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Evanescent wave fluorescence biosensor combined with DNA bio-barcode assay for platelet genotyping

Marie Trévisan[a], Manfred Schawaller[b], Gerald Quapil[c], Eliane Souteyrand[a], Yves Mérieux[d], Jean-Pierre Cloarec[a,*]

[a] Université de Lyon, Institut des Nanotechnologies de Lyon, UMR 5270, site Ecole Centrale de Lyon, 36 avenue Guy de Collongue, 69134 Ecully Cedex, France
[b] Diamed, Cressier, Switzerland
[c] Leuze Electronic GmbH+ Co. KG, In der Braike 1, D-73277 Owen, Germany
[d] Établissement Français du Sang Rhône-Alpes, 1-3 rue du Vercors, 69007 Lyon, France

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ABSTRACT

An evanescent wave fluorescence biosensor was combined with a DNA bio-barcode assay to resolve problems met in detection of poor biologic samples. Human platelet antigen (HPA) genotyping was used as a demonstrator. Our bio-barcode assay was based on magnetic carboxylatex particles and non-magnetic carboxylatex particles, both functionalized with oligonucleotides. It was assessed for detecting 84mer synthetic oligonucleotides as targets. The assay allows to specifically detect single nucleotide polymorphism with a detection limit of 2 pM of target nucleic acids. The fluorescence detection is achieved in 150 s.

1. Introduction

Alloimmunization against human platelet antigens (HPAs) may cause three clinical conditions: neonatal alloimmune thrombocytopenia (NAIT), post-transfusion purpura (PTP) and platelet transfusion refractoriness (PR), mostly due to HPA-1a- and 5b-specific antibodies (Kiefel et al., 2001). In these conditions, HPA typing of patients is needed, so that the diagnosis can be confirmed, HPA-matched products can be transfused if required and, in NAIT, parents can be counseled for the risks associated with possible future pregnancies. The assays must be rapid, accurate and sensitive due to the lack of biological material.

Until the early 1990s, platelet typing was performed by serologic assays. These assays required the use of polyclonal antisera coming from patients, which were relatively uncommon. In addition the majority of platelet immunized individuals have human leukocyte antigen (HLA) class-I antibodies. Therefore, typing that could be performed was limited, and many laboratories were only able to phenotype for HPA-1a. The publication of more advanced assays, such as monoclonal antibody-specific immobilization of platelet antigens assay (MAIPA), permitted more extensive phenotyping, but did not resolved the lack of antisera.

The appropriate solution is polymerase chain reaction (PCR) used as a routine technique for platelet typing (Hurd et al., 2002; Merieux et al., 1997; Metcalfe and Waters, 1993). This technique is suitable to type donors and build a library of compatible donors. In some other situations, amount of genomic material is not sufficient and alternative solutions have to be explored. This is the case in NAIT (Winters et al., 1998) where another solution is required for baby's blood sample or amniotic fluid. An alternative technique is widely studied to detect a low-concentration DNA target: the bio-barcode assay (BBC). Based on the assay described by Mirkin et al. (Nam et al., 2003; Stoeva et al., 2006), this technique is based on the formation of a sandwich which on the one hand allows to capture targets with magnetic beads, and on the other hand permits an indirect amplification with the multiplicity of detection markers grafted on non-magnetic beads which are also able to recognize the target. Firstly, the DNA target is captured by a specific probe grafted on magnetic beads, and then non-magnetic beads formed sandwiches with the immobilized targets. Afterward, sandwiches are separated from non-reacted target and non-magnetic beads by a magnetic separation. The detection markers are then analyzed by the chosen technique. These markers can be of different nature such as fluorescent (Hill and Mirkin, 2006; Nie et al., 2009; Thaxton et al.,

* Corresponding author. Tel.: +33 47 218 6252; fax: +33 47 218 6250.
E-mail address: jcloarec@ec-lyon.fr (J.-P. Cloarec).

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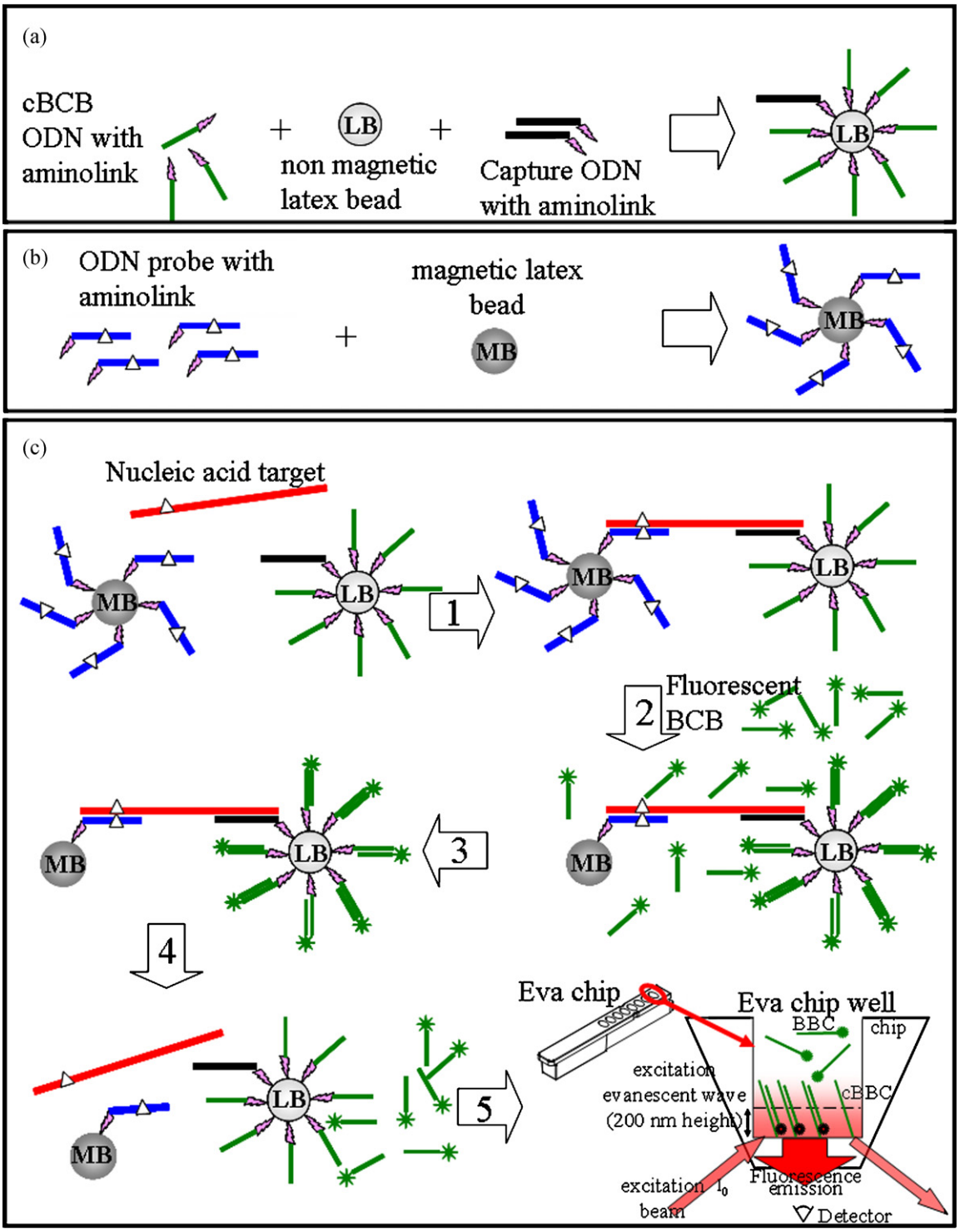

Fig. 1. Bio-barcode assay schematic diagram: (a) preparation of non-magnetic latex beads (MB) by simultaneous grafting of capture OligoDeoxyriboNucleotide (ODN) and ODN complementary to bio-barcode ($_C$BBC); (b) preparation of non-magnetic latex beads (LB) by grafting of ODN probes designed for HPA1 SNP detection; (c) bio-barcode assay schematic diagram. (1) Hybridization of DNA target to LB and MB, (2) hybridization of bio-barcode ODN (BBC) labeled with Cy5, (3) magnetic separation of MB/target/LB/BCB sandwiches from excess non-hybridized BBC and targets, (4) denaturation of sandwiches, and (5) detection of fluorescent BBC ODN in EVA chip with EWF sensor.

2005; Zhang et al., 2009a) or electrochemical as can be the detection techniques such as scanometric method (Hill and Mirkin, 2006; Hill et al., 2007; Nam et al., 2004; Stoeva et al., 2006; Thaxton et al., 2005), DNA chip (Nie et al., 2009), mass spectrometry (Qiu et al., 2008), capillary electrophoresis (He et al., 2008) or electrochemical detection (Ding et al., 2009; Du et al., 2009; Hu et al., 2008; Zhang et al., 2009b; Zhu et al., 2008).

In the present work, a bio-barcode assay (Fig. 1) was coupled with an evanescent wave fluorescence (EWF) biosensor allowing a rapid platelet genotyping. Our EWF sensor allows exciting fluorophores at the very proximity of a surface, within the first 200 nm (Fig. 1c5). This surface is biofunctionalized with Oligonucleotides (ODN) probes able to specifically capture fluorescent bio-barcode ODN. The sensor monitors the capture of BBC ODN onto the sur-

face by measuring the kinetics of evolution of the fluorescence. The detection device can simultaneously analyze eight independent wells in a plastic chip, used for independent non-competitive assays. For each well, kinetic analysis provides a result in 10 min without washing step, thus considerably simplifying the analysis. A first evaluation of our BBC assay/fluorescence biosensor was carried out using 84mers synthetic oligonucleotides as targets. These targets correspond to real sequences routinely used for platelet genotyping (Metcalfe et al., 2003; Bres et al., 2005). After identification of beads biofunctionalization conditions, selectivity and sensitivity of the assay were investigated and compared to literature.

2. Experimental

2.1. Reagents and chemical

1X PBS (0.01 M phosphate buffered saline, [NaCl] = 0.138 M, [KCl] = 0.0027 M, pH 7.4), 20X SSC (0.3 M sodium citrate, pH 7.0, [NaCl] = 3 M), N-hydroxysuccinimide (NHS), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (EDCI), Bovine serum albumin (BSA), magnetic microparticles carboxylate-modified (1 μm diameter, 20 mg/mL, 5×10^8 particles/mg, [COOH] = 5 μmol/mL of solution), latex beads carboxylate-modified polystyrene (900 nm diameter; 30% solids content, 7.5×10^{11} particles/mL) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Ultrapure water (18.2 MΩ) was purified by Purelab ultra purification water system. A NdFeB magnet was used for magnetic separation. All oligonucleotides were obtained from Eurogentec (Belgium). Oligonucleotide sequences, presented in Table A (in additional data), correspond to HPA1 biallelic system (Metcalfe et al., 2003).

2.2. Apparatus

2.2.1. Chip

The EVA chip (Fig. 1), from the lab-on-a-chip concept, is made by injection molding of polystyrene. The polystyrene is identical to the common used polystyrene that is the basis of ELISA microplate molding. A high activity binding polystyrene surface is made form molded chips then by irradiating the polystyrene EVA chips with a Co-60 gamma source at 25 kGray, very much like standard high binding ELISA plates are made from low binding ELISA plates, for example Nunc Polysorb Nunc Maxisorb.

The EVA chip has two areas; the upper part is eight polystyrene wells for the biochemicals that are used in the assay procedure and the analysis. The lower part is an optical prism with a special design to be used in the EVA reader.

EVA chips wells were biofunctionalized using the following procedure: each well was coated with 50 μL of a 10 μg/mL avidin solution in 0.1 M $NaHCO_3$ buffer (pH 9.3). Chips were incubated at room temperature overnight in a water-saturated atmosphere. EVA chip wells were then washed four times with 1X PBS solution and dried by shaking. 80 μL per well of blocking buffer (1X PBS, 1% BSA, 0.25% Tween 20) were added and left to incubate for 1 h at room temperature. Wells were washed twice with 1X PBS then twice with a sucrose solution (1% w:w in water) and dried by gentle shaking. Finally outside part of chips was cleaned with distilled water and dried using optical paper to avoid scratching. After this step chips could be stored for months at room temperature for further immobilisation of nucleic acid probes.

cBBC probes bearing a 3′ terminal biotin were immobilized onto avidin modified wells with the following protocol: 25 μL of a 25 μM biot-cBBC in 1X PBS was added in each well and left to incubate at room temperature for 45 min in a water-saturated atmosphere. Non-reacted cBBC probes were pipetted off. 40 μL of 1X PBS + BSA 4% were added for 30 min. Wells were washed three times with 40 μL of 1X PBS then three times with 40 μL of distilled water. After drying EVA chips were ready for BBC analysis.

2.2.2. Evanescent wave fluorescence biosensor

The evanescence biosensor is based on Evanescence excitation of fluorophores bound to the EVA chip surface (Fig. 1).

The interface between the liquid in the upper well and the lower prism is called the evanescent surface. A diode laser light beam at a wavelength of 635 nm is directed against the side wall of the prism part, is then refracted due to difference in the refractive index of air $n = 1.00$ and polystyrene $n = 1.595$ according to classical geometrical optics. The light beam then further travels in the prism to hit the evanescent surface at the bottom of the well where total internal reflection takes place and the light beam exits then on the other side of the optical prism. The laser light does not enter the liquid above the well but follows classical optic rules of refraction and total internal reflection. As the light beam does not enter the liquid on a whole, it still penetrates into the liquid inside the well which usually is an aqueous solution with a refractive index of n = 1.33. The molecules present in the lower 200 nm layer (evanescent field) of this liquid is then excited by the 635 nm laser light. The majority of unbound fluorescent molecules is outside of this 200 nm evanescent field and is not excited by the incoming photons. In other words the EVA chips as designed allow an excitation of bound fluorophores in the presence of the same fluorophore not bound to the surface.

The set-up in the EVA instrument is conceived in such a way that only the photons that are emitted through the bottom of the prism are collected and filtered with interference filters down to 660+/-5 nm and those resulting photons are counted in time dependant mode. Individual first measurement is then 1 s for well 1, 1 s for well 2 and so on till well 8. Afterwards the second cycle of measurement is then identical to measurement cycle one. Photon counts for well 1 are then plotted in a graph *X*-axis is time and *Y*-axis the photons measured (Additional data, Fig. A). The other seven wells are plotted in the same way as for well one. The increase of bound photons is directly proportional to the amount of fluorophores present initially in the liquid. Apparently the reaction at the bottom of the well is a diffusion controlled reaction and the basic laws of diffusion apply. As all parameters are kept constant, except the concentration of analyte the application of Ficks second law shows a direct proportionality between signal and analyte concentration.

This correlation is given by the "dC" parameter which is linear regression slope of the fluorescence curve. dC is calculated between 10 and 150 s.

2.3. Immobilisation of ODN probes on latex beads

Latex beads were received in a storage buffer solution and were washed before their use according to the following procedure: 10 μL of commercial solution were transferred into a BSA-coated Eppendorf tube and suspended in 490 μL water. Latex particles were then centrifuged for 10 min at 14000 rpm, allowing aspiration of supernatant. Supernatant was carefully pipetted off, leaving particles undisturbed. Particles were then resuspended with 500 μL of water. This purification procedure was repeated three times. Particles were finally resuspended in 450 μL of 1X PBS. Carboxyl functions of the beads were then chemically activated using a classical NHS/EDCI (carbodiimide) chemistry: activation solution was prepared by mixing 2 mg of EDCI with 2 mg of NHS solution in 500 μL of 1X PBS. 50 μL activation solution was added to resuspended particles and they were incubated for 2 h at 25 °C under shaking. Activated particles were washed three times with 1X PBS buffer (pH 7.4), following the washing procedure described above, replacing water with 1X PBS buffer.

Particles were resuspended in 400 μL of 1X PBS. 50 μL of capture probe in 1X PBS and 50 μL of 25 μM cBBC solution in 1X PBS were added with different ratios [capt]/[cBBC], as described in Section 3. The incubation took place overnight at 25 °C under constant shaking. Grafted particles were washed three times with 5X SSC buffer, using the washing procedure described above. They were finally resuspended in 200 μL of 5X SSC buffer.

2.4. Immobilisation of ODN probes on magnetic beads

Magnetic beads were washed following the same procedure that for latex beads, except the fact that centrifugation was replaced by magnetic separation.

Before oligonucleotide grafting, particles were suspended in 450 μL of 1X PBS. 50 μL of 25 μM probe «a» or probe «b» in 1X PBS were added at a 2.5 μM concentration. Incubation took place overnight at 25 °C under constant shaking. Grafted particles were washed three times with 5X SSC buffer, following the washing procedure described above. They were finally resuspended in 450 μL of 5X SSC buffer.

2.5. Bio-barcode assay and EVA reader analysis

In a typical bio-barcode assay, 450 μL of grafted magnetic particles were mixed with 50 μL of target DNA (final concentrations of target are detailed in Section 3). Hybridization was carried out for 2 h at 66 °C. Magnetic particles were then pulled to the side of the Eppendorf tube by using a magnet, supernatant was suppressed. The magnetic field was removed and particles were resuspended with 500 μL of 5X SSC buffer. This purification procedure was repeated three times to remove unspecific adsorption and non-hybridized target. Magnetic particles were then resuspended in 300 μL of 5X SSC buffer. Grafted latex particles (200 μL) were then added to magnetic beads suspension. Sandwich hybridization was performed such as capture probe was hybridized to the end of target DNA at 66 °C for 2 h. As previously, sandwiches were separated from supernatant then washed three times with 5X SSC and resuspended in 450 μL of 5X SSC. Afterward, 50 μL of 2.5 μM Cy5-BBC were added and hybridized with sandwiches for 30 min at 50 °C. As previously described, resulting sandwiches were separated from supernatant then washed three times first with 5X SSC, then 2X SSC and finally 0.2X SSC. Sandwiches were resuspended in 500 μL of 3 mM SSC buffer. The mixture was heated for 5 min at 90 °C for denaturation. Magnetic and latex particles were then separated from Cy5-BBC by centrifugation during 10 min at 14,000 rpm. Supernatant containing bio-barcode was pipetted and transferred in a new Eppendorf tube. 30 μL of supernatant were then injected in a 384-well microplate. This step was repeated with seven other supernatants. 25 μL of each supernatant were then simultaneously pipetted with a multichannel micropipette, put in EVA chip and immediately analyzed by Evareader at 29 °C.

3. Results and discussion

3.1. Design of the HPA1 BBC assay

Oligonucleotide probes used for the presented BBC assay were first qualified using biochip technology, as described in a previous work (Bres et al., 2005). Sequences corresponding to BBC/cBBC, capture/target and probe/target were proved not to cross-hybridize.

Fig. B in additional data shows preliminary results obtained for non-optimized BBC assay conditions. The assays carried out with bare centrifuge tubes exhibited a dC values similar for a perfect match BBC assay ($6.1E^4 \pm 3.8E^4$ a.u), and a single mismatch negative control assay ($8.6E^4 \pm 2.7E^4$ a.u.). Coefficients of variation were, respectively, 31 and 62%. On the contrary, using centrifuge tubes coated by a 4% w:w BSA in 1X PBS solution, and incubated for 2 h at 37 °C, allowed to measure a dC signal 2.6-fold higher for perfect match BBC assay than for single mismatch negative control. Moreover, the measurements gained better repeatability, the coefficient of variation for perfect match BBC assay decreasing to 5% for BSA blocked tubes. BSA blocking of tubes was thus assessed to minimize false positive signals, and improve repeatability of assays, by preventing BBC oligonucleotides to adsorb on tubes during assays. Notice that the importance of an efficient surface blocking of vessels on the quality of the results has been already mentioned (Goluch et al., 2006).

3.2. Specificity of the HPA1 BBC assay

The ability of the HPA1 BBC assay to discriminate SNP was assessed by comparing signals returned by the EWF biosensor for four different cases, as described in Table B in additional data.

Fig. 2 shows dC values obtained for BBC assays tested with 2.5 μM target solution, with a non-optimized [cBBC]/[capture] ratio of 1000 (see below). The dC value for the complementary probe «b» – target «b» perfect match duplex was twice higher than for the one-base mismatched probe «b» – target «a» system. The dC value was ten times higher for the complementary probe «a» – target «a» system than the one-base mismatched probe «a» – target «b» system. In these first testing conditions, probe «a» exhibited a better SNP discrimination than probe «b», showing that hybridization conditions can still be improved.

3.3. Influence of probes relative densities on BBC sensitivity

Because cBBC and capture oligonucleotides have two different functions (detection and capture, respectively) (see Fig. 1) a compromise in their relative surface densities onto latex beads has to be found for improving the sensitivity of HPA1 BBC assay. Latex beads were functionalized for [cBBC]/[capture] ratios of 100/1, 1000/1, 2000/1, 10,000/1, 25,000/1 and 50,000/1. BBC assays were carried out for target concentration of 250 nM for the probe "a" – target "a" duplex. Fig. C in additional data shows a maximum signal was reached for 2000/1 ratio. This ratio was used for afterwards assays.

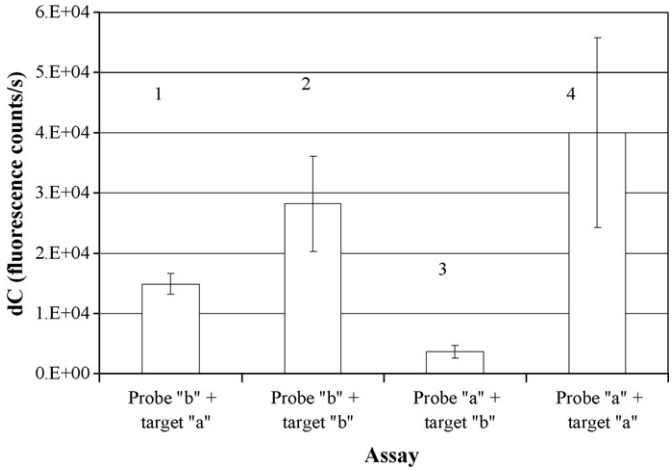

Fig. 2. Measurement of dC for a selectivity assay. (1) Probe «b» + target «a»; (2) probe «b» + target «b»; (3) probe «a» + target «b» and (4) probe «a» + target «a». All assays used a [capture]/[cBBC] ratio equals to 1/1000. Final target DNA concentrations are 2.5 μM. Error bars indicate standard deviation. Each point on the graph corresponds to measurements in duplicate.

Table 1
Comparison between literature and our work performance. "ILOD" indicates inferior limit of detection; "DT" corresponds to detection time of the analysis method used after BBC assay.

Reference	Detection method	ILOD	DT	Type of target	Specificity	Advantages	Drawbacks
Chang et al. (2006)	Electrochemical	1 fM	2000 s	DNA			
Ding et al. (2009)	Electrochemical	4.2 fM	NA	DNA	Detection of two bases mismatch		CdS NPs labeled bio-barcode DNA: unusual markers. Time of hybridization between target and bio-barcode modified beads: 12 h.
Du et al. (2009)	Electrochemical	5 fM	NA	DNA	Detection of two bases mismatch		PbS NPs labeled bio-barcode DNA: unusual markers.
He et al. (2008)	Capillary electrophoresis	5 fM	NA		Recognition of four completely different virus DNA	Whole analysis time: 40 min. The whole process can be automated and can be completed in less than 1 h with high throughput	
Hill et al. (2007)	Scanometric	2.5 fM	6 h	Double-strand genomic DNA			
Hu et al. (2008)	Electrochemical	28 aM	NA		Single base mismatched DNA	The NPG-based biosensor could be regenerated	Hexaammineruthenium(III) chloride markers. The DNA-modified NPG electrode could be stored in the refrigerator for 1 week with negligible loss of the immobilized probe DNA.
Nam et al. (2004)	Scanometric	500 zM	6 h		Single base mismatch DNA		
Nie et al. (2009)	Fluorescence	1 pM	NA	DNA	Single nucleotide polymorphism		Bio-barcode analysis on DNA chip: time consuming detection.
Qiu et al. (2008)	Mass spectrometry	100 pM	NA			Multiplexing can be easy. Detection of 100 pM: not optimized conditions	
Stoeva et al. (2006)	Scanometric	500 fM	6 h	DNA			
Thaxton et al. (2005)	Scanometric	7 aM	6 h	DNA			
Thaxton et al. (2005)	Fluorescence	7 pM	NA	DNA		Fluometer can analyse a 96-well microplate	
Zhang et al. (2009a)	Fluorescence	2.15E−16 mol	NA	DNA		Detection of 2.15E−16 mol target DNA.	
Zhang et al. (2009b)	Electrochemical	1.5 pM	NA	DNA		Detection of about 1.5 pM target DNA	Accumulation time is 240 s.
Zhu et al. (2008)	Electroluminescence	1 fmol	NA			Fast detection (fluorescence count)	Detection of 1 fmol in 25 μL (assay volume)

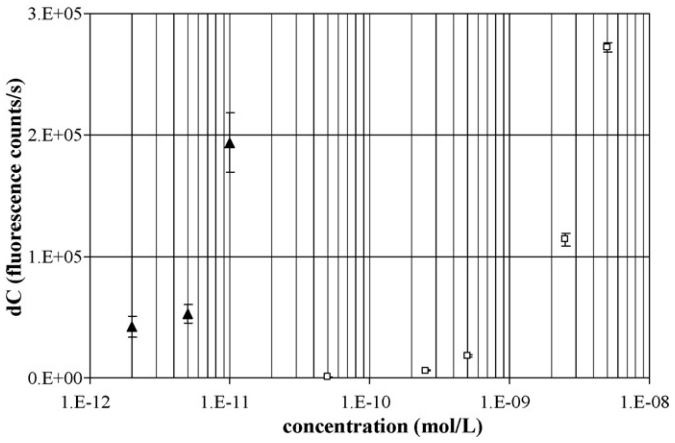

Fig. 3. calibration curves for bio-barcode assay and direct analysis of BBC oligonucleotides. Black triangles: bio-barcode detected in EWF biosensor after BBC assay for perfect match (probe «b» + target «b»). White squares: direct detection of fluorescent targets in EWF biosensor, without BBC assay. For triangles, *X*-axis represents the target DNA concentration. For squares, *X*-axis corresponds to bio-barcode concentration. [cBBC]/[capture] = 2000.

3.4. Assay performances

Eva chips bearing immobilized cBBC probes were used to directly detect fluorescent targets. The calibration curve characterizing the EWF biosensor is presented in Fig. 3 (white squares). Inferior limit of detection (ILOD) is approximately $2.5E^{-10}$ mol/L of fluorescent nucleic acids. When used in combination with BBC assay, ILOD is improved to $2E^{-12}$ mol/L of targets (Fig. 3, black triangles). In the present conditions, BBC assay therefore enhances ILOD by a factor of approximately 250.

Fig. 4 compares signal calibration curves obtained with BBC assays for perfect match and single nucleotide mismatch. For the lowest target concentration tested, SNP detection is assessed by a ratio of 3.5 between perfect match and single mismatch. Considering the variation coefficients of 20–25% for both assays (three measurements were taken for each dot), limit of detection of our method is expected to be further improved. The limit of detection should be further lowered and still requires investigations. Use of a fluorophore with better fluorescence characteristics than Cy5, optimization of BBC components are for instance ways for improving the HPA1 BBC assay inferior limit of detection, while keeping a short time of detection.

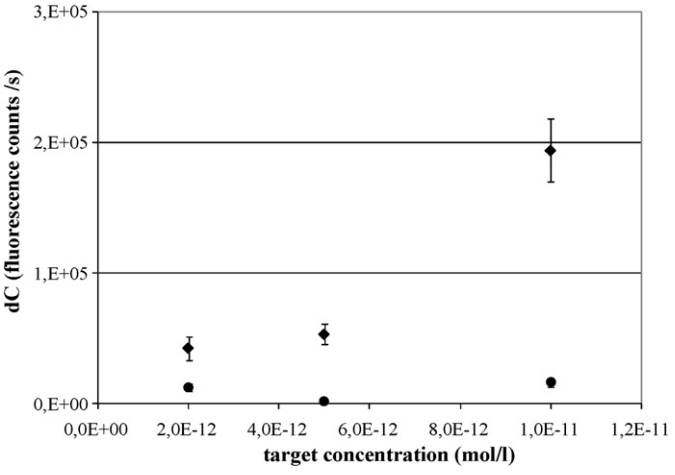

Fig. 4. Calibration curve of bio-barcode assay. Rhombus: perfect match (probe «b» + target «b»); circles: single nucleotide mismatch (probe «b» + target «a»). All BBC assays were carried out with [cBBC]/[capture] = 2000. Error bars indicate standard deviation. Each point on the graph corresponds to three different measurements.

3.5. Comparison of HPA1 BBC assay with literature

Table 1 summarizes analytical performances and duration of final analysis. Scanometric method (Hill and Mirkin, 2006; Hill et al., 2007; Nam et al., 2004; Stoeva et al., 2006; Thaxton et al., 2005) for bio-barcode detection exhibits a far better limit of detection ranging from 500 fM to 500 zM. However, this strategy is time consuming, the detection time being 6 h (not including the BBC assay itself). In the case of NAIT, it is necessary to obtain a genotyping in a time as shortest as possible, as for some other biological analysis such as nosocomial infections. Most of electrochemical methods are faster than our fluorescence detection strategy, and have a better lower limit of detection. However, to our knowledge, no example of SNP discrimination was assessed with electrochemical detection system in a time competitive with our methodology (Ding et al., 2009; Du et al., 2009). Our evanescent wave fluorescent biosensor inferior limit of detection (ILD) is at least comparable with other fluorescence detection systems combined with BBC assays. However, our real time fluorescence analysis approach allows us to proceed to hybridization on a very short time, and avoids any washing step, which is an advantage over more classical biochip strategies (Nie et al., 2009; Thaxton et al., 2005).

4. Conclusion

In the present work, an evanescent wave fluorescent biosensor was combined with a bio-barcode assay for human platelet genotyping. The BBC assay can discriminate SNP, and allows enhancing inferior limits of detection by a factor of 250–500. EWF biosensor returns a final result in 150 s without washing step, thanks to real time analysis. The inferior limit of detection reached for now, without full optimization, is 2 pM of nucleic acid target. This analytical strategy was assessed using single stranded 84mer oligonucleotides as targets. Next steps of this work will consist in optimising the inferior limit of detection, and adapting the strategy to real biological samples.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.08.038.

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