A nanoparticle-based sensor for visual detection of multiple mutations

2011 Nanotechnology 22 155501

(http://iopscience.iop.org/0957-4484/22/15/155501)

Nanotechnology **22** (2011) 155501 (9pp) doi:10.1088/0957-4484/22/15/155501

A nanoparticle-based sensor for visual detection of multiple mutations

Dimitrios S Elenis[1], **Penelope C Ioannou**[1,3] **and Theodore K Christopoulos**[2]

[1] Department of Chemistry, University of Athens, Athens 15771, Greece
[2] Department of Chemistry, University of Patras, Patras 26500, Greece

E-mail: ioannou@chem.uoa.gr

Received 25 October 2010, in final form 24 January 2011
Published 10 March 2011
Online at stacks.iop.org/Nano/22/155501

Abstract

Disposable dipstick-type DNA biosensors in the form of lateral flow strips are particularly useful for genotyping in a small laboratory or for field testing due to their simplicity, low cost and portability. Their unique advantage is that they enable visual detection in minutes without the use of instruments. In addition, the dry-reagent format minimizes the pipetting, incubation and washing steps. In this work, we significantly enhance the multiplexing capabilities of lateral flow strip biosensors without compromising their simplicity. Multiplex genotyping is carried out by polymerase chain reaction (PCR) followed by a single primer extension reaction for all target alleles, in which a primer is extended and biotin is incorporated only if it is perfectly complementary to the target. Multiallele detection is achieved by multiple test spots on the membrane of the sensor, each comprising a suspension of polystyrene microspheres functionalized with capture probes. The products of the primer extension reaction hybridize, through specific sequence tags, to the capture probes and are visualized by using antibiotin-conjugated gold nanoparticles. This design enables accommodation of multiple spots in a small area because the microspheres are trapped in the fibres of the membrane and remain fixed in site without any diffusion. Furthermore, the detectability is improved because the hybrids are exposed on the surface of the trapped microspheres rather than inside the pores of the membrane. We demonstrate the specificity and performance of the biosensor for multiallele genotyping.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Genotyping is a challenging problem for the analyst. It requires the identification of single base changes amidst the 3×10^9 base pairs of the human genome. Genotyping methods comprise the following. (a) Exponential amplification (usually by polymerase chain reaction, PCR) of the target genomic DNA sequence. (b) An allele discrimination step, which is based on restriction fragment length polymorphism analysis, hybridization with allele-specific oligonucleotides, melting curve analysis or enzyme reactions (oligonucleotide ligation, primer extension and invasive cleavage). (c) Detection of the products.

With regard to allele discrimination, the use of specific oligonucleotides requires fine adjustment of the stringency of hybridization and washing, whereas the enzyme-based methods are more robust and easily applicable. Detection of the genotyping products is based on fluorescence [1, 2], bio(chemi)luminescence [3–5], surface plasmon resonance [6], quartz crystal microbalance [7] and electrochemical methods [8]. Technological advances in recent years (such as DNA microarrays) have led to the development of sophisticated high-throughput systems that enable genotyping of hundreds of thousands of polymorphisms in parallel. While these systems are essential for carrying out large-scale association studies, it is anticipated that eventually only a few polymorphisms per patient will be assessed routinely in the molecular diagnosis laboratory. As a result, the development of DNA biosensors

[3] Author to whom any correspondence should be addressed.

has received considerable attention. Electrochemical, optical or gravimetric biosensors [6–9] offer simplicity, low cost and portability, and are therefore more suitable for a small laboratory or for field testing (point-of-care).

During the last few years, dry-reagent dipstick-type biosensors in the form of lateral flow strips have been developed for the genotyping of single nucleotide polymorphisms (SNP). The key advantage of these sensors is that they enable visual detection within minutes without the use of instruments. In addition, they are disposable and the dry-reagent dipstick format eliminates several pipetting and washing steps because the reagents form an integral part of the sensor. Hybridization with allele-specific oligonucleotides, primer extension reaction and oligonucleotide ligation reaction have all been used for allele discrimination in combination with dipstick-type biosensors [10–12]. The general strategy for visual detection involves capture of the genotyping products on the membrane of the biosensor (e.g. primer extension reaction products) through biotin/avidin, antibody/hapten interaction or DNA hybridization and the use of gold nanoparticles or coloured polystyrene microspheres as reporters; these are functionalized either with antibody or oligonucleotide. The presence of an allele is indicated by the visualization of a red band on the strip (test line).

In the first reports, a dipstick-type biosensor could detect a single allele [10–12]. Consequently, for the genotyping of a biallelic polymorphism, two biosensors were needed. Subsequently, biosensors with two test lines (a combination of immobilized avidin and antibody) or four test lines (immobilized oligonucleotides) were developed for simultaneous detection of two or four alleles, respectively [13, 14].

In the present work, we enhance significantly the multiplexing capabilities of dipstick-type biosensors while maintaining their simplicity. The multiallele visual detection is achieved by preparing multiple test spots (around 1 mm diameter) through simple pipetting on the strip. Various capture oligonucleotide probes are first attached, covalently, to polystyrene microspheres and then the suspension is pipetted to the membrane of the biosensor. A single primer extension reaction is performed, in which multiple allele-specific primers are extended simultaneously and biotin is incorporated only if there is perfect complementarity with the target sequence. The products are captured via hybridization at the corresponding spots of the sensor and detected by using antibiotin-functionalized gold nanoparticles. This biosensor design (i) enables the accommodation of multiple spots in a small area of the strip, because the capture probe-conjugated microspheres are trapped in the fibres of the membrane and remain fixed in the application site without any diffusion, and (ii) offers higher detectability compared to direct immobilization of the capture oligonucleotides, because the hybrids are exposed on the surface of the trapped microspheres rather than inside the pores of the membrane.

We carried out extensive studies to optimize the multiallele biosensor and demonstrate its specificity and performance.

2. Experimental details

2.1. Instrumentation

PCR and primer extension (PEXT) reactions were carried out in a Hybaid Omni-E thermal cycler (Middlesex, UK). The digital camera Kodak DC 120 and the gel analyser software for DNA documentation were purchased from Kodak (New York, NY, USA). The microcentrifuge Mikro-20 was from Hettich (Tuttlingen, Germany). The Linomat 5 TLC applicator and the software WinCats were from Camag (Muttenz, Switzerland). The UV lamp was from Vilber Lourmat (Marne la Valée, France). Images of the strips were captured by scanning the strips using a simple desktop scanner (ScanJet 4300C, Hewlett-Packard).

2.2. Materials

Hotstar Taq Plus DNA polymerase was purchased from Qiagen (Hilden, Germany). DNA polymerase Vent (exo-) was from New England Biolabs (Beverly, MA, USA). Ultrapure 2-deoxyribonucleoside 5-triphosphates (dNTPs) were from HT Biotechnology (Cambridge, UK) and biotin-11-dUTP, EDC (1-ethyl-3-[dimethylaminopropyl]carbodiimide hydrochloride) and MES [2-(*N*-morpholino)ethanesulfonic acid] were from Applichem (Darmstadt, Germany). Carboxylate-modified white microspheres, 2 μm in diameter, were purchased from Polysciences (Warrington, PA, USA). DNA molecular weight markers and terminal deoxynucleotidyl transferase (TdT) were from MBI Fermentas (Vilnius, Lithuania). Immunopure goat antibiotin was from Pierce (Rockford, IL, USA). Bovine serum albumin (BSA) was purchased from Serva (Heidelberg, Germany). Immunopore FP membrane (5 μm pore size) was from Whatman (Florham Park, NJ, USA) and the plastic adhesive backing, wicking pad, glass-fibre conjugate pad and absorbent pad were from Schleicher & Schuell (Dassel, Germany). Gold nanoparticles (40 nm, 9×10^{10} particles ml^{-1}) were purchased from British Biocell (BB International, Cardiff, UK). Formamide was from Roth (Karlsruhe, Germany). All other common chemicals were from Sigma (St Louis, MO, USA).

All oligonucleotides used in this work (table 1) were synthesized by Thermo Electron (Ulm, Germany). Three pairs of primers were designed to amplify a TLR4 gene fragment (containing the A896G and C1196T SNPs), the promoter region of the MBL2 gene (containing the −550 (c. −619 G > C) and −221 (c. −290 G > C) polymorphisms) and the region of exon 14 of the JAK2 gene containing the JAK2V617F somatic point mutation. Ten allele-specific primers were used in a 10-plex PEXT reaction for the genotyping of the five SNPs. Ten oligonucleotides, with a $-NH_2$ group at their 5′ end via a 6-carbon spacer arm, comprised the capture probes.

The running buffer contained 5.5 mM sodium citrate, pH 7.0, 55 mM NaCl, 10 ml l^{-1} Tween-20, 10 g l^{-1} BSA, 1 g l^{-1} SDS and 200 ml l^{-1} formamide.

2.3. Conjugation of oligonucleotides to microspheres

A 12.8 μl aliquot of a 5.6×10^6 μl^{-1} suspension of 2 μm white microspheres (0.12 fmol) in 150 μl of 0.1 M MES, pH 4.5,

Table 1. Oligonucleotide sequences used as primers and probes.

Oligonucleotide	Name	Sequence (5′ → 3′)	Length (bp)	Label
Allele-specific primers for the 10-plex PEXT genotyping reaction for MBL2, JAK2 and TLR4 genes				
G-221 allele (MBL2)	N221	**CTCTACTCCACCCCCATCCACATT** CCGAATTCTCTCGGTCCCATTTGTT CTCACTGCCACG	61	—
-221C allele (MBL2)	M221	**GTGGCGATGGGTGCAGCGGTATCC** CCGAATTCTCTCGGTCCCATTTGTT CTCACTGCCACC	61	—
G-550 allele (MBL2)	N550	**AGTCGTAGTCAGAAGTTCAGCAAG** CCGAATTCTCTCAATGCTTACCCAGG CAAGCCTGTG	60	—
-550C allele (MBL2)	M550	**ACGTCACCATCCGAACTTAAAACG** CCGAATTCTCTCAATGCTTACCCAGG CAAGCCTGTC	60	—
G1849 allele (JAK2)	NJAK2	**GATGCGTGACATTACTGTGAGGCG** CCGAATTCTCTCTTACTTACTCTCGT CTCCACAGAC	60	—
1849T allele (JAK2)	MJAK2	**CAGTCCAGCATCCACCAGTCGGCT** CCGAATTCTCTCTTACTTACTCTCGT CTCCACAGAA	60	—
A896 allele (TLR4)	N896	**GATAGTGTATTACATGGCTCTTCA** CCGAATTCTCTCAAACAATTAAATA AGTCAATAATAT	61	—
896G allele (TLR4)	M896	**ACAGAGCAGTGTTTAGGTGAGCTA** CCGAATTCTCTCAAACAATTAAATA AGTCAATAATAC	61	—
C1196 allele (TLR4)	N1196	**CACAGTCATCATAGTATATAGGGG** CTTATATCGAGTCTAAAGTGATTTT GGGACAAC	57	—
1196T allele (TLR4)	M1196	**CTATCTTTAAACTACAAATCTAAC** CCGAATTCTCTCAAAGTGATTTT GGGACAAT	55	—
Capture probes				
N221	N221-PR	**AATGTGGATGGGGGTGGAGTAGAG**	24	5′-NH_2
M221	M221-PR	**GGATACCGCTGCACCCATCGCCAC**	24	5′-NH_2
N550	N550-PR	**CTTGCTGAACTTCTGACTACGACT**	24	5′-NH_2
M550	M550-PR	**CGTTTTAAGTTCGGATGGTGACGT**	24	5′-NH_2
NJAK2	NJAK2-PR	**CGCCTCACAGTAATGTCACGCATC**	24	5′-NH_2
MJAK2	MJAK2-PR	**AGCCGACTGGTGGATGCTGGACTG**	24	5′-NH_2
N896	N896-PR	**TGAAGAGCCATGTAAAGCACTATC**	24	5′-NH_2
M896	M896-PR	**TAGCTCACCTAAGGTCTGCACACT**	24	5′-NH_2
N1196	N1196-PR	**CCCCTATATACTATGATGACTGTG**	24	3′-NH_2
M1196	M1196#2-PR	**GTTAGATTTGTAGTTTAAAGATAG**	24	5′-NH_2
Primers for the amplification of MBL2, JAK2 and TLR4 genes fragments				
5′ MBL	MBL-up	AGGGCCAACGTAGTAAGA	18	—
3′ MBL	MBL-dwn	GGTAGGCACTATGATGAGC	19	—
5′ JAK2	JAK2-up	TGGGCATTGTAACCTTCTA	19	—
3′ JAK2	JAK2-dwn	AAGGGACCAAAGCACATTG	19	—
5′ TLR4	TLR4-up	GTAAAACGACGGCCAGTAGTCCATC GTTTGGTTCTGGGAGA	25	—
3′ TLR4	TLR4-dwn	*CAGGAAACAGCTATGAC* GCCATTGAAAGCAACTCTGTG	38	—

was centrifuged for 2 min at 15 000 g and the supernatant was discarded. The microspheres were resuspended in 160 μl of 0.1 M MES, pH 4.5, by vortexing and sonication for 10 s and mixed with 400 pmol of the appropriate oligonucleotide probe that carries an amino group at the 5′-end. An 8 μl aliquot of a freshly prepared 200 g l^{-1} EDC solution, in 0.1 M MES, was added and the mixture was incubated at ambient temperature for 30 min under occasional vortexing. The addition of EDC was repeated with a fresh EDC solution and the mixture was incubated for another 30 min followed by the addition of 2 μl of 100 ml l^{-1} Tween-20. The microspheres were pelleted by centrifugation at 15 000 g for 2 min and washed twice with 100 μl of a Tris-EDTA solution containing 2 ml l^{-1} Tween-20. Finally, the microspheres were resuspended in 64 μl of the

Tris-EDTA solution. Assuming that no loss of beads occurs during the washing steps, the concentration of microspheres in this suspension is 1.1×10^6 μl^{-1}.

2.4. Tailing of oligonucleotides attached to the surface of microspheres

Oligonucleotide probes conjugated to microspheres were tailed with biotin-dUTP by using terminal deoxynucleotidyl transferase. The tailing reactions were carried out in a total volume of 20 μl containing 0.2 M potassium cacodylate, 0.125 M Tris-HCl, pH 7.2, 0.1 ml l^{-1} Triton X-100, 1 mM $CoCl_2$, 0.5 mM of each dNTP and 25 μM of biotin-dUTP, 25 U of TdT and 3.6×10^7 conjugated microspheres. After incubation for 1 h at 37 °C with occasional mixing, the microspheres were separated from the excess of biotin-dUTP by centrifugation, washed and, finally, resuspended in 128 μl Tris-EDTA (final concentration 2.8×10^5 microspheres μl^{-1}).

2.5. Construction of the dry-reagent strip

The dry-reagent strip (40 mm × 70 mm) comprising a wicking pad, a glass-fibre conjugate pad, a nitrocellulose membrane, an absorbent pad and a plastic adhesive backing was assembled as described previously [11]. Subsequently, a 0.5 μl aliquot containing 5.5×10^5 microspheres was applied to the membrane by using a pipette to form the test spots. An equal volume of a suspension containing 1.4×10^5 microspheres conjugated to biotinylated tailed oligos was applied to the top of the membrane to form the control spots. The membrane was air dried for 5 min. Antibiotin-functionalized gold nanoparticles (2.25 fmol μl^{-1}) were prepared by physical adsorption of the protein [13]. The nanoparticles were loaded by spotting on the conjugate pad of the strip at a density of 9 fmol/4 mm and left to dry.

2.6. PCR amplification

PCR was performed in a total reaction volume of 50 μl containing 2 U of HotStar Taq DNA polymerase, 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, MBL-up and MBL-dwn, JAK2-up and JAK2-dwn or TLR4-up and TLR4-dwn for the amplification of the corresponding fragment and 50–100 ng of genomic DNA. The cycling parameters were as follows: initial denaturation at 95 °C for 5 min and 35 cycles of 95 °C for 30 s, 55 °C for 30 min and 72 °C for 1 min, and a final extension step at 72 °C for 8 min.

2.7. Multiplex PEXT reaction

PEXT 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^{-1} Triton X-100, 2 mM $MgSO_4$, 1 U of Vent(exo-) DNA polymerase, 25 fmol each of the three amplification products, 250 fmol each of the 10 primers, 10 μM each of dATP, dCTP, and dGTP, 5 μM dTTP, and 5 μM biotin-11-dUTP. Formamide was added at a final concentration of 125 ml l^{-1}. The cycling parameters were as follows: an initial denaturation step at 95 °C for 5 min followed by 10 cycles of denaturation at 95 °C for 15 s, annealing at 40 °C for 15 s and extension at 72 °C for 15 s. PEXT reaction products were subjected to a final denaturation step at 95 °C for 5 min and placed immediately on ice before the dipstick assay.

2.8. Visual detection by a multiallele dipstick-type DNA biosensor

A 6 μl aliquot of the denatured product of the extension reaction was mixed with an equal volume of running buffer. The mixture was then applied to the upper part of the conjugate pad above the functionalized nanoparticles (i.e. closer to the membrane). The wicking pad was then immersed into a microcentrifuge tube containing 300 μl of running buffer. Visual detection of the extension reaction products was complete within 10 min.

2.9. Interpretation of results/genotype assignment

The dry-reagent strip was configured in such a way that the capture probes corresponding to the five wild-type (normal) alleles were spotted on the left side of the membrane, whereas capture probes specific for the five variant (mutant) alleles were immobilized on the right side of the membrane, thus facilitating the interpretation of the test results. Allelic variants were positioned as follows (from the bottom to the top of the membrane): MBL2 (G221C), MBL2 (G550C), JAK2 (G1849T), TLR4 (A896G) and TLR4 (C1196T). The genotype is assigned by observing each pair of spots. For any particular SNP, a wild-type individual will give a red colour at the left spot only, whereas a variant homozygote will only give colour at the right spot of the pair. Both spots will be red if the sample comes from a heterozygote for this polymorphic site.

3. Results and discussion

A schematic presentation of the multiplex genotyping method is shown in figure 1. The whole assay comprises the following steps in sequence: (i) isolation of genomic DNA, (ii) PCR amplification of the DNA segments containing the SNPs of interest, (iii) multiplex primer extension reaction of the amplified products without prior purification and (iv) rapid simultaneous visual detection of all extension products by the dipstick-type biosensor, again without prior purification.

For the primer extension reaction, two allele-specific primers are designed per polymorphic site. Thus, for genotyping of five SNPs, the extension reaction mixture contains 10 primers (a 10-plex reaction). Each primer consists of two main segments: the 3′ region that is adjacent to the polymorphic site, with the 3′-end nucleotide complementary to the allelic variant, and the 5′ region that contains a unique sequence tag that enables capture of the primers by hybridization with the complementary capture oligonucleotides that are immobilized at the test spots of the biosensor. A 12-nucleotide (nt) spacer is introduced between the two segments to avoid steric hindrance that may have an impact on the interactions along the diagnostic membrane of

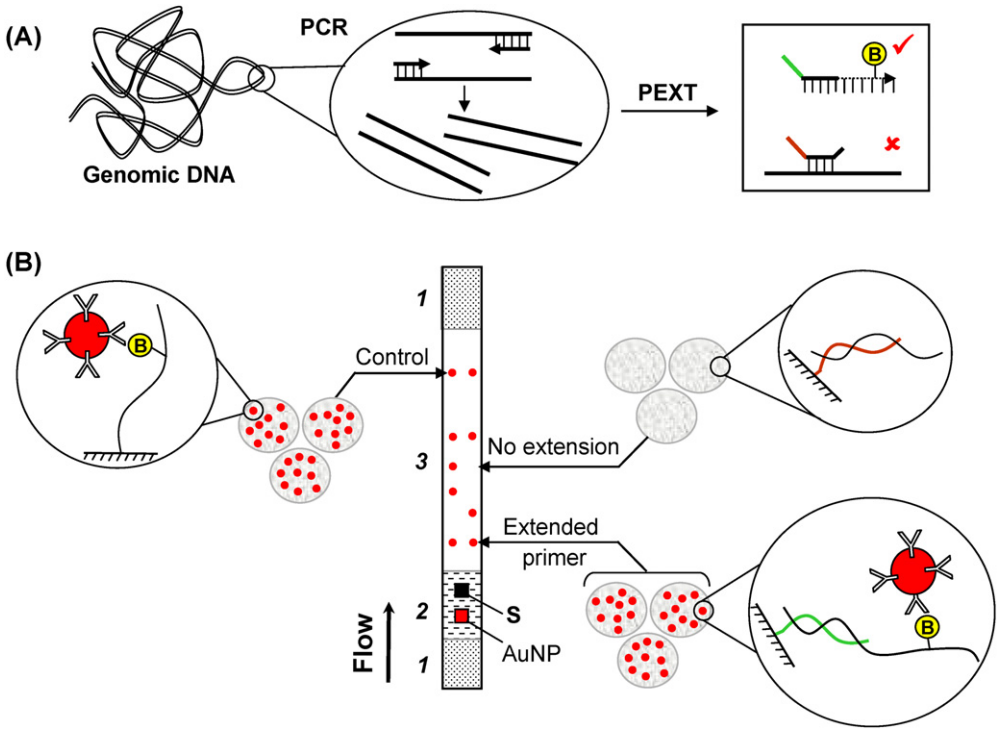

Figure 1. Schematic illustration of the principle of simultaneous multiallele visual detection by a dipstick-type disposable DNA biosensor. (A) The segments of the genes that contain the nucleotide variations (single nucleotide polymorphisms) of interest are amplified by PCR. PCR products are subjected to a multiplex primer extension reaction (PEXT) using allele-specific primers. Only the primer whose 3′ end is fully complementary to the target is extended by the DNA polymerase. Biotin (circled B) is incorporated in the extended fragment. The extension product is directly applied to the dipstick-type biosensor. (B) The biosensor comprises the following. (1) The immersion pad. (2) The conjugate pad where the antibiotin-conjugated gold nanoparticles (AuNP) are deposited in dry form. The extension product is applied to the conjugate pad, above the nanoparticles (position S). (3) The diagnostic membrane that contains spots of deposited microspheres with capture probes attached to their surface. The extended primer hybridizes to the corresponding capture probe and is linked to the gold nanoparticles via biotin/antibiotin interaction. If the primer is not extended then it still hybridizes to the capture probe but is not linked to the nanoparticles. The appearance of red spots at the left or the right side of the membrane indicates the presence of the wild-type or the variant allele in the sample, respectively. Two control spots consisting of biotinylated oligos conjugated to microspheres capture the excess of nanoparticles.

the sensor. A DNA polymerase lacking the 5′ → 3′ and the 3′ → 5′ exonuclease activities is used to extend a primer only if its 3′-end is complementary with the allele. Biotin is incorporated in the extended primer by the addition of biotin-dUTP to the dNTP mix. If there is a mismatch at the 3′ end, then extension and, consequently, biotin incorporation do not occur. Consequently, the 10-plex extension reaction produces extended primers containing a characteristic sequence tag at their 5′ end and a biotinylated extension at the 3′ end.

The visual detection of extension reaction products by the dipstick-type DNA biosensor could be considered as a rapid hybridization assay, in which, steps such as hybridization, washing out the excess of reagents and detection with strongly coloured particles is accomplished by the impact of the continuous flow of running buffer. Following the application of the extension reaction mixture and the immersion of the wicking pad of the biosensor, the flow of running buffer forces the primers (extended or not) to migrate along the membrane. Ten oligonucleotide probes (capture probes) complementary to the sequence tags of the PEXT primers have been positioned at 10 spots (∼1 mm in diameter) on the membrane of the biosensor. Therefore, the allele-specific primers are captured via hybridization to specific sites of the membrane. For immobilization, the capture probes were first conjugated to polystyrene microspheres and then simply pipetted onto the membrane. Due to their size, the microspheres are locked in the deposition site by the fibres of the membrane. After air drying for <5 min, the membrane is ready to use. In this way, the capture probes are easily allocated to the corresponding sites and accessible for hybridization with the specific sequence tags of the allele-specific primers. The antibiotin-functionalized gold nanoparticles migrate along the membrane by capillary action and bind to the biotin moieties of the captured extension products. This leads to accumulation of nanoparticles and the formation of red spots detectable with the naked eye. A non-extended primer is still captured through its 5′ sequence tag but not detected because it lacks biotin. Two control spots are formed at the top of the membrane by applying a suspension of microspheres conjugated with biotinylated oligonucleotides. Control spots confirm the proper performance of the strip.

Parameters that affect the performance of the biosensor were investigated in order to optimize the specificity and detectability of the biosensor. Optimization studies were performed by using 50 fmol of biotinylated (tailed) allele-specific primers as a model.

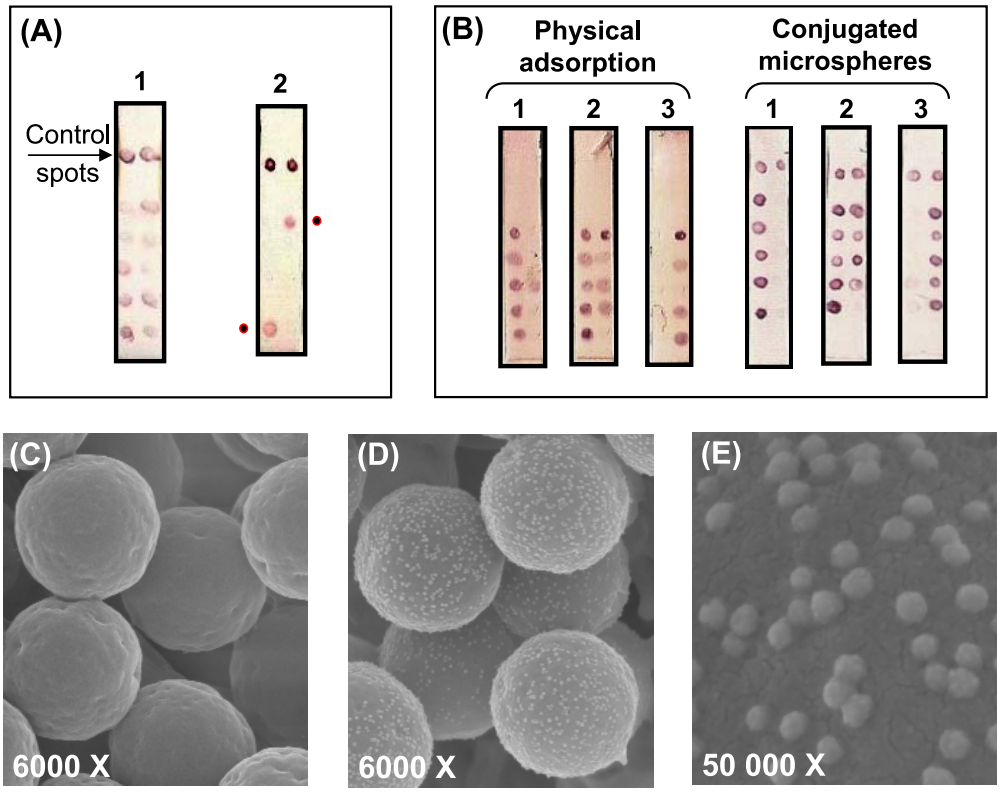

Figure 2. (A) In sensor (1) the running buffer contains 75 mM NaCl. No sample was applied to the conjugate pad. In sensor (2) the running buffer contains 55 mM NaCl and 20% formamide. The two red dots, next to the membrane, indicate where red spots should be formed due to the presence of the corresponding target sequences in the sample (50 fmol of each target). (B) Comparison of passive adsorption versus conjugated microspheres for immobilization of the capture oligonucleotide probes to the membrane of the biosensor. The two immobilization approaches were compared on three samples, i.e. (1) a 'normal' for all SNPs, (2) a heterozygote for all but the first SNP that is normal and (3) a 'mutant' for all SNPs. (C) Scanning electron microscopy (SEM) images of the microspheres deposited on the diagnostic membrane. (D) SEM images of microspheres with nanoparticles on their surface following capture of extended products through hybridization and subsequent binding of antibiotin antibody–gold nanoparticle conjugates to the hybrids. (E) Same as in (D) but at a larger magnification.

Our first experiments showed that the carboxylate-activated microspheres used as a means of oligonucleotide immobilization on the membrane constitute a source of non-specific interactions with antibiotin-conjugated gold nanoparticles. When a negative sample containing no target DNA was tested by the sensor using a running buffer containing low salt concentration (75 mM), regardless of the salt used (NaCl, $MgCl_2$, KCl or tetramethylammonium chloride), accumulation of nanoparticles was observed on all 10 spots. The non-specific binding was practically eliminated at 55 mM salt concentration in the running buffer by including 20% formamide and 1% bovine serum albumin (figure 2).

The immobilization of oligonucleotide probes on the diagnostic membrane by means of microsphere conjugates was compared to the previously used procedure of passive adsorption of the capture probes on the membrane [14]. The sensors prepared by the two immobilization methods were used for the detection of 10-plex extension reaction products. Six microlitres of the same extension reaction product from samples representing the three genotypes (wild-type, heterozygote and mutant) were analysed by the proposed sensor and the one prepared by passive adsorption of the capture probes. According to the results (figure 2) the performance of the sensor prepared with microsphere-conjugated capture probes is much better in terms of specificity and detectability.

Figure 2 also presents scanning electron microscopy (SEM) images of the microspheres before and after the formation of the hybrids. It is clearly observed that hybridization of the extended product to the capture probe leads to the accumulation of gold nanoparticles on the surface of the microspheres.

The effect of the capture probe to microsphere ratio on the colour density of the test spots of the strip was studied by using a constant number of 5.5×10^5 microspheres/spot that were conjugated with various amounts of capture probes, i.e. 0.78, 3.12, 12.5 and 62.5 pmol of probes. We observed that the colour density of the spots increased with the amount of probe up to 3.12 pmol. This corresponds to a molar ratio of 3.5×10^6 probes/microsphere in the conjugation reaction. At higher ratios, the colour density remained constant.

Next, we studied the effect of the number of immobilized microspheres on the colour density of the spots. Various numbers of microspheres (5.5×10^5, 1.1×10^6 and 2.2×10^6) were conjugated with capture probes at a constant molar ratio of 3.5×10^6 probes/microsphere and immobilized on

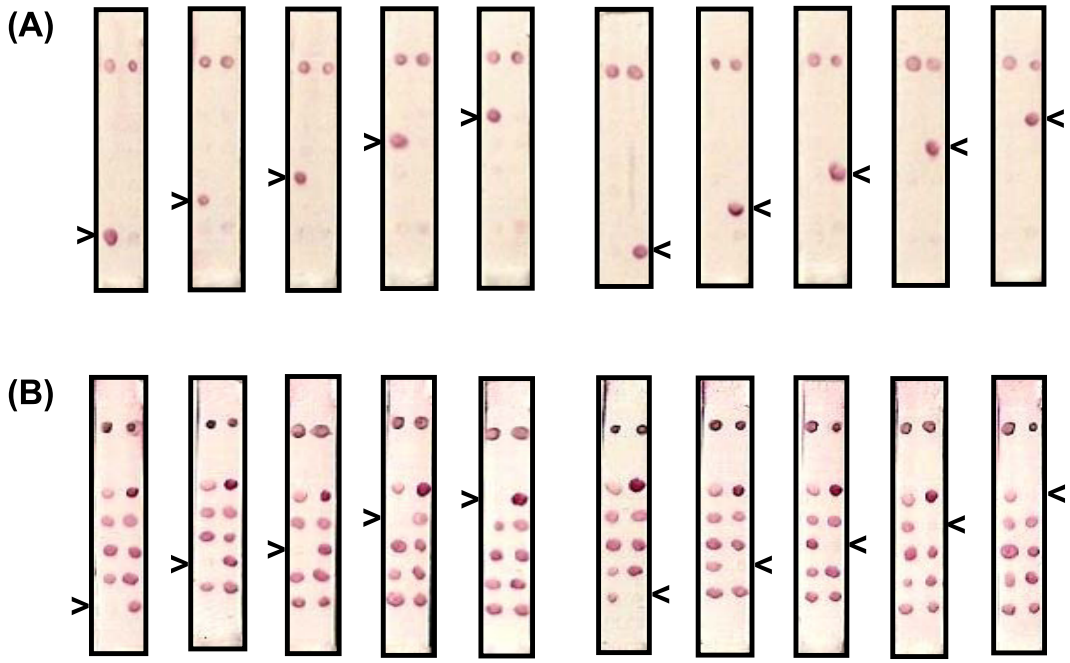

Figure 3. Study of the specificity of the dipstick-type multiallele biosensor. (A) Specificity of the biosensor with one target DNA present each time in the sample. (B) Specificity of the biosensor with one target DNA missing each time. The arrow shows where a red spot should be formed.

the membrane (0.5 μl/spot). We found that the increase in the number of microspheres per spot resulted in higher non-specific signals without increasing the colour density. Thus, 5.6×10^5 microspheres conjugated with 3.12 pmol of capture probe was selected as the optimum for immobilization on the membrane.

The study of the specificity of the response of the biosensor is the most critical aspect for multiallele detection. In order to confirm that each extended primer (PEXT product) hybridizes only to the corresponding capture probe on the membrane and not to any other immobilized probes, we prepared a series of 10 solutions, each containing only one extended primer (50 fmol). The solutions were tested by the biosensor and the results are presented in figure 3. We observe that a red spot appears only at the position that contains the corresponding capture probe, thus confirming that the extension products are captured only to the assigned positions without cross-hybridization. To strengthen these results, we prepared a series of 10 solutions each containing nine extended primers (50 fmol of each) and missing only one primer (different each time). These solutions were also tested by the biosensor and the results are shown in figure 3. We observe that, for each solution, the biosensor shows nine red spots at exactly the appropriate positions. No red spot is formed at the position that corresponds to the missing extended primer. The above experiments prove the high specificity of the sensor.

The detectability of the multiallele assay was examined by analysing serial dilutions of a mixture containing the 10 extended primers and the results are shown in figure 4. As a negative control, a mixture of the 10 non-biotinylated PEXT primers containing 100 fmol of each primer was used. As shown, as little as 2 fmol of extended primer could be detected by naked eye and the analytical range of the assay extends to 80 fmol. At higher amounts of extended primer the density of the spots remains constant due to saturation of the capture oligonucleotides.

Next, we studied the effect of the amount of primers in the extension reaction. A series of extension reactions was performed in the presence of equimolar amounts of all 10 primers in the range of 62.5–1000 fmol. The results are presented in figure 4. An increase in the colour density was observed by increasing the amount of each primer up to 250 fmol. At higher amounts of primers the colour density decreases. This was attributed to competition between extended and non-extended primers for hybridization to the immobilized capture probe. With regard to the amount of PCR product introduced in the extension reaction, we found that the addition of 10–100 fmol of amplified DNA has no effect on the specificity and detectability.

The multiallele genotyping assay was evaluated by analysing mixtures of PCR products representing different genotype combinations of the aforementioned five SNPs (10 alleles). The results (figure 5) were in full concordance with genotyping results obtained by DNA sequencing of the samples, thereby demonstrating the high accuracy of the multiallele visual detection test.

Data pertaining to the reproducibility of the multiallele biosensor are presented in figure 6. Two samples, a 'normal' (N) and a heterozygote (H) for all the SNPs, were tested three times. The first series of results refers to the reproducibility of the detection step only, whereas the second series corresponds to the reproducibility of both the primer extension reaction and the biosensor detection.

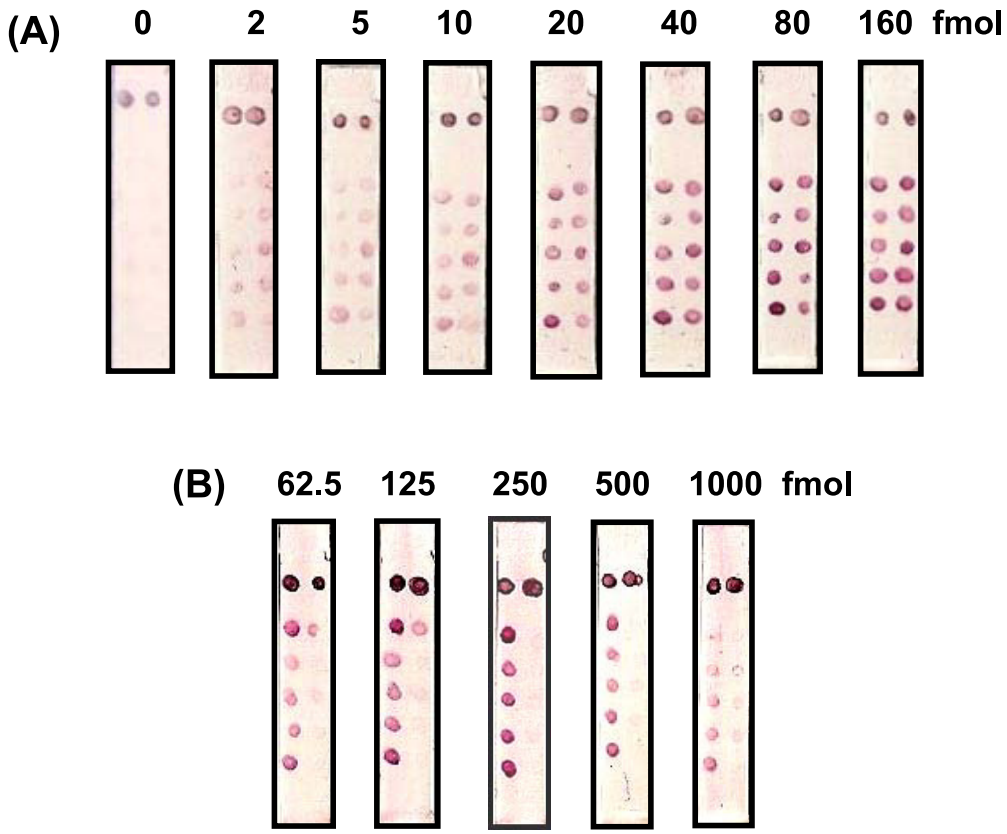

Figure 4. (A) Detectability of the biosensor assay. Solutions containing all 10 biotinylated target DNAs at equimolar amounts were tested by the biosensor. The amount of each target, in fmol, is shown above each biosensor. (B) Effect of the amount of primers in the PEXT reaction. The amount of primer, in fmol, is shown above each biosensor.

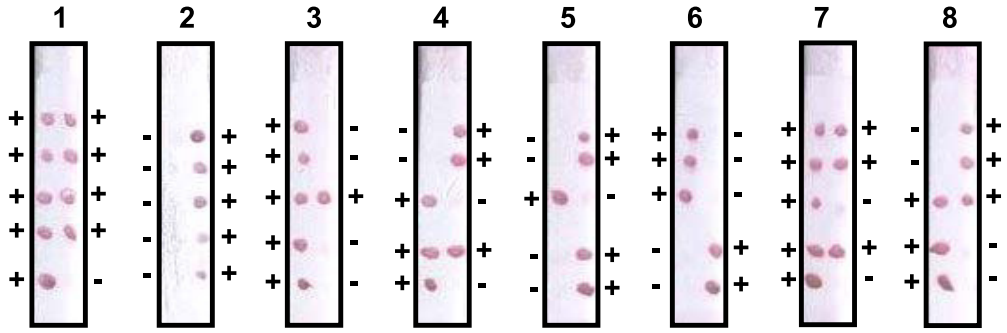

Figure 5. Genotyping of characterized DNA samples. The '+' and '−' signs next to the diagnostic membrane indicate the presence or absence, respectively, of the corresponding allele.

4. Conclusions

The multiplexing capability of the dipstick-type biosensor was enhanced significantly without compromising its simplicity, specificity and reproducibility. This was accomplished through an alternative method for immobilization of capture probes on the membrane of the sensor. Compared to passive adsorption for oligonucleotide immobilization, this method provides improved hybridization efficiency leading to higher detectability because the oligonucleotides are attached to the surface of the membrane and thus are easily accessible for hybridization with the target DNA in the flow stream. The application of microsphere conjugates on the membrane is simple and the test spots are of a strict non-diffused round shape. This allows simultaneous multiallele detection without cross-talking. For biosensor construction, there is no need for prolonged drying at elevated temperatures (only 5 min in air is needed) or cross-linking under UV illumination, which is required for passive adsorption of oligonucleotides. The conjugation reaction is simple, fast and reproducible. The conjugates could be prepared at a large scale within 70 min and stored at 4 °C for several months. During 10 cycles of the

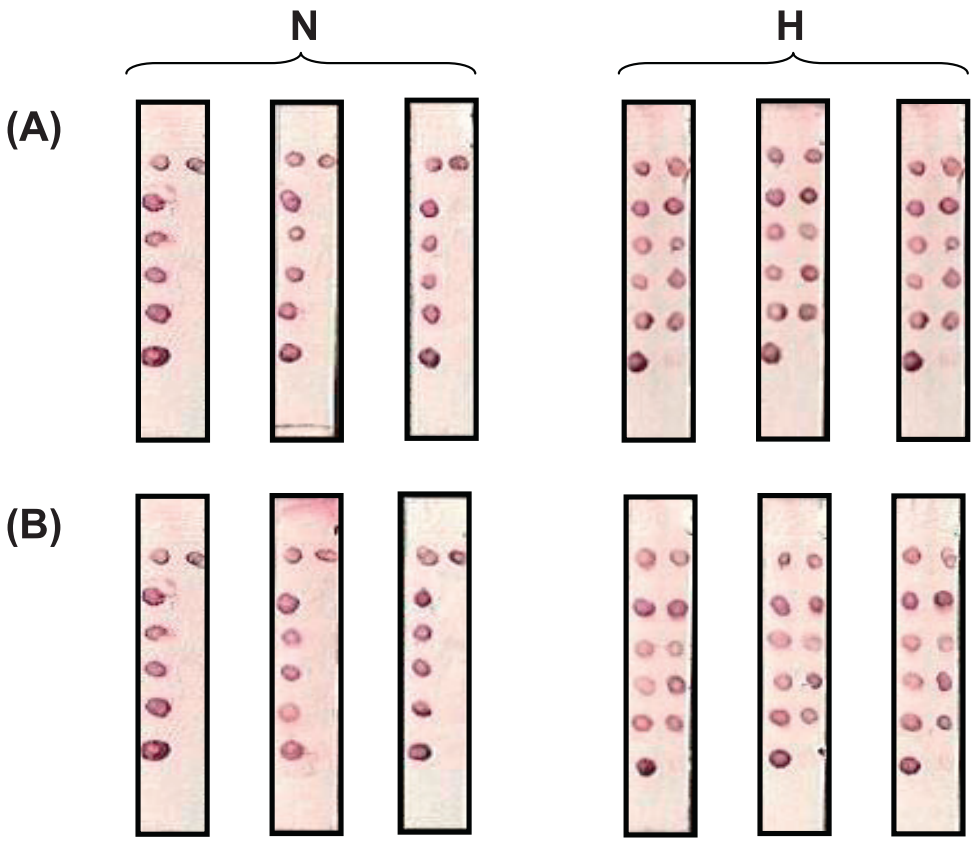

Figure 6. Study of the reproducibility of the assay. (A) Reproducibility of the detection using the biosensor. (B) Reproducibility of both the primer extension reaction and the biosensor detection steps.

genotyping reaction (13 min only), the amount of accumulated extension products is sufficient for visual detection by the biosensor, which requires about 15 min. It is conceivable that the number of test spots, and therefore the number of alleles, can be increased by simply increasing the dimensions of the membrane. To our knowledge this is the first report that enables simultaneous visual genotyping of at least 10 alleles in a single biosensor.

References

[1] Gerion D, Chen F, Kannan B, Fu A, Parak W J, Chen D J, Majumdar A and Alivisatos A P 2003 *Anal. Chem.* **75** 4766–72
[2] Consolandi C *et al* 2004 *Hum. Mutat.* **24** 428–34
[3] Konstantou J, Ioannou P C and Christopoulos T K 2007 *Anal. Bioanal. Chem.* **388** 1747–54
[4] Glynou K, Kastanis P, Boukouvala S, Tsaoussis V, Ioannou P, Christopoulos T K, Traeger J and Kanavakis E 2007 *Clin. Chem.* **53** 384–91
[5] Zerefos P G, Ioannou P C, Traeger-Synodinos J, Dimissianos G, Kanavakis E and Christopoulos T K 2006 *Hum. Mutat.* **27** 279–85
[6] Mannelli I, Lecerf L, Guerrouache M, Goossens M, Millot M C and Canva M 2007 *Biosens. Bioelectron.* **22** 803–9
[7] Wang R, Tombelli S, Minunni M, Spiriti M M and Mascini M 2004 *Biosens. Bioelectron.* **20** 967–74
[8] Ozkan-Ariksoysal D, Tezcanli B, Kosova B and Ozsoz M 2008 *Anal. Chem.* **80** 588–96
[9] Marquette C A and Blum L 2008 *Anal. Bioanal. Chem.* **390** 155–68
[10] Matsubara Y and Kure S 2003 *Hum. Mutat.* **22** 166–72
[11] Litos I K, Ioannou P C, Christopoulos T K, Traeger-Synodinos J and Kanavakis E 2007 *Anal. Chem.* **79** 395–402
[12] Toubanaki D K, Christopoulos T K, Ioannou P C and Gravanis A 2008 *Hum. Mutat.* **29** 1071–8
[13] Toubanaki D K, Christopoulos T K, Ioannou P C and Flordellis C S 2009 *Anal. Chem.* **81** 218–24
[14] Litos I K, Ioannou P C, Christopoulos T K, Traeger-Synodinos J and Kanavakis E 2009 *Biosens. Bioelectron.* **24** 3135–9