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Short communication

Multianalyte, dipstick-type, nanoparticle-based DNA biosensor for visual genotyping of single-nucleotide polymorphisms

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ABSTRACT

DNA biosensors involve molecular recognition of the target sequence by hybridization with specific probes and detection by electrochemical, optical or gravimetric transduction. Disposable, dipstick-type biosensors have been developed recently, which enable visual detection of DNA without using instruments. In this context, we report a multianalyte DNA biosensor for visual genotyping of two single-nucleotide polymorphisms (SNPs). As a model, the biosensor was applied to the simultaneous genotyping of two SNPs, entailing the detection of four alleles. A PCR product that flanks both polymorphic sites is subjected to a single primer extension (PEXT) reaction employing four allele-specific primers, each containing a region complementary to an allele and a characteristic segment that enables subsequent capture on a test zone of the biosensor. The primers are extended with dNTPs and biotin-dUTP only if there is perfect complementarity with the interrogated sequence. The PEXT mixture is applied to the biosensor. As the developing buffer migrates along the strip, all the allele-specific primers are captured by immobilized oligonucleotides at the four test zones of the biosensor and detected by antibiotin-functionalized gold nanoparticles. As a result, the test zones are colored red if extension has occurred denoting the presence of the corresponding allele in the original sample. The excess nanoparticles are captured by immobilized biotinylated albumin at the control zone of the sensor forming another red zone that indicates the proper performance of the system. The assay was applied successfully to the genotyping of twenty clinical samples for two common SNPs of MBL2 gene.

1. Introduction

The development of biosensors for rapid, simple and inexpensive nucleic acid analysis, suitable for the small laboratory or for field testing, has progressed greatly in recent years. In DNA biosensors, molecular recognition is achieved via hybridization of the target sequence with complementary oligonucleotide probes. Detection is carried out by electrochemical (Lucarelli et al., 2008), optical (Mannelli et al., 2007) or gravimetric transduction (Dell'Atti et al., 2006).

Recently, our group and others have reported the development of disposable dipstick-type biosensors that enable visual detection of DNA without the use of instruments (Glynou et al., 2003; Matsubara and Kure, 2003; Kalogianni et al., 2008). The dipstick-type dry-reagent format eliminates multiple incubation and washing steps, which are often necessary in DNA assays, and minimizes the requirements for highly qualified personnel. The presence of the analyte (target DNA sequence) triggers the formation of a biotinylated hybrid, which is captured at the test zone of the sensor by immobilized streptavidin and linked to oligonucleotide-functionalized, strongly colored nano- or microparticle reporters (e.g. gold nanoparticles and polystyrene microspheres). Up to now, the dipstick-type biosensor has been applied to the detection of genetically modified organisms in food (Kalogianni et al., 2006), detection of pathogens in biological fluids (Glynou et al., 2003; Kalogianni et al., 2007a), screening for leukemia-related chromosomal translocations (Kalogianni et al., 2007b) and genotyping of single-nucleotide polymorphisms (SNPs) (Litos et al., 2007a,b; Toubanaki et al., 2008).

The aim of the present work is to increase the number of target DNA sequences that can be detected visually on a single biosensor strip, i.e., to develop multianalyte dipstick-type DNA biosensors. In SNP genotyping methods, an allele-specific primer extension reaction (PEXT) or an oligonucleotide ligation reaction (OLR) lead

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to the formation of biotinylated allele-specific sequences that are captured on the test zone by immobilized streptavidin. Consequently, one biosensor strip is used for the detection of each allele. Dual-allele biosensors were developed by incorporating a second test zone containing immobilized antidigoxigenin antibody (Konstantou et al., 2008). However, the number of high-affinity antibody/hapten pairs that can be incorporated into a biosensor strip is very limited to allow multianalyte testing. On the contrary, the interaction between complementary DNA strands offers a much greater variety of combinations. In this context, we report the development of a quadruple-analyte DNA biosensor comprising four test zones and one control zone in a single strip. The biosensor was applied to the genotyping of two SNPs, which entails the simultaneous detection of four alleles.

2. Materials and methods

Apparatus and reagents are described in supplement.

2.1. Simultaneous genotyping of two SNPs

2.1.1. Genomic DNA isolation and amplification of the MBL2 gene

Genomic DNA was isolated from 200 μL of human whole blood using the QiaAmp DNA blood minikit. PCR was performed in a total reaction volume of 50 μL containing 2 U of HotStar Taq DNA polymerase, 1 × PCR buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer U_{479} and D_{479} and 50–100 ng of genomic DNA. The cycling parameters were as follows: initial denaturation at 95 °C for 5 min and 32 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 40 s, and, finally, incubation at 72 °C for 10 min.

2.1.2. Primer extension (PEXT) reaction

All primer extension reactions were carried out in a total volume of 10 μL containing 20 mM Tris–HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 1 mL/L Triton X-100, 2 mM $MgSO_4$, 0.5 U of Vent (exo-) DNA polymerase, 0.2 pmol of amplified DNA, 1 pmol of N550, M550 and M221 primers, 0.25 pmol of N221 primer, 2.5 μM each of dATP, dCTP and dGTP, and 1.25 μM each of dTTP and biotin-11-dUTP. PEXT reactions for the detection of SNPs at positions −550 and −221 were performed in a thermal cycler as follows: an initial denaturation step at 95 °C for 5 min followed by three cycles of denaturation at 95 °C for 15 s, primer annealing at 65 °C for 15 s, and primer extension at 72 °C for 15 s. All primer extension reactions were subjected to a final denaturation step at 95 °C for 5 min and placed immediately on ice before the dipstick assay.

2.1.3. Visual multianalyte detection of primer extension reaction products

A 2.5 μL aliquot of the PEXT reaction product from the previous step was mixed with 7.5 μL hybridization buffer (2x SSC buffer, pH 7.0, 40 mL/L glycerol, 10 mL/L Tween-20 and 0.5 g/L SDS) and applied to the conjugate pad of the sensor close to the nanoparticles. The wicking pad of the sensor was then dipped into a microcentrifuge tube containing 200 μL of hybridization buffer. The visual detection of the extension products was complete within 10 min.

3. Results and discussion

3.1. Assay principle

Fig. 1 illustrates the principle of the multianalyte DNA biosensor that allows simultaneous visual detection of four alleles involved in the genotyping of two biallelic polymorphisms.

The biosensor was applied to the genotyping of two SNPs in the promoter region of the MBL2 gene at positions −550 (c.−619G > C) and −221 (c.−290G > C). A 479 bp fragment containing polymorphisms was amplified from genomic DNA by PCR. The PCR product was subjected (without prior purification) to a single PEXT reaction using four allele-specific oligonucleotide primers, N550, M550, N221 and M221 (N and M stand for normal and mutant alleles, respectively). Each allele-specific primer comprises: (a) a sequence that is adjacent to the polymorphic site, with the 3′-end nucleotide complementary to the allelic variant, (b) an upstream 12 nt spacer arm, and (c) a 24 nt sequence at the 5′-end, which is different for each primer and enables capture of the primers by hybridization with the complementary oligonucleotides (N1, M1, N2 and M2,

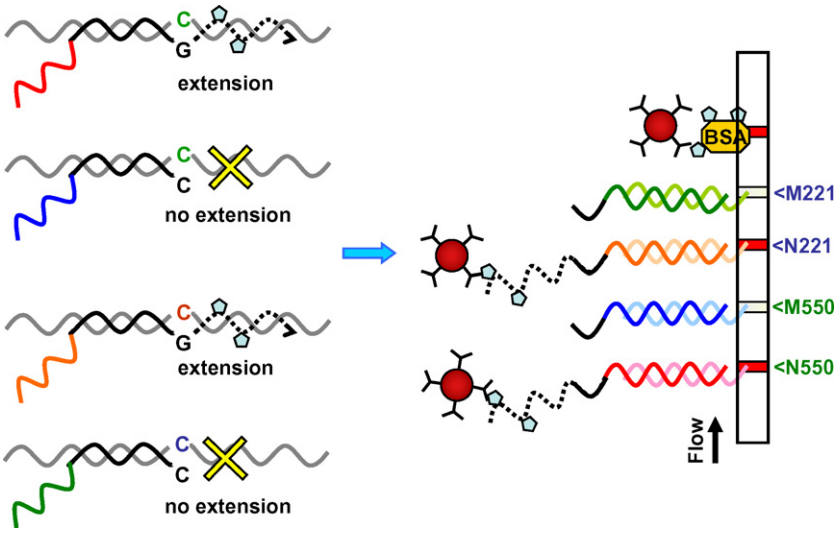

Fig. 1. Schematic illustration of the principle of multianalyte DNA biosensor for the visual genotyping of SNPs. As a model, the assay was developed for two SNPs at positions −550 and −221 of the MBL2 gene, involving four alleles (N550, M550, N221, M221, where N and M stand for normal and mutant, respectively). A PCR product that flanks both polymorphic sites is subjected to a single PEXT reaction employing four allele-specific primers, each containing a region complementary to an allele and a characteristic segment, at the 5′-end, that enables subsequent capture on a test zone of the sensor. The primers are extended with dNTPs and biotin-dUTP only if there is perfect complementarity with the interrogated sequence. PEXT products are captured via hybridization with complementary oligonucleotides that are immobilized on the four test zones of the biosensor. Visual detection is accomplished through antibiotin antibody-functionalized gold nanoparticles. Characteristic red lines indicate the presence of the corresponding alleles. The control zone of the sensor comprises immobilized biotinylated bovine serum albumin (BSA). Each test zone is named after the specific allele (N550, M550, N221 and M221) that is detected by the presence of a color line in the zone.

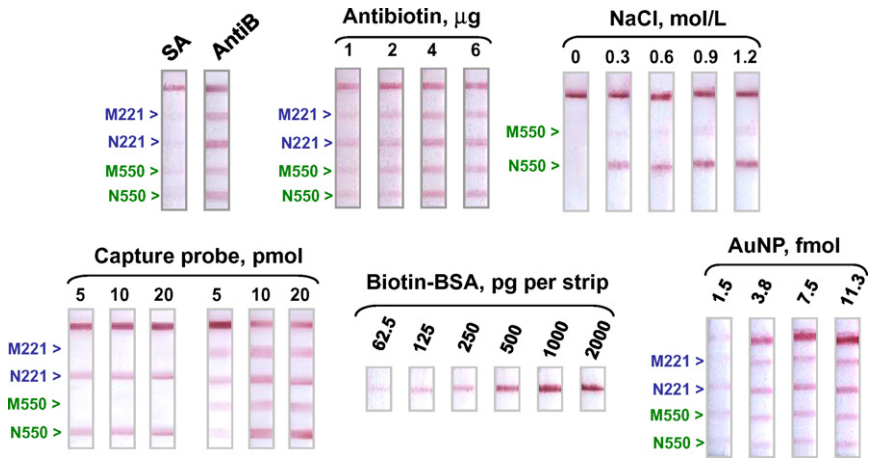

Fig. 2. Study of the functionalization of the gold nanoparticles and construction of the multianalyte DNA biosensor. A sample that was heterozygous for both SNPs was used for comparison of gold nanoparticle reporters conjugated with streptavidin or antibiotin as well as for the optimization of the amount of antibody in the conjugation reaction. Each test zone is named after the specific allele (N550, M550, N221 and M221) that is detected by the presence of a color line in the zone. A normal sample (−550N/N genotype) was used as a target for the optimization of the salt concentration in the capture probe application solution. Two samples were used for the study of the effect of the amount of capture oligonucleotide probes applied to test zones of the strip: a normal sample for both SNPs (N/N genotype for −550 and −221) and a heterozygote for both polymorphisms (N/M genotype for −550 and −221). A heterozygote for both polymorphisms (N/M genotype for −550 and −221) was used for the study of the effect of the amount of conjugated gold nanoparticle reporters (AuNP) applied to the strip.

respectively) that are immobilized at the test zones of the sensor. All the genotyping primers are designed to avoid cross-hybridization and have similar T_m values, using the Primer Premier 5 software. Only three cycles of PEXT reaction are performed during which biotin-dUTP is introduced into the extended primer(s). Primer extension takes place only if the 3′-end of the primer is perfectly complementary with the interrogated DNA sequence. The primers are then separated from the interrogated DNA by heat denaturation and the mixture is applied to the sensor. As the developing buffer migrates along the strip, all the allele-specific primers (extended and non-extended) are captured by the immobilized oligos at the four test zones of the sensor and the antibiotin-functionalized gold nanoparticles bind only to those allele-specific primers that have been extended with biotin-dUTP. As a result, the test zones are colored red if extension has occurred denoting the presence of the corresponding allele in the original sample. The excess antibiotin-conjugated nanoparticles are captured by immobilized biotinylated BSA at the control zone of the sensor forming another red zone that indicates the proper performance of the system.

The biosensor described and evaluated in this work has a fundamentally different configuration from those reported recently by our group (Kalogianni et al., 2009; Konstantou et al., 2009). In the previous reports, the DNA target (PCR product or PEXT reaction product) was captured by streptavidin and antidigoxigenin antibody immobilized at the test zone(s) of the biosensor. In the present biosensor, capture of the PEXT reaction products is accomplished via hybridization to oligonucleotides immobilized at the test zones. This configuration is more suitable for multianalyte DNA genotyping. Moreover, in the previous reports oligonucleotide-functionalized gold nanoparticles or polystyrene microspheres were used as reporters, whereas in the present sensor detection is accomplished through antibody-coated nanoparticles.

3.2. Functionalization of gold nanoparticles

As detection reagent, we compared gold nanoparticles functionalized either with antibiotin antibody or with streptavidin (the streptavidin-AuNP conjugate was prepared according to Geoghegan and Ackerman, 1977). Both functionalizations were carried out by physical adsorption of the proteins. The two conjugates were used as reporters for the genotyping of various samples. Typical results, obtained from a sample that is heterozygous for both SNPs (all four alleles are present), are shown in Fig. 2. We observe that the antibiotin-AuNP conjugate gives a higher signal than streptavidin-AuNP, in spite of the fact that the biotin/streptavidin interaction is much stronger than the binding of biotin to the antibody. Perhaps the larger size of the antibody (the molecular mass of an antibody is about 150,000 compared to 60,000 for streptavidin) leads to a greater distance of the biotin-binding site from the surface of the nanoparticles thus facilitating the interaction.

The amount of antibiotin antibody that gives the highest color densities at the test zones of the sensor was estimated by adding antibody amounts ranging from 1 μg to 6 μg into 1 mL of the nanoparticle solution (0.15 pmol of particles). Aliquots (5 μL) of the conjugates were then dried on the conjugate pad and the resulting sensors used for genotyping of a heterozygote sample for both SNPs. The highest signals were obtained when at least 4 μg of antibody was used for functionalization (Fig. 2).

3.3. Construction of the biosensor

We first investigated the composition of the application solution for the capture probes in order to ensure efficient immobilization of the oligonucleotides at the test zones of the strip. Studies were performed using application solutions containing 20 pmol of probe N1 or M1 diluted either in water or in 1× to 8× SSC buffers (150–1200 mM NaCl). Sensors containing two test zones (comprising the capture probes N1 and M1) along with a control zone were constructed. A normal sample (−550 N/N genotype) was used as a target for the optimization experiments (Fig. 2). We observe no signal when water is used as a diluent. The density increases with the SSC content up to 6× SSC (900 mM NaCl) and then remains constant. As the ionic strength of the application solution increases there is more effective masking of the negative charges of the nitrocellulose membrane and the oligonucleotides, thus facilitating the approach and binding to the membrane. Since the M1 probe does not hybridize with the normal allele, the M550 zone provides an estimate of the background, i.e., the nonspecific binding of the functionalized nanoparticles to the test zones of the sensor. The background is unaffected by the salt concentration.

The effect of the amount of applied capture probe on the color density of the test zone was studied in the range of 5–20 pmol oligonucleotide per strip (Fig. 2). A normal sample for both SNPs (N/N genotype for −550 and −221) and a heterozygote sample for

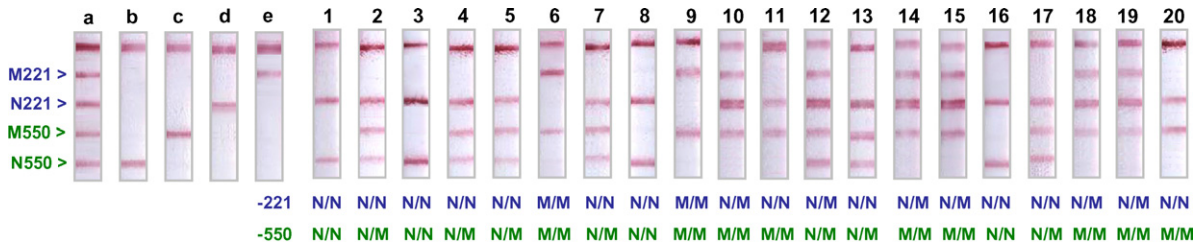

Fig. 3. Cross-hybridization study and genotyping of 20 clinical samples for −550 and −221 polymorphisms of MBL2 gene by PEXT reaction followed by visual detection of the products using the multianalyte biosensor. For the cross-hybridization study, a sample that was heterozygote for both SNPs (−550 and −221) was subjected to five PEXT reactions in the presence of: (a) all 4 allele-specific primers; (b) only primer N550; (c) only primer M550; (d) only primer N221; (e) only primer M221. Each test zone is named after the specific allele that is detected by the presence of a color line in the zone. For genotyping of clinical samples, a single PEXT reaction was performed using all four allele-specific primers. The products were detected simultaneously by the biosensor.

both SNPs (N/M genotype) were used for this study and the sensors contained all four test zones. The signal is low at 5 pmol probe and increases with the amount of probe (without an increase of the background) because a larger number of nanoparticles accumulate at the test zone. The order in which the capture probes are immobilized on the membrane of the sensor has no effect on the results of the assay.

For an indication of the proper performance of the sensor, biotin-BSA conjugate (B-BSA) was immobilized on the control zone of the membrane. The effect of the amount of applied B-BSA on the color density of the control zone was studied in the range of 62.5–2000 pg per strip (Fig. 2). The signal increases with the amount of loaded B-BSA up to 500 pg per strip. Furthermore, we optimized the amount of antibiotin-AuNP applied to the conjugate pad of the sensor. Samples heterozygous for both SNPs were used for this study. Various amounts ranging from 1.5 fmol to 11.25 fmol were tested (Fig. 2) and 7.5 fmol per strip was chosen as the optimum quantity of reporters that gives clear red lines in all test zones and the control zone.

3.4. Performance of the biosensor

3.4.1. Cross-hybridization study

It is essential for the accuracy of the visual genotyping assay that each allele-specific primer from the PEXT reaction hybridizes only with the complementary capture probe, which is immobilized on the cognate test zone of the biosensor. Cross-hybridization, if present, would generate non-specific signals at various test zones leading to false genotypes. In order to investigate whether there was any cross-hybridization of the immobilized capture probes with the allele-specific primers we performed the following experiment. A PCR product obtained from a heterozygote for both polymorphisms was subjected to five separate PEXT reactions. The first reaction was performed in the presence of all allele-specific primers and gave all four extended products. The other extension reactions (four reactions) were performed by using only one of the primers N550, M550, N221 or M221. The products of the five PEXT reactions were assayed by the DNA biosensor and the results are presented in Fig. 3. The data show that each allele-specific primer is captured only by the cognate test zone and there is no detectable cross-hybridization.

3.4.2. Simultaneous visual genotyping of two SNPs in clinical samples

The multianalyte DNA biosensor was applied to the genotyping of two SNPs in the MBL2 gene at positions −550 and −221. Twenty samples were genotyped, i.e., a total of forty variants. A 479 bp fragment containing both polymorphisms was amplified by PCR from genomic DNA isolated from whole blood. The PCR product was subjected (without purification) to a single PEXT reaction using the allele-specific primers N550, M550, N221 and M221. The PEXT products were detected by the DNA biosensor containing immobilized complementary capture probes. The results (Fig. 3) were fully concordant with those obtained by direct DNA sequencing of the same samples. The samples show a variety of combinations of genotypes that are visually detected by the biosensor without the use of instruments.

For comparison, we also constructed dual-analyte biosensors for the genotyping of each SNP separately in a number of previously characterized clinical samples. Each biosensor contained only two test zones for detection of either the N550, M550 alleles (N1, M1 capture probes) or the N221, M221 alleles (N2, M2 capture probes). Following PCR, two separate PEXT reactions were performed, each using only one pair of allele-specific primers for the −550 or the −221 SNP. The results were identical with those of the multianalyte biosensor (Figure S1, supplement). This confirms that (a) the PEXT reaction does not produce nonspecific extension products due to mispriming events and (b) each test zone of the biosensor is specific for the cognate extension product.

3.4.3. Reproducibility

The reproducibility of the detection of the alleles by the multianalyte biosensor was assessed by analyzing six times the PEXT reaction product obtained from a sample that was heterozygous for both SNPs. The color density of the test zones was determined by scanning the strips (Hewlett-Packard, ScanJet 3400C desktop scanner). The coefficients of variation of the densitometric signals for N550, M550, N221 and M221 test zones were 7.7%, 7.3%, 5.9% and 6.8%, respectively ($n = 6$). The overall reproducibility of the method (including the PEXT reaction and the biosensor assay) was found to be 9.3%, 9.8%, 7.4% and 9.4% for N550, M550, N221 and M221, respectively ($n = 6$).

4. Conclusions

Our biosensor allows simultaneous visual detection of four alleles without the use of instrumentation. The entire genotyping assay is inexpensive, very simple to perform and rapid (it takes less than 1.5 h). The biosensor is disposable. No purification of the PCR or PEXT products is required. The continuous migration of developing buffer along the sensor strip by capillary action ensures efficient washing of the four test zones as well as the control zone, thus eliminating washing and incubation steps that are necessary for other genotyping methods. Because of its simplicity the assay does not require the training of highly qualified personnel. We believe that the multianalyte DNA biosensor will find wide applications in small molecular diagnosis laboratories and in point-of-care testing (field testing). In a previous report from our group (Kalogianni et al., 2009), we have shown that colored polystyrene microspheres can also be used as reporters instead of gold nanoparticles for detection of PEXT products. Therefore, it can be realized that in the present biosensor antibiotin-coated colored microspheres could be used as an alternative reporter.

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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.2009.03.010.

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