Analytica Chimica Acta 658 (2010) 193–196

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Spatially addressable bead-based biosensor for rapid detection of beta-thalassemia mutations

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ARTICLE INFO

Article history:
Received 3 December 2008
Received in revised form 4 May 2009
Accepted 10 November 2009
Available online 18 November 2009

Keywords:
Beta-thalassemia mutations
Beta-globin gene
Spatially addressable
Bead-based biosensor
Rapid detection

ABSTRACT

A simple glass-polymer bead-based biosensor was validated for the detection of beta-thalassemia mutations. Different bead types, each carrying allele-specific probes targeting a particular mutation on the beta-globin gene, were immobilized and distinguished on the chip based on their spatial addresses. Genomic DNA samples carrying various single nucleotide transitions and transversions in the beta-globin gene were subjected to polymerase chain reaction and asymmetric amplification in the presence of Cy3-labeled primers, followed by hybridization onto the chip and detection under an epifluorescent microscope. Mutations that were heterozygous or homozygous were easily detected on the device based on the signal intensity difference (or similarity) between the wildtype and mutant probes. This device successfully detected all six common beta-globin gene mutations within 30 min. The number of targeted mutations on this chip can be easily expandable through the introduction of additional probe sets.

1. Introduction

Beta-thalassemia is a common monogenic disease characterized by anemia of varying severity and is typically caused by point mutations or small deletions or inserts within the beta-globin gene. It affects an estimated 50 000 infants each year with a higher prevalence among Mediterranean, Middle Eastern, African and Asian populations. This correlates with a corresponding high carrier frequency ranging from 1 to 20% among these populations [1]. So far, more than 200 different mutations associated with the disease have been identified, even though a much lesser number may be sufficient to characterize the majority of beta-thalassemia genotypes within certain populations [2]. For instance, <20 mutations account for the majority of beta-thalassemia alleles in South-East Asia (SEA). Still, methods to detect these mutations can be inefficient and costly, particularly for poorer developing countries.

Conventional molecular methods for beta-thalassemia screening usually involve an initial polymerase chain reaction (PCR) step followed by subsequent analysis to detect the relevant mutations using techniques such as temperature gradient gel electrophoresis [3], denaturing high performance liquid chromatography [4], amplification refractory mutation system [5], direct sequencing [6] and minisequencing [7]. Other methods, such as allele-specific PCR [8] and melting curve analysis using specific probes [9], simplify the detection process by combining PCR and mutation analysis into a single-step reaction. Depending on the method, these analyses are commonly performed on platforms such as the 96-well plate, polyacrylamide gel block and chromatography column, which can be low-throughput, cumbersome, and time-consuming. Harnessing microfabrication technology, beta-thalassemia mutations have recently been carried out on chip-based platforms like the oligonucleotide microarray [10] and microelectronic chip [11], which offer the advantages of enhanced throughput, reduced sample requirement, and improved sensitivity. In spite of that, fabrication process for these devices can be complicated and costly, while its reaction time can still be several hours long.

We previously reported a spatially addressable bead-based biosensor and demonstrated its ability to discriminate single-base mismatches using purified synthetic oligonucleotides to represent the target sample DNA [12]. Here, we show the real-world applicability of this device by validating it for the detection of beta-thalassemia mutations from patient genomic DNA samples. Allele-specific probes targeting various regions of the beta-globin gene are surface-bound to polystyrene microbeads, which are then sequentially spotted onto a polymer matrix at the surface of the biosensor (Fig. 1). The polymer matrix causes the first set of spot-

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doi:10.1016/j.aca.2009.11.020

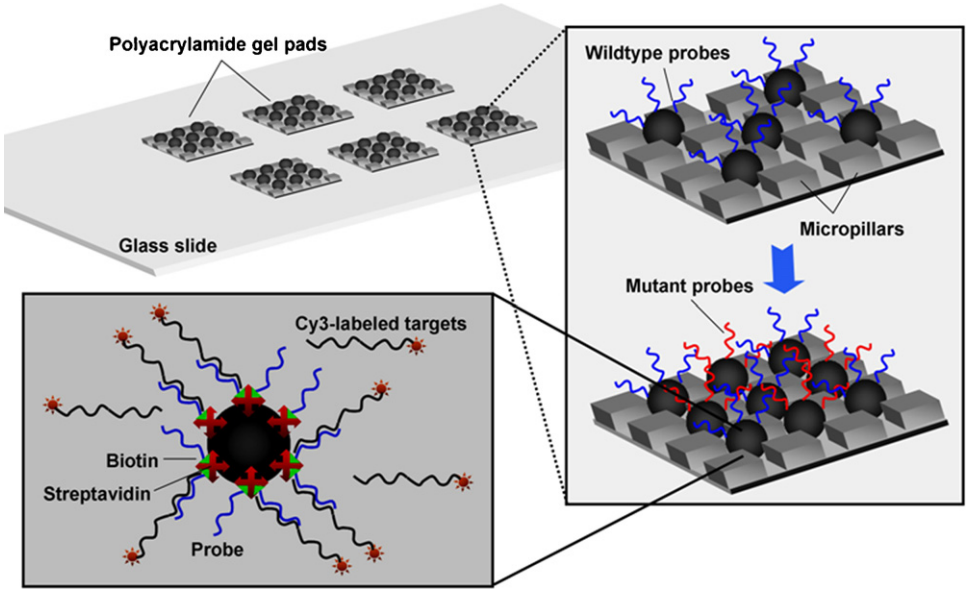

Fig. 1. Schematic representation of the spatially addressable bead-based biosensor.

ted beads to be immobilized at unique locations, thus acquiring spatial addresses then can be recorded via an image. The process is then repeated for spotting and distinguishing subsequent bead types, with each bead type carrying probes for targeting either the wildtype or mutant allele of a sample. This allows the different probe-bead types to be identified without the need for prior encoding of the microbeads. Genomic DNA samples are sequentially amplified by duplex-PCR and asymmetric PCR, followed by hybridization and detection. Hybridization is accomplished within 30 min, and the SEA beta-globin genotype can be determined within 3 h, demonstrating its potential as a simple tool for rapid beta-thalassemia carrier screening.

2. Experimental

2.1. Fabrication of device

The spatially addressable bead-based biosensor had previously been described [12]. Briefly, it consisted of a polyacrylamide gel matrix fabricated onto the surface of a glass slide using a photopolymerization process [13]. The gel matrix comprised an array of square pads for immobilizing the beads, which rested in between the evenly spaced micropillars that made up each pad. Beads were spotted onto the gel pads either manually using a micropipette or automatically using a robotic arrayer. Spatial addresses of the spotted beads were then recorded via a phase contrast image captured under a microscope (BX51, Olympus) at 200× magnification. All the bead types, each targeting a specific mutation site, were spotted onto the same gel pads.

2.2. Oligonucleotides probes and genomic targets

Six common Southeast Asian beta-globin gene point mutations, representing both transitions (A/G, C/T) and transversions (G/T, G/C), were selected for this study: −28 A→G, −29 A→G, IVSI5 G→C, IVSI1 G→T, Cd26 GAG→AAG, and IVSII654 C→T. Common in/del mutations such as the codon 41/42 ΔTCTT 4 bp deletion and the codon 71/72 + A 1 bp insertion were excluded, since perfect match and mismatch probe-fragment duplexes involving single nucleotide substitutions are more challenging to discriminate compared to those involving in/del mutations.

For each mutation, allele-specific probes were designed to hybridize with perfect complementary to either the wildtype or mutant variant allele (Table 1). A biotin moiety was added to the 5′ end of each probe, and probes were then conjugated to 9.95 μm streptavidin-modified polystyrene beads according to previously described protocol [12].

PCR was carried out to amplify two fragments of the beta-globin gene from genomic DNA, with the first fragment (319 bp) encompassing exon 1 which included all the targeted mutations except for IVSII654 C→T, which was contained in the second fragment (128 bp). Primer sequences were: Frag1-F: 5′-Cy3-ACggCTgTCATCACTTAgAC-3′ (HUMHBB nucleotide 62010–62029 [7]); Frag1-R: 5′-CCCAgTTTCTATTggTCTCC-3′ (HUMHBB nucleotide

Table 1
Probe sequences for targeting each of the six beta-globin gene mutations.

Probe name	Allele targeted	Sequence (5′–3′)	Size (bp)	Tm (°C)
−28,−29_WT	−28/−29 WT	CCTGACTTTTATGCCCAG	18	54
−28_MT	−28 MT	CCTGACTTCTATGCCCAG	18	56
−29_MT	−29 MT	CCTGACTTTCATGCCCAG	18	56
IVSI5,1_WT	IVSI5/1 WT	CTTGATACCAACCTGCCC	18	56
IVSI5_MT	IVSI5 MT	CTTGATAGCAACCTGCCC	18	56
IVSI1_MT	IVSI1 MT	CTTGATACCAAACTGCCC	18	54
Cd26_WT	Cd26 WT	GGGCCTCACCACCAAC	16	52
Cd26_MT	Cd26 MT	GGGCCTTACCACCAAC	16	52
IVSII654_WT	IVSII654 WT	TTGCTATTGCCTTAACCC	18	52
IVSII654_MT	IVSII654 MT	TTGCTATTACCTTAACCC	18	50

WT: wildtype; MT: mutant; Tm = 2(A/T) + 4(G/C).

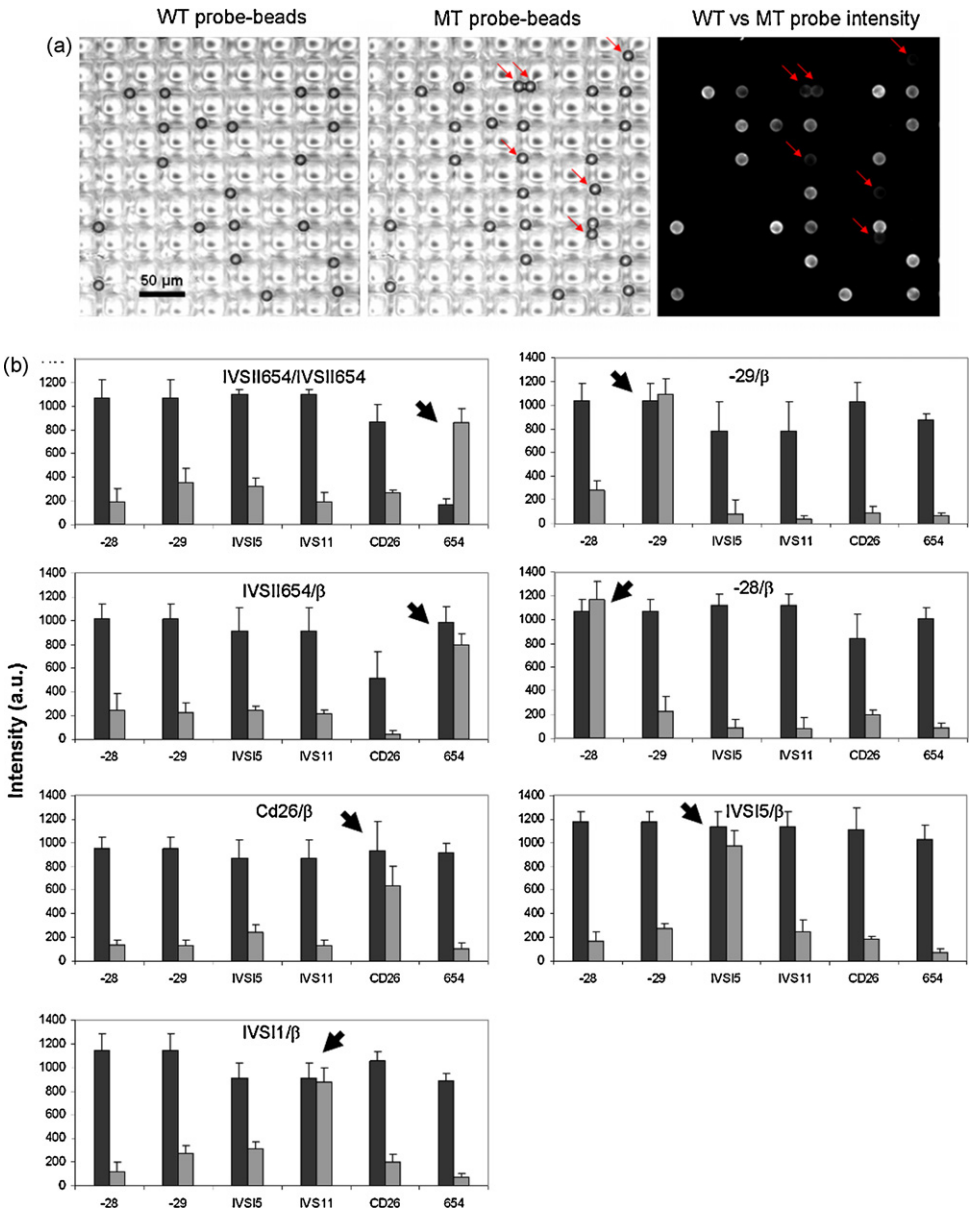

Fig. 2. Allele-specific hybridization on the device. (a) Typical example of the beads spotted onto a gel pad. Probe-beads targeting Cd26 wildtype variant were spotted onto a gel pad, followed by those targeting the mutant variant (red arrows). Difference in probe intensities showed sample to be of homozygous Cd26 normal genotype. (b) Results of the six human DNA samples heterozygous for −28, −29, IVSI5, IVSI1, Cd26, and IVSII654, and one homozygous for IVSII654 C → T analyzed using the bead-based biosensor. Each graph shows the signal intensities from the wildtype (■) and mutant (■) probe-bead targeting each mutation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

62328–62309); Frag2-F: 5′-Cy3-TgTATCATgCCTCTTTgCACC-3′ (HUMHBB nucleotide 63227–63247); and Frag2-R: 5′-CAATATg-AAACCTCTTACATCAg-3′ (HUMHBB nucleotide: 63354–63332). Genomic DNA (100 ng) was amplified in a total volume of 50 μL containing 0.5 μM each of the two sets of primers, 200 μM of each deoxynucleotide triphosphate, and 1 U of HotStarTaq polymerase in 1× supplied PCR buffer (Qiagen). Amplification was carried out in an iCycler thermal cycler (BioRad) with an initial denaturation at 95 °C for 15 min, followed by 35 cycles at 98 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. After purification (Microcon YM-30, Millipore), 100 ng of the products were re-amplified in an asymmetric PCR using only the forward primers to generate ssDNA for allele-specific hybridization. All other conditions were identical to the first PCR step.

2.3. Hybridization and signal detection

Re-amplified PCR products were purified using the Microcon YM-30 filter device (Millipore) before being diluted to a 10 μL hybridization solution containing 500 mM NaCl and 30% formamide. Hybridization was carried out by pipetting the solution over the spotted beads. After 30 min incubation, device was rinsed briefly with a solution containing only 500 mM NaCl and 30% formamide, and signal capture was carried by fluorescence imaging. The imaging system comprised an epifluorescent microscope (BX51, Olympus), 100 W mercury lamp and fluorescence filter set 41007 (Chroma Technology). MetaMorph 5.0 (Molecular Devices) was used to control acquisition of 12-bit monochrome bead images at 2 s exposure from a SPOT-RT Slider cooled-CCD camera (Diagnostic Instruments), and bead signals were quanti-

tated using the modified version of a software developed in-house previously [14].

3. Results and discussion

To demonstrate detection of the six beta-globin gene mutations, six human DNA samples heterozygous for −28 A→G, −29 A→G, IVSI5 G→C, IVSI1 G→T, Cd26 GAG→AAG, and IVSII654 C→T, and one homozygous for IVSII654 C→T were analyzed using the bead-based biosensor. All samples were genotyped previously by direct sequencing or multiplex minisequencing [7]. Wildtype and mutant probes targeting each mutation were conjugated to distinct bead sets, spotted onto a particular gel pad on the device, and distinguished based on their spatial addresses (Fig. 2a). Probes were designed with the targeted mutation as near as possible to the center of the oligonucleotide probe to increase the discrimination between matched and mismatched duplexes. Due to the proximity between the −28 and −29 mutations, as well as between the IVSI1 and IVSI5 mutations, each pair of mutations must be detected simultaneously on a single gel pad by four sets of probes to cover all possible genotypes. However, due to the lack of patient samples compound heterozygous for −28/−29 and IVSI1/IVSI5, only three sets of probes were required in this study for each pair of mutations.

Fig. 2b shows the signal intensity from the wildtype and mutant probes used to target each mutation. All seven different samples were correctly genotyped using the device. For heterozygous mutations, signal intensities from the wildtype probes did not differ significantly from that of the mutant probes, attaining student *t*-test *p*-values > 0.05 for all except IVSII654 which had a slightly lower *p*-value of about 0.01. In the absence of a mutation, the wildtype probe intensities were significantly higher than that of the mutant probes, with *p*-values < 0.001. For the homozygous IVSII654 mutation, the mutant probe intensity was significantly higher than the wildtype probe, attaining a *p*-value < 0.0001. This similarity or significant difference between wildtype and mutant probe intensities allowed correct identification of the heterozygous mutant and homozygous wildtype (or mutant) samples respectively.

The spatially addressable bead-based biosensor offers an alternative tool for simple yet efficient and rapid detection of beta-thalassemia mutations. The device is comprised simply of a glass slide fabricated with a thin polyacrylamide matrix on its surface using a photopolymerization process that is faster (∼45 min) and far less complicated than conventional photolithographic techniques for making silicon chips [15]. The main advantage of the device is its ability to distinguish different bead types without the need for prior time-consuming and laborious techniques such as color-encoding [16]. This is due to the natural immobilization of the beads into the polyacrylamide gel pads, thus allowing the beads to acquire unique spatial addresses. Detection is achieved by applying the solution of PCR-amplified targets over the region of the spotted beads for passive hybridization to occur, which obviates the need for microfluidic mixing and thus microchannels. This further simplifies the fabrication process, lowers the cost of the device, and reduces the sample volume required (<10 μL). Despite the lack of microfluidic mixing, detection is achieved in 30 min, although this might possibly be even faster, given that we have achieved hybridization on this device within 10 min, albeit with synthetic targets [12].

4. Conclusion

Although the bead-based biosensor was demonstrated for only six mutations, this can be easily increased by designing more probe sets to target other mutations on the gene fragments amplified in this study. For instance, probes can be designed to target Cd0 T→G, Cd8/9 + G, Cd17 A→T, Cd19 A→G and Cd27/28 + C, which are common mutations also found within the exon 1 fragment, to enhance the device's throughput. Future work will involve demonstrating the device's ability to detect all mutations, including homozygous and compound heterozygous genotypes. Blinded analysis of a large number of different samples will also verify the reliability and robustness of the device. One disadvantage of this device, however, is the need for a prior PCR step to generate the amplified targets for hybridization, which might make it less attractive as compared to one-step PCR-based techniques [8,9]. Furthermore, certain probes gave good hybridization signals only when single-stranded PCR products are generated (results not shown). Nonetheless, for the majority of analyses that still require a PCR step (and asymmetric PCR for generating ssDNA) prior to detection, this device has the potential to be a simple, easy-to-use tool for rapid (total time <3 h) detection of mutations and is amenable to diseases other than beta-thalassemia.

Acknowledgement

This work was supported by a research grant from the Biomedical Research Council of Singapore to SSC & WTL (BMRC 04/1/21/19/310).

References

[1] D.J. Weatherall, J.B. Clegg, Genes Immun. 3 (2002) 331–337.
[2] D.J. Weatherall, J.B. Clegg, The Thalassaemia Syndromes, Blackwell Science, Oxford, 2001.
[3] R.V. Shaji, E.S. Edison, B. Poonkuzhali, A. Srivastava, M. Chandy, Clin. Chem. 49 (2003) 777–781.
[4] S.N. Bournazos, A. Tserga, G.P. Patrinos, M.N. Papadakis, Am. J. Hematol. 82 (2007) 168–170.
[5] J.A. Tan, J.S. Tay, L.I. Lin, S.K. Kham, J.N. Chia, T.M. Chin, N.B. Aziz, H.B. Wong, Prenat. Diagn. 14 (1994) 1077–1082.
[6] G. Hussein, M. Fawzy, T.E. Serafi, E.F. Ismail, D.E. Metwally, M.A. Saber, M. Giansily, J.F. Schved, S. Pissard, P.A. Martinez, Hemoglobin 31 (2007) 49–62.
[7] W. Wang, S.K. Kham, G.H. Yeo, T.C. Quah, S.S. Chong, Clin. Chem. 49 (2003) 209–218.
[8] D.L. Quek, Y.Y. Ng, W. Wang, A.S. Tan, G.I. Tang-Lim, I.S. Ng, H.Y. Law, S.S. Chong, Clin. Biochem. 40 (2007) 427–430.
[9] C. Vrettou, J. Traeger-Synodinos, M. Tzetis, G. Malamis, E. Kanavakis, Clin. Chem. 49 (2003) 769–776.
[10] Y. Bang-Ce, L. Hongqiong, Z. Zhuanfong, L. Zhengsong, G. Jianling, Haematologica 89 (2004) 1010–1012.
[11] B. Foglieni, L. Cremonesi, M. Travi, A. Ravani, A. Giambona, M.C. Rosatelli, C. Perra, P. Fortina, M. Ferrari, Clin. Chem. 50 (2004) 73–79.
[12] J.K. Ng, E.S. Selamat, W.T. Liu, Biosens. Bioelectron. 23 (2008) 803–810.
[13] D. Proudnikov, E. Timofeev, A. Mirzabekov, Anal. Biochem. 259 (1998) 34–41.
[14] J.K. Ng, W.T. Liu, Bioinformatics 21 (2005) 689–690.
[15] G.S. Fiorini, D.T. Chiu, BioTechniques 38 (2005) 429–446.
[16] K. Braeckmans, S.C. De Smedt, M. Leblans, R. Pauwels, J. Demeester, Nat. Rev. Drug Discov. 1 (2002) 447–456.