the detection of pathogenic bacteria. Although four well-known pathogenic bacteria models were tested, more bacterial and fungal pathogenic species could be added to this system by using genus- and species-specific probes.

Detection of SNP in Wilson's disease

Based on the aforementioned results, we assumed that streptavidin-displayed spores could also be used for the detection of SNP in Wilson's disease through DNA

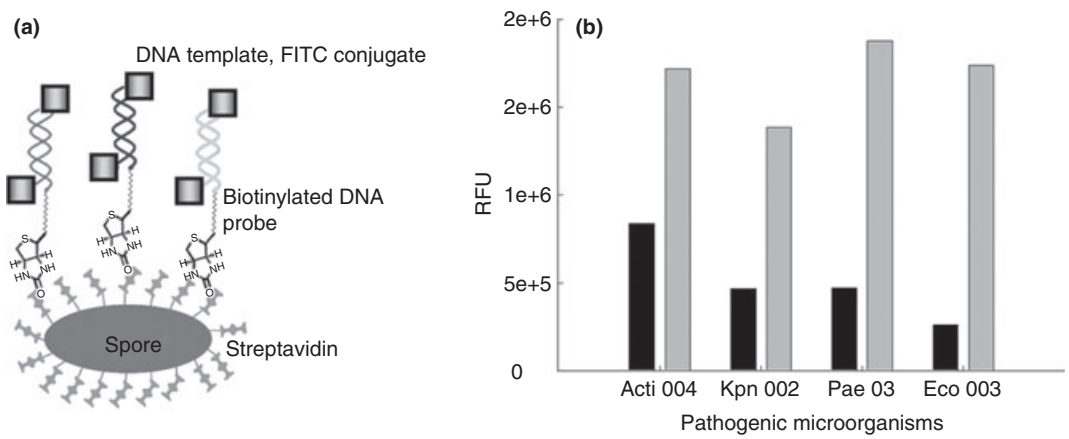

Figure 1 (a) Schematic representation of a spore-display system for DNA hybridization. (b) Fluorescence assay for the detection of various pathogenic micro-organisms after DNA hybridization to non-displaying spores (black bars) and after DNA hybridization to streptavidin-displayed spores (grey bars). Acti004: *Acinetobacter baumannii*, Kpn002: *Klebsiella pneumoniae*, Pae03: *Pseudomonas aeruginosa* and Eco003: *Escherichia coli*.

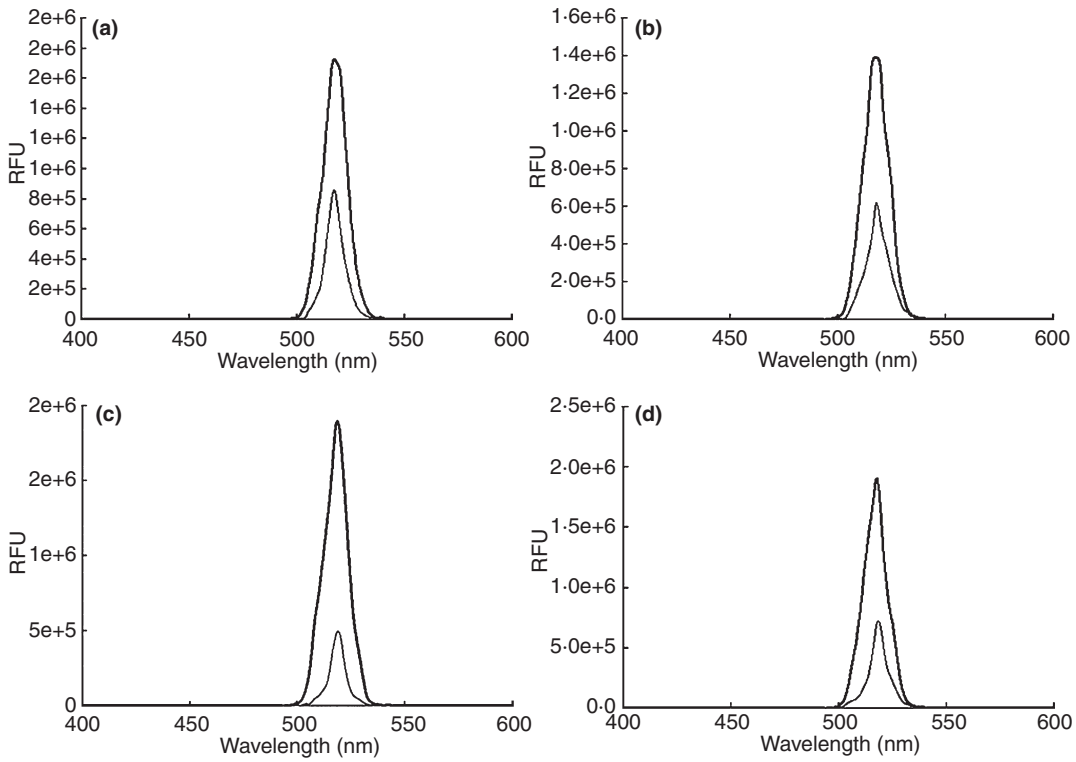

Figure 2 Emission spectra for pathogen detections of (a) *Acinetobacter baumannii*, (b) *Klebsiella pneumoniae*, (c) *Pseudomonas aeruginosa* and (d) *Escherichia coli coli* after DNA hybridization to streptavidin-displayed spores (thick lines) and after DNA hybridization to control spores (thin lines), respectively.

hybridization. To verify our hypothesis, we used four sets of probe sequences that are normal DNA (R778R) and a high frequency of single-mismatched DNA sequences (R778P G → C substitution, R778L G → T substitution and R778Q G → A substitution) containing biotin at the 3′-end as shown in Table 4. Then, biotinylated probes of 5 μmol l^{-1} were incubated with streptavidin-displayed spores of $1{\cdot}4 \times 10^8$ CFU ml^{-1} in PBS solution for 1 h at room temperature. DNA capturing machinery in the presence of biotinylated probes as molecular recognition elements was applied to detect different types of Wilson's disease mutation variants by fluorescence spectroscopy. It is not surprising that R778R was perfectly hybridized to normal DNA, which comes from healthy patients. As expected, the DNA capturing machinery with streptavidin-displayed spores could be used for detecting R778L and R778Q mutations as shown in Fig. 3, while the detection of R778P was rather lower than those two. It is not clear, but we assumed that the changes were probably due to different conformations when hybridized to the target DNAs.

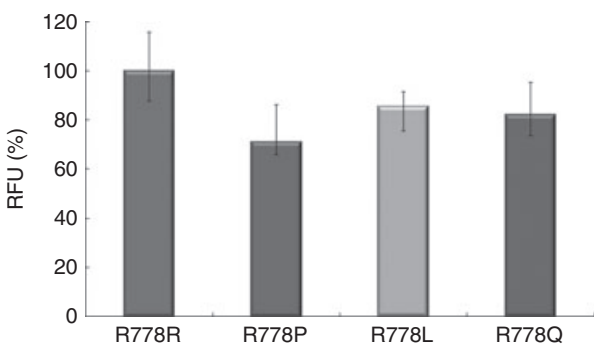

Figure 3 Fluorescence assay for SNP detection against patients with Wilson's disease. Each phenotype indicates the amino acid mutation of ATP7B patient.

Discussion

The streptavidin–biotin interaction is one of the strongest interactions between proteins and ligands for studying biomolecular interactions in the development of biosensors. As has previously been demonstrated (Kim *et al.* 2005), this model system is a useful tool for studying molecular recognition in solution and on chips, for biosensor development. Figure 1(a) shows a schematic description of the DNA capturing machinery for the detection of pathogenic bacteria and SNP in Wilson's disease as model system. By using this technique, four well-known pathogenic bacteria were tested and the feasibility

of our system was validated. The fluorescence intensity of streptavidin displayed on the surface of the spores is much higher, while the fluorescence of nonstreptavidin-displayed spores is quite low (Fig. 1b). This indicates that streptavidin was successfully displayed on the surface of *B. thuringiensis* spores and reacted with biotinlyated DNA targets. It is known that one streptavidin can bind to 4–5 individual biotin molecules (Kim *et al.* 2005; Park *et al.* 2011). According to our previous reports (Park *et al.* 2009, 2011), we assumed that at least 700–800 streptavidin molecules could be displayed on individual *Bacillus* spores used in this study. More notably, the order of mean fluorescence intensity was as follows (Figs 1b and 2): *P. aurogenosa* (Pae03) > *E. coli* (Eco003) > *Ac. baumannii* (Acti004) > *Kl. pneumonia* (Kpn002). *Pseudomonas aeruginosa* is one of the leading causes of pathogenic bacteria, which have morbidity and mortality rate ranging from 25 to 50% (Pfaller *et al.* 1989). *Acinetobacter baumannii, Kl. pneumonia* and *E. coli* have also served as important bacteria (Yoo *et al.* 2010). This finding is not clear, but it may be due to the specificity of the probe sequences tested. Despite the emergence of advanced technology for the detection of pathogens (Deer *et al.* 2010; Dwivedi and Jaykus 2011) using ELISA, PCR, real-time PCR, nanoparticle-based assay and other techniques, accurate and simple methods are highly desirable. Our strategy has broad spectrum against pathogenic bacteria and is also available within 30 min of DNA hybridization. Therefore, it can provide a simple method for the detection of pathogenic bacteria.

Wilson's disease is an autosomal recessive disorder of the copper transport metabolism that leads to an accumulation of copper in hepatocytes (Kim *et al.* 2008). Much effort has been made to identify a series of new mutations for detecting Wilson's disease in Japanese, Taiwanese, Chinese and Korean patients (Kim *et al.* 1998, 2008; Ferenci 2006). Among these mutations, Arg778Leu is one of the highest frequency mutations in Korea, causing mutations in the *ATP7B* gene coding copper transport P-type ATPase at exon 8 (Kim *et al.* 1998). R778R was perfectly hybridized to normal DNA that comes from healthy patients. As expected, streptavidin-displaying spores could be detected by R778L and R778Q mutations (Fig. 3), while the detection of R778P is rather lower than those two. R778L and R778P replace a hydrophobic amino acid, and R778Q changes a hydrophilic amino acid. Although more general mutation variants were not tested in this study, our system has the potential to carry out mutational analysis in genetic diseases if allele-specific probes are used.

In conclusion, we described an efficient new approach for DNA capturing machinery, which can recognize pathogenic bacteria and a set of mutation variants in Wilson's disease on the surface of bacterial spores. The spore surface display technique is well established and can be used to express any of the proteins or peptides used in many applications. The most important reason for this is that the bacterial spore is thermally and chemically stable. In addition, any proteins or other targets to be displayed on the spore can be mass produced. Such interesting characteristics, along with genetic modification, enable the use of bacterial spores as a molecular machinery in biosensor applications. Our strategy can also be applied to the diagnosis of other pathogens and genetic diseases. The functionalized bacterial spores as carriers with DNAs and proteins could be useful in many biomedical and biosensor applications, including molecular recognition studies of diverse DNA–DNA and antigen–antibody interactions.

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