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TECHNICAL NOTE

Portable chemiluminescence multiplex biosensor for quantitative detection of three B19 DNA genotypes

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Abstract A miniaturized multiplex biosensor exploiting a microfluidic oligonucleotide array and chemiluminescence (CL) lensless imaging detection has been developed for parvovirus B19 genotyping. The portable device consists of a reaction chip, comprising a glass slide arrayed with three B19 genotype-specific probes and coupled with a polydimethylsiloxane microfluidic layer, and a charge-coupled device camera modified for lensless CL imaging. Immobilized probes were used in DNA hybridization reactions with biotin-labeled targets, and then hybrids were measured by means of an avidin-horseradish peroxidase (HRP) conjugate and CL detection. All hybridization assay procedures have been optimized to be performed at room temperature through the microfluidic elements of the reaction chip, with sample and reagents delivery via capillary force exploiting adsorbent pads to drive fluids along the microchannels. The biosensor enabled multiplex detection of all B19 genotypes, with detectability down to 80 pmolL^{-1} for all B19 genotype oligonucleotides and 650 pmolL^{-1} for the amplified product of B19 genotype 1, which is comparable with that obtained in traditional PCR-ELISA formats and with notably shorter assay time (30 min vs. 2 h). The specificity of the assay has been evaluated by performing DNA–DNA hybridization reactions among sequences with different degrees of homology, and no cross hybridizations among B19 genotypes have been observed. The clinical applicability has been demonstrated by assaying amplified products obtained from B19 reference serum samples, with results completely consistent with the reference PCR-ELISA method. The next crucial step will be integration in the biosensor of a miniaturized PCR system for DNA amplification and for heat treatment of amplified products.

Keywords Biosensor · Chemiluminescence lensless imaging · Point-of-care testing · Gene probe hybridization · Multiplex assay · Parvovirus B19 genotyping

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Introduction

Portable, minimally or non-instrumented analytical devices are under development for several biomedical and clinical diagnostic applications to enable point-of-care (POC) testing. Multiplexed analysis is also an important trend in diagnostics, driving the development of POC devices for marker panel tests, including cancer markers, cardiac health markers, and infectious agents [1]. Biospecific binding assays exploiting chemiluminescence (CL) detection represent one of the most promising approaches towards miniaturized multiplex assays characterized by high detectability and rapidity [2]. Recently, a versatile and portable bioanalytical device relying on CL lensless charge-coupled device (CCD)-based imaging was developed to perform hybrid multiplexed assays, enabling detection of enzymes, antigens,

and nucleic acids [3]. Digoxigenin-labeled amplified products from parvovirus B19 DNA, selected as a model nucleic acid analyte, were detected by hybridization with an immobilized capture probe and hybrids detection employing a horseradish peroxidase (HRP)-conjugate antibody and a CL substrate. However, the limit of detection (LOD) of the assay was 50 nmol L^{-1}, thus not adequate for diagnostic applications. Herein, we report our progresses on multiplex DNA detection, describing an optimized assay displaying improved detectability and the ability to distinguish between three different B19 virus genotypes [4]. The reaction chip was designed employing capillary forces instead of pumping devices to drive the flows of samples and reagents in order to obtain a minimally instrumented portable device. In this assay, three different amino-modified probes, each specific for a B19 genotype, were covalently immobilized onto a glass slide and used in hybridization reactions with biotin-labeled targets. Hybrids were then detected by means of an avidin-HRP conjugate, upon addition of a CL HRP substrate. At first, artificial oligonucleotide targets, perfectly matching in sequence and length the corresponding probe sequence, were used for method development. Subsequently, biotin labeled amplified products obtained from a B19 control plasmid and from reference clinical samples were assayed to assess diagnostic applicability.

Materials and methods

Oligonucleotide array biosensor

A disposable reaction chip for B19 genotyping was obtained by assembling a microscope borosilicate glass slide (26× 76 mm^2, Thermo Fisher Scientific, Waltham, MA) with a polydimethylsiloxane (PDMS) microfluidic module (Fig. 1). The disposable microfluidic module consisted of three channels (40 mm length, 1 mm width, and 0.1 mm height) with 100-μL inlet reservoirs, while the common outlet reservoir was a 4-cm^2 adsorbent cellulose pad (Whatman CF7, Whatman plc, Maidstone, UK). To perform bioprobe immobilization, the slide glass surface was activated with polyetheramine (JEFFAMINE® ED-600, generously provided by Huntsman Corporation, Everberg, Belgium) according to a reported procedure [5] with slight modifications (see Electronic supplementary material (ESM)). The genotype specific oligoprobes were then spotted (0.2 pmol/spot) in triplicate in an array of nine (3×3) spots, each one not exceeding 1 mm in diameter and 5 mm spacing.

B19 probes and artificial targets

B19 probes and targets were designed based on genomic reference sequences available in the National Center for Biotechnology Information nucleotide data base (sequences are reported in Table S1 in the ESM). The 25-mer B19 probes were amino-modified (NH_2) at 5′-end using a spacer arm of 12 carbon atoms. The complementary 25-mer B19 artificial targets were biotin labeled at the 5′-end. A biotin-labeled 25-mer B19-negative control target was also designed basing on a previously published synthetic sequence [6].

B19 amplified plasmid DNA and clinical samples

Biotin-labeled amplified products (145 base pairs) were obtained with a multiplex PCR assay enabling the simultaneous amplification of the three B19 genotypes. A biotin-modified forward primer and two different reverse primers (see ESM) were used for amplification, as reported in a published procedure [7]. Amplified products were prepared

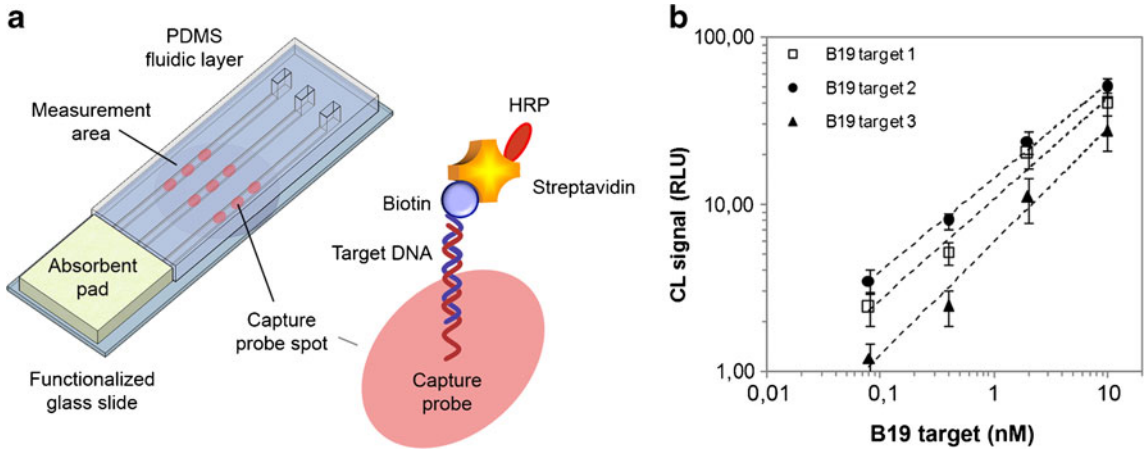

Fig. 1 (**a**) Layout of the disposable reaction chip combining a glass slide with arrayed B19 genotype-specific oligonucleotide capture probes (3×3 array) and a PDMS microfluidic layer. Measurement area corresponds to the reaction chip area that is positioned on the fiberoptic taper surface. (**b**) CL calibration curves obtained by DNA–DNA hybridization reactions between fully complementary B19 capture probe and artificial oligonucleotide targets. The coefficient of determination (R^2) is 0.99 for genotype 1 and 0.98 for genotypes 2 and 3. CL data represent the mean values±SD of three separate experiments. *RLU* relative light unit

either from a B19 genotype 1 plasmid DNA or from ten purified serum samples (five positive and five negative), which were previously assayed with a reference PCR-ELISA method [8]. To enable construction of a calibration curve, amplified products obtained from the plasmid DNA were purified using the Wizard® PCR Clean-Up System (Promega Corporation, Indianapolis, IN) and the concentration was fluorimetrically estimated (QuantiFluor™ ds DNA System, Promega Corporation). The products were denatured by incubation at 95 °C for 5 min, and then iced before hybridization procedure.

Assay procedure

Phosphate-buffered saline (PBS) at pH 7.6, sodium dodecyl sulfate (SDS), 0.05 M borate buffer at pH 9.6, and saline-sodium citrate (SSC) buffer were used in hybridization procedure (buffers composition is described in the ESM).

For analysis, 50 μL of hybridization buffer (5× SSC, 0.1 % SDS) containing the artificial targets at different concentrations (from 1.6×10^{-2} to 10 nmol L^{-1}), or the denatured and purified product of amplification from plasmid DNA (diluted at concentrations from 8×10^{-2} to 50 nmol L^{-1}), or 10 μL of the denatured product of amplification from a clinical sample, were dispensed in the inlet reservoir and let flowing through the channels. Then, washing was performed with 100 μL of 2× SSC containing 0.1 % SDS to remove nonspecific DNA–DNA hybrids. Upon changing the adsorbent pad, 50 μL of HRP-labeled avidin (from Sigma-Aldrich, St. Louis, MO; 50 μg L^{-1} in PBS containing 0.1 % (*w*/*v*) BSA) was added in each channel, then a further washing was performed with 100 μL of PBS. Upon adsorbent pad removal, 20 μL of SuperSignal ELISA Femto (Thermo Fisher Scientific, Waltham, MA) was dispensed in the inlet reservoir, the reaction chip was positioned directly on the taper surface of the CCD-based device according to the reported procedure, and the CL signal was imaged (30 s acquisition time) [3]. Samples and reagents dispensed in the inlet reservoir flowed through the channels at a 15- to 20-μL min^{-1} flow rate, each assay step requiring 2–3 to 5–7 min depending on the dispensed volume (50 or 100 μL, respectively).

Data analysis

Quantitative analysis of CL images was performed using the WinLight software v. 1.2 (Berthold Technologies GmbH & Co KG, Bad Wildbad, Germany). The mean photon emission measured in the area corresponding to probe spots was corrected by subtracting the CL substrate background signal (measured in the absence of target analyte) and expressed in relative light units. For production of calibration curves, the net value of CL signal was plotted against the target concentration. The LOD was calculated as the background signal +3 standard deviations. The same value was selected as cut-off of the assay to evaluate the clinical samples.

Results and discussion

Three different parvovirus B19 genotypes have been recently identified, varying up to 15 % in nucleotide sequence [4]. Although differences in clinical manifestations have not been yet demonstrated, several nucleic acids based technologies failed to detect or underestimated such genotypes leading to B19 misdiagnosis [9]. Thus, more specific analytical methods are required both for research and for diagnostic purposes.

Reaction chip optimization

The immobilization procedure of B19-specific oligoprobes was optimized with respect to the previously reported feasibility study [3] to improve the signal-to-noise ratio and thus assay detectability. First, a polyetheramine-based surface activation strategy was adopted to reduce non-specific DNA binding and to increase oligonucleotide probe accessibility for specific target hybridization [5]. Data related to the optimization of surface activation are reported as ESM (Fig. S1). The optimal amount of immobilized capture probes was determined by spotting different amounts (0.04, 0.2, and 1 pmol) of each genotype specific probe along a channel, then performing hybridization procedures with the corresponding target (10 nmol L^{-1}). For all genotypes, the highest CL signal was observed for 0.2 pmol of deposited capture probe (Fig. S2 in the ESM) while higher amounts showed a lower CL signal. This effect can be ascribed to steric hindrance of capture probes, which are closely packed together in the spot and therefore less exposed to target hybridization [10]. Indeed, 0.2 pmol/spot of probe corresponds to approximately 10^5 molecules μm^{-2}, which is in the range of the optimal surface printing density previously reported for oligonucleotide arrays [11].

Calibration curves

The calibration curves produced for each B19 genotype employing complementary biotin-labeled artificial targets in the concentration range from 1.6×10^{-2} to 10 nmol L^{-1} are reported in Fig. 1. The dynamic range of the assay extended from 0.1 to 10 nmol L^{-1} and the LOD was 80 pmol L^{-1} for all B19 targets, corresponding to 5.0×10^7 molecules μL^{-1}. When the same reagents were assayed in a conventional PCR-ELISA format, a LOD value of 100 pmol L^{-1} was obtained. Remarkably, the LOD obtained in the present work is significantly lower than the previously reported one [3] due to the optimization of surface functionalization strategy, biospecific

capture probes design and assay experimental conditions. Figure 1 also shows that the calibration curve obtained for genotype 3 presents a different slope than those obtained for genotypes 1 and 2. This can be ascribed to the different percentage content of GC couples in hybrids, which is 56 % for genotypes 1 and 2, while it is 52 % for genotype 3.

Assay specificity

Specific B19 capture probes were carefully selected to achieve a significant discrimination of mismatches between different B19 genotypes. Assay specificity was established by analyzing B19 targets (10 nmolL^{-1}) of the different genotypes. As shown in Fig. 2, CL signals above the cut-off value were observed only when the target analyte hybridized with the corresponding capture probe. In addition, no significant CL signal was detected when the biotin-labeled B19 negative control target was assayed with each capture probe.

Analysis of amplified samples

To evaluate the detectability of PCR-amplified products, the product of amplification obtained from a B19 genotype 1 plasmid DNA was purified from reaction mix, fluorimetrically quantified, and then differently diluted (from 8×10^{-2} to 50 nmolL^{-1}) prior to analysis. A CL signal above the cut-off value was detected only in the correspondence of the B19 genotype 1 capture probe, thus confirming assay specificity on amplified products. The LOD value (650 pmol L^{-1}) was slightly higher than that obtained for B19 targets (80 pmolL^{-1}), presumably due to a lower hybridization efficiency between a 25-mer oligonucleotide probe and a 145-base pair amplified product, with respect to hybridization between single-stranded sequences of the same length. Finally, amplified products obtained from ten reference serum samples were directly analyzed after PCR and scored as negative and positive, based on the cut-off value of the reaction chip. The results (five negative and five B19 genotype 1-positive samples) were completely consistent with the reference PCR-ELISA method.

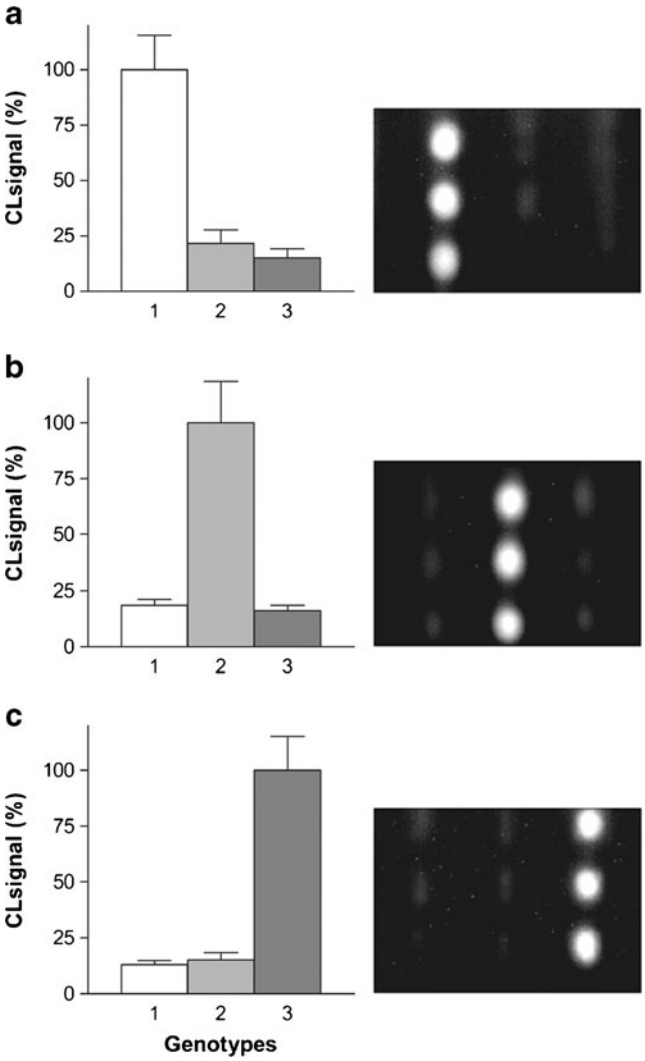

Fig. 2 CL-normalized signals (*left*) and CL images (*right*) obtained from hybridization reactions between the three amino-linked B19 DNA probes and the three biotin-labeled artificial oligonucleotide B19 targets. CL signals represent the mean values±SD obtained for three separate experiments. The images show three reaction chips, each one arrayed with the three B19 genotype-specific probes (three separate spots per genotype; genotype 1 along the *left*, genotype 2 along the *middle*, and genotype 3 along the *right microchannel*) and hybridized with a defined B19 biotin-labeled target ((**a**) genotype 1, (**b**) genotype 2, and (**c**) genotype 3)

Conclusions

In the present study, an improved portable biosensor able to detect and genotype B19 DNA exploiting CL lensless imaging as a detection system was described. A microfluidic reaction chip was designed comprising a PDMS microfluidic layer coupled with a glass slide on which genotype specific B19 capture oligonucleotide probes had been arrayed. Adsorbent pads were employed to draw the samples and reagents along the channels exploiting capillary forces, thus realizing a self-powered non-instrumented reaction chip.

The miniaturized analytical biosensor allowed detecting and genotyping viral targets in an overall hybridization assay time of 30 min, which is remarkably shorter with respect to the microtiter plate DNA hybridization step of reference PCR-ELISA method (2 h). Target detectability was 80 pmolL^{-1} for the three different B19 genotype oligonucleotide targets and 650 pmolL^{-1} for the amplified product of DNA genotype 1, which is comparable to that obtained with standard laboratory methodologies (e.g., PCR-ELISA) and significantly improved with respect to the previously reported feasibility study [3]. The applicability of the developed experimental platform for

B19 genotyping in clinical samples was assessed on ten serum samples, and the obtained results were confirmed by a reference PCR-ELISA method.

The developed biosensor thus represents a minimally instrumented system for the simultaneous and accurate quantification of various DNA sequences in one sample (multiplexing) employing relatively inexpensive instrumentation, even in POC applications. The next crucial step to obtain full POC applicability will be the integration of a miniaturized PCR system [12] to perform on-chip both target DNA amplification and product denaturation prior to hybridization analysis. Thanks to its simple configuration, the biosensor could be also tailored to other applications by changing the arrayed probes, which makes it a valuable tool for viral infection diagnosis. Indeed, for various viruses (e.g., hepatitis C virus, hepatitis B virus, human papillomavirus, and influenza viruses) genotype determination provides important information to assess therapeutic intervention and to predict the outcome of the disease.

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