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Short communication

Dipstick-type biosensor for visual detection of DNA with oligonucleotide-decorated colored polystyrene microspheres as reporters

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ABSTRACT

In recent years, there is a continuously growing interest in the development of biosensors for rapid, simple and inexpensive DNA tests suitable for the small laboratory or for on-site testing. Detection is accomplished through electrochemical, optical or gravimetric transduction. We report on the development of disposable dipstick-type DNA biosensors that employ oligonucleotide-decorated colored polystyrene microspheres as reporters and enable visual detection of DNA sequences without the use of instrumentation. The biosensors have been designed to detect DNA molecules that contain both, a biotin moiety and a segment that is complementary to the oligonucleotide attached on the surface of blue or red microspheres. Capture of the hybrids by immobilized streptavidin at the test zone results in the formation of a colored line. The biosensors were applied to: (a) detection of single-stranded DNA, (b) detection of PCR-amplified double-stranded DNA and (c) genotyping of single nucleotide polymorphisms (SNP). The results were compared with sensors based on gold nanoparticle reporters. It is also demonstrated that the microspheres offer the potential for multicolor detection of specific DNA sequences.

1. Introduction

Nucleic acid hybridization assays have evolved significantly to allow a wide spectrum of applications for the routine laboratory or for field testing. In recent years research efforts aim at: (a) High analyte-throughput systems enabling parallel detection of thousands sequences in one or a few samples (e.g., DNA microarrays). (b) High sample-throughput assays based on microtiter wells or spectrally encoded beads that allow analysis of a large number of samples, each for a few target sequences. (c) There is a continuously growing activity on the development of biosensors for rapid, simple and inexpensive DNA tests suitable for the small laboratory or for on-site testing.

The principle of a DNA biosensor entails the immobilization of a DNA probe on the surface of the sensor, hybridization and detection by electrochemical (Lucarelli et al., 2004; Ozsoz et al., 2003), optical (Minunni et al., 2001; Mannelli et al., 2007) or gravimetric transduction (Tombelli et al., 2005).

The types of DNA biosensors listed above require instrumentation and, in most cases, involve several incubation and washing steps. Recent reports from our group and others (Glynou et al., 2003; Matsubara and Kure, 2003; Kalogianni et al., 2006, 2007a,b; Litos et al., 2007; Toubanaki et al., 2008) have described the development of novel disposable biosensors that enable visual detection of DNA without the use of instruments. Moreover, these biosensors are based on a dry-reagent dipstick format that eliminates multiple incubation and washing steps and minimizes the requirements for highly qualified personnel. The transduction principle exploits the characteristic red color of gold nanoparticles (AuNP) that is due to the plasmon resonance peak at 520 nm (Hayat, 1989). Hybridization triggers the accumulation of AuNP at the test zone of the sensor enabling visual detection.

In the present work we report on the development of dipstick-type DNA biosensors that employ oligonucleotide-decorated red and blue colored polystyrene beads as reporters. The performance of the biosensors is evaluated by: (a) the detection of single stranded target DNA, (b) detection of polymerase chain reaction (PCR)-amplified double-stranded DNA and (c) genotyping of single-nucleotide polymorphisms (SNP). In all cases the new sensors are compared with AuNP-based biosensors. In addition, we demonstrate that the bead-based sensors allow dual-color detection of

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specific DNA sequences, such as the color-coded identification of bacteria.

2. Materials and methods

The apparatus and reagents are presented in Supplement.

2.1. Coupling of oligonucleotides to microspheres

Microspheres (17 fmol) suspended in 50 μL of 0.1 M MES buffer, pH 4.5, were centrifuged for 3 min at 10,000 × *g* and washed twice with 50 μL of the same solution. The microspheres were resuspended in 100 μL of 0.1 M MES, pH 4.5, sonicated for 5 min and mixed with 100 pmol of 5′ amino-labelled $(dT)_{30}$ oligonucleotide. A 2-μL aliquot of freshly prepared 100 g/L EDC solution, in water, was added and the mixture was incubated at ambient temperature for 30 min. The addition of EDC was repeated with a fresh solution followed by 30-min incubation. The microspheres were pelleted by centrifugation at 10,000 g (3 min) and washed twice with 100 μL of 0.2 mL/L Tween-20. The $(dT)_{30}$-conjugated microspheres were resuspended in 20 μL of tailing reaction mixture (0.2 M potassium cacodylate, 25 mM Tris, pH 7.2, 0.1 mL/L Triton X-100, 2.5 mM $CoCl_2$, 0.5 mM dTTP) and sonicated for 20 s. Terminal deoxynucleotidyl transferase (30 U) was added and the mixture was incubated at 37 °C for 1 h. The microspheres were centrifuged as above and washed once with 100 μL of 0.2 mL/L Tween-20 and once with 100 μL of 1 g/L sodium dodecyl sulphate (SDS). The microspheres were sonicated for 10 s during each washing step. The poly(dT)-conjugated microspheres were finally resuspended in 85 μL of 15% sucrose, 45 mM NaCl, 2.5 g/L SDS and 2.5 mL/L Tween-20 solution.

2.2. Preparation of the dipstick-type DNA biosensor

The dry-reagent strip (4 mm × 70 mm) consisted of an immersion pad, a conjugate pad, a laminated membrane and an absorbent pad assembled on a plastic adhesive backing that provides rigidity. Streptavidin (27 pmol) and poly(dA) (1.2 pmol) were immobilized on the test zone and control zone of the strip, respectively. Blue or red microspheres coupled with poly(dT) strands (1 fmol), were placed on the conjugate pad.

2.3. Visual detection of PCR products by using the DNA biosensor

A 225-bp segment of maize-specific invertase (IVR) gene was amplified from genomic DNA by PCR (Elenis et al., 2007). The product was first hybridized with (dA)-tailed IVR-specific probe. Tailing of the probe was performed as above (Section 2.1) using 0.5 mM dATP and 100 pmol probe. For hybridization, 10 μL of PCR product was mixed with 1 μL of 0.9 M NaCl and 1 pmol probe. The mixture was heated at 95 °C (2 min) and then incubated at 37 °C (5 min). A 5-μL aliquot was applied to the conjugate pad of the sensor. The strip was then immersed into the developing solution containing 40 mL/L glycerol, 10 g/L SDS and 0.5 mL/L Tween-20 in PBS, pH 7.4. SDS and Tween-20 were added in order to avoid the appearance of nonspecific lines at the test zone of the sensor. A colored line (blue or red, depending on the color of the microspheres) at the test zone of the sensor denotes the presence of the amplified product. The control zone is always colored to confirm proper function of the sensor.

2.4. Genotyping of single nucleotide polymorphisms

Genomic DNA was isolated from 200 μL human whole blood using the QiaAmp DNA blood mini-kit. PCR amplification of MBL2 gene was performed as described previously (Litos et al., 2007). PEXT reactions (20 μL) were performed in the thermal cycler in a mixture containing 20 mM Tris–HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 1 mL/L Triton X-100, 1 mM $MgSO_4$, 0.25 U of Vent (exo-) DNA polymerase, 0.2 pmol amplified DNA, 2 pmol of the appropriate probe (normal or mutant), 2.5 μM each of dATP, dCTP, and dGTP, 1.9 μM dTTP, and 0.62 μM biotin-11-dUTP. The reaction conditions for the SNP at position -550 (c.-619G > C) were: initial denaturation at 95 °C for 5 min, followed by three cycles of denaturation at 95 °C (15 s), primer annealing at 65 °C (10 s) and extension at 72 °C (15 s). For detection of the SNP at position -221 (c.-290G > C), the cycling parameters were as above except that the annealing step was at 55 °C (10 s). PEXT products were subjected to a final denaturation step at 95 °C (5 min) and placed immediately on ice. A 5-μL aliquot of denatured product was applied onto the conjugate pad next to the microspheres. The wicking pad was then dipped into a microcentrifuge tube containing 200 μL of 10 mM phosphate buffer (pH 7.4), 75 mM NaCl, 30 mL/L glycerol, and 10 g/L SDS. The products were detected visually in about 10 min.

2.5. Dual-color biosensor for detection of bacteria

A fragment of the 23S rRNA gene was amplified by PCR using universal primers (Kalogianni et al., 2007b). Specific probes for *Escherichia coli* and *Staphylococcus aureus* were coupled to blue and red microspheres, respectively, as described in Section 2.2. Biotinylated PCR products were denatured at 95 °C (5 min) and a 5-μL aliquot (about 50 fmol) was added to a mixture of the conjugated microspheres (blue and red) (1 fmol of each) in 0.2 M NaCl, 0.1 M Tris, pH 8.0 and 0.8 mL/L Triton X-100. The mixture was incubated for 10 min at 37 °C. The microspheres were pelleted by centrifugation for 3 min at 10,000 g, resuspended in 10 μL of 15% sucrose, 45 mM NaCl, 2.5 g/L SDS, 2.5 mL/L Tween-20 and applied to the sensor. A 2.5-μL aliquot of blue microspheres (0.5 fmol) coupled with $(dT)_{30}$ probe was also applied to the sensor strip. The sensor was then immersed into the developing solution. A blue or red line was formed at the test zone of the sensor indicating the presence of *E. coli* or *S. aureus*, respectively, in the sample. A second line (blue) was formed at the control zone of the strip confirming the proper function of the sensor.

3. Results and discussion

A schematic illustration of the principle of the dipstick-type biosensor for visual detection of DNA by using oligonucleotide-decorated colored polystyrene microspheres as reporters is presented in Fig. 1. Three assay configurations are also presented in Fig. 1: (A) detection of ssDNA, (B) detection of PCR-amplified dsDNA and (C) detection of SNP genotyping products. The biosensor has been designed to detect DNA molecules that contain both a biotin moiety and a segment that is complementary to the oligonucleotide attached on the surface of the microspheres. The sample is placed on the conjugate pad of the sensor, which is then immersed in the developing buffer. The buffer migrates along the strip by capillary action. The DNA is linked, through hybridization, to the microspheres and captured at the test zone through biotin/streptavidin interaction. The proper function of the biosensor is confirmed at the control zone in which immobilized poly(dA) strands capture poly(dT)-decorated microspheres. The size of 300 nm for the microspheres was chosen because it is the optimum for the porosity of the nitrocellulose membrane (5 μm) thereby allowing smooth flow of the microspheres with the developing solution.

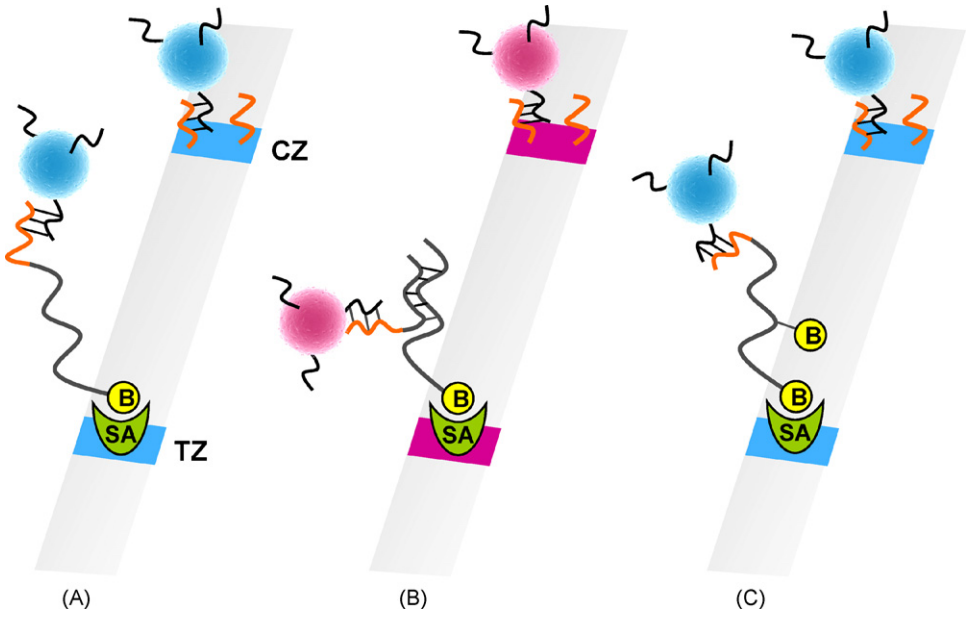

Fig. 1. Schematic illustration of the principle of the dipstick-type biosensor for visual detection of DNA by using oligonucleotide-decorated colored polystyrene microspheres as reporters. Three assay configurations are presented. (A) The target sequence is a ssDNA fragment that is biotinylated at the 5′ end and carries a poly(dA) segment at the 3′ end. The target is captured from immobilized streptavidin at the test zone of the sensor and detected through dA/dT hybridization with the microspheres. (B) The target sequence is a 5′ biotinylated PCR product that is denatured and hybridized, briefly, with a poly(dA)-tailed oligonucleotide probe. (C) Genotyping of single nucleotide polymorphisms (SNP) by primer extension (PEXT) reaction followed by visual detection of PEXT products using the DNA biosensor. The primer contains a $(dA)_{30}$ segment at the 5′ end and is extended with dNTPs and biotin-dUTP only if it is perfectly complementary with the interrogated sequence. Extended products are captured from immobilized streptavidin at the test zone and detected by hybridization with the oligo(dT)-microspheres. The control zone of the sensor comprises immobilized oligo(dA). SA = streptavidin; B = biotin; TZ = test zone; CZ = control zone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.1. Oligonucleotide–microsphere conjugates

We optimized the $(dT)_{30}$-to-microspheres molar ratio in the coupling reaction. Aliquots containing from 25 to 400 pmol oligonucleotide were mixed with a constant amount of 17 fmol blue microspheres (10^{10} particles). The final concentration of EDC was 4 g/L. All optimization studies were carried out by applying to the strip 10 fmol of 5′ biotinylated single stranded DNA target that carries a poly(dA) tail at the 3′ end. The results are presented in Fig. S1 (Supplement). The density of the test zone increases with the $(dT)_{30}$-to-microspheres molar ratio up to a ratio of 6000. At higher ratios the color density drops. The decrease in the signal at high oligonucleotide-to-microsphere ratios was also reported, very recently, by another group (Sheng and Ye, 2008) that studied various methods for probe attachment to μm-size glass beads. The effect was attributed to a decrease in hybridization efficiency when the bead surface becomes overloaded with oligonucleotide probes.

The dependence of signal density on EDC concentration was studied in the range of 1–6 g/L, while the $(dT)_{30}$-to-microspheres molar ratio was kept constant at 6000. The highest color density at the test zone was obtained using 4 g/L EDC (Fig. S1). At 6 g/L EDC, the density drops. The conjugation reaction is carried out in one step, in which the microspheres, the oligo and the carbodiimide are added simultaneously to the reaction mixture. At high concentrations there are enough EDC molecules to react separately with the microspheres and the oligonucleotide thus decreasing the oligonucleotide–microsphere conjugation efficiency.

The effect of the amount of poly(dT)-conjugated microspheres was studied in the range of 0.2–2 fmol microspheres per strip (Fig. S1). As the number of microspheres increases, the color density of both the test zone and the control zone becomes higher, because more beads are available for binding to the target DNA at the test zone and reacting with immobilized poly(dA) strands at the control zone of the strip. The optimum amount is 1–1.5 fmol microspheres.

We investigated whether the tailing of $(dT)_{30}$ improves the detectability of the biosensor due to the fact that longer dT strands attached on the surface of the microspheres would allow more effective hybridization. Aliquots containing 0, 0.5, 1, 2.5, 5 and 10 fmol of biotinylated (dA)-tailed oligonucleotide (as target DNA) were assayed in parallel by using strips containing blue microspheres coupled either with $(dT)_{30}$ or with poly(dT) strands prepared by tailing with dTTP after the conjugation reaction. The color density was consistently higher when poly(dT) was used instead of $(dT)_{30}$.

We performed direct comparison studies of sensors constructed by using either microspheres (blue or red, 1 fmol per strip) or gold nanoparticles (40 nm in diameter) as reporters. All reporters were conjugated to poly(dT) strands. The gold nanoparticle-based strips were prepared as described previously and 7.5 fmol of functionalized nanoparticles were applied per strip (Glynou et al., 2003). The three types of strips were used for detection of 0, 0.5, 1, 2.5, 5 and 10 fmol of biotinylated (dA)-tailed oligo. The results are presented in Fig. 2. We observe that the colored polystyrene microsphere reporters give the same detectability as gold nanoparticles (about 0.5 fmol biotinylated poly(dA) target).

3.2. Visual detection of PCR products

To assess the ability of the DNA biosensor to detect double-stranded DNA, we applied various amounts of PCR products (225 bp, IVR-amplified DNA) ranging from 0 to 200 fmol per strip. Strips constructed with blue and red microspheres were compared directly with those based on AuNP (Fig. 2). As low as 2.5 fmol PCR product can be detected by all three types of sensors, regardless of the particle used as reporter. It is worth noting that the detectability

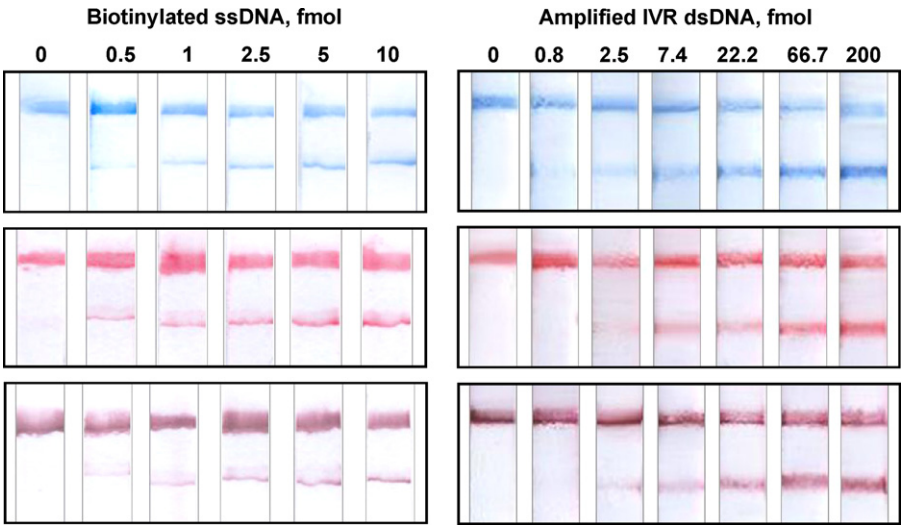

Fig. 2. Effect of the amount of target DNA on the color density of the test zone of the biosensor. A 5′ biotinylated ssDNA fragment with a poly(dA) tail at the 3′ end and a PCR-amplified dsDNA (225-bp) fragment were used as targets. Blue microspheres (top), red microspheres (middle) and gold nanoparticles (bottom) are compared directly as reporters. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

is higher with ssDNA targets (see Section 3.1) than dsDNA due to the fact that the reannealing of the target strands in dsDNA competes with probe–target hybridization.

3.3. Reproducibility

To determine the reproducibility of the biosensor, samples containing 15 and 30 fmol PCR product were tested using strips based on blue or red microspheres. The strips were scanned with a simple desktop scanner (Hewlett–Packard ScanJet 3400C) followed by densitometry. The CVs (CV is the coefficient of variation, i.e., the relative standard deviation) were found to be 7.5% and 8.4% (blue microspheres-based strips), respectively. The corresponding CVs obtained from red microsphere-based strips were 5% and 9.4%. Thus both colored microspheres have very similar performances.

3.4. Genotyping of single nucleotide polymorphisms

Fig. 1 illustrates the principle of SNP genotyping by PEXT reaction and visual detection of the products using the DNA biosensor. Aliquots of the PCR product were subjected to two PEXT reactions using allele-specific primers. Each primer comprised a sequence adjacent to the SNP site with the 3′ nucleotide complementary to the allelic variant and a $(dA)_{30}$ segment at the 5′ end that allows hybridization with the poly(dT)-conjugated microspheres. Extension by DNA polymerase and biotin-dUTP incorporation takes place only if the primer matches the interrogated sequence. The denatured PEXT product is captured by immobilized streptavidin at the test zone and linked to the colored microspheres through poly(dA)/(dT) hybridization. If the primer has not been extended (because of a 3′ mismatch) then the hybrids are not captured at the test zone due to lack of biotin.

As a model, we have genotyped SNPs at positions -550 and -221 of MBL2 gene (20 samples). All genotyping experiments were carried out using three types of biosensors based on blue microspheres, red microspheres and gold nanoparticles as reporters. The results for each polymorphic site are presented in Fig. 3 and Figs. S2 and S3 (Supplement). The three types of sensors give identical genotypes. The results are fully concordant with sequencing data.

3.5. Dual-color detection of bacteria

A dual-color biosensor was developed to discriminate *E. coli* from *S. aureus*. PCR-amplified DNA from *E. coli* or *S. aureus* was hybridized with a mixture containing blue and red microspheres conjugated with *E. coli* and *S. aureus* probes, respectively. A blue zone was observed if *E. coli* was present in the sample, whereas a red zone indicated the presence of *S. aureus* (Fig. 3). In both assays, negative controls were included that contained water instead of target DNA.

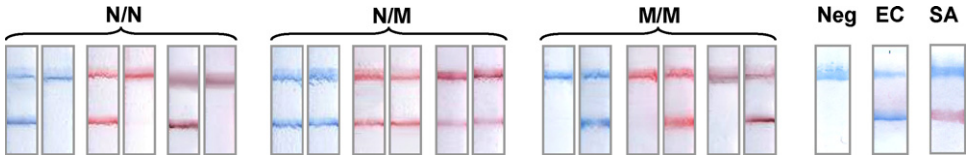

Fig. 3. Application of the biosensor to genotyping of single-nucleotide polymorphisms and to dual-color detection of bacteria. The genotyping of -221 polymorphism of MBL2 gene was carried out by primer extension (PEXT) reaction followed by visual detection of the products using biosensors based on blue microspheres (left pair of strips), red microspheres (middle pair of strips) and gold nanoparticles (right pair of strips) as reporters (for comparison). For each sample, two PEXT reactions were performed, one with the "normal" primer (left strip of each pair) and the other with the "mutant" primer (right strip of the pair). N = normal allele; M = mutant allele. The dual-color detection of bacteria was applied to the discrimination of *Escherichia coli* (EC) from *Staphylococcus aureus* (SA) by the color change of the test zone of the sensor. PCR-amplified 5′ biotinylated DNA from EC or SA was hybridized with a mixture of blue and red microspheres conjugated with EC-specific and SA-specific probes, respectively. A blue color at the test zone denotes the presence of *E. coli*, whereas a red color at the test zone signifies the presence of *S. aureus* in the sample. Neg = negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Conclusions

Disposable dipstick-type DNA biosensors based on oligonucleotide-decorated colored polystyrene microspheres offer equivalent performance with AuNP reporters both in the detection of PCR products and genotyping of single nucleotide polymorphisms. Because of the high detectability of the biosensor, visual genotyping requires only three cycles of primer extension reaction and subsequent detection, directly, without prior purification of the DNA after the PCR step or the PEXT reaction step. The assays are simple, rapid (visual detection within minutes), inexpensive and require no specialized instruments. The dipstick format eliminates multiple incubation and washing steps and minimizes the need for qualified personnel. The color of the microspheres (blue or red) does not affect the detectability and the performance of the biosensor.

Polystyrene is a well-established solid substrate for the immobilization of reagents (antibodies, other binding proteins and DNA) in microtiter well-based immunoassays and nucleic acid hybridization assays. The advantage of polystyrene microspheres over AuNP, however, arises from their superior stability in various buffers and the rapid preparation of conjugates with oligonucleotides. AuNP require a long (over 48-h) salt aging process in order to avoid aggregation at the ionic strength of the hybridization buffer. On the contrary, the oligonucleotide-decorated polystyrene microspheres are prepared in less than 3 h. In addition, polystyrene microspheres allow the development of dual-color or multicolor biosensors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.08.056.

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