



Detection of the sickle hemoglobin allele using a surface plasmon resonance based biosensor

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ABSTRACT

Sickle Cell Disease (SCD) is a monogenic hereditary blood disorder caused by a single point mutation (β^S) in the β globin gene resulting in an abnormal hemoglobin (HbS) that can polymerize within the erythrocytes, inducing their characteristic sickle shape. This causes hemolytic anemia and occlusive vessels for the most severe clinical status. Molecular analysis is crucial for fast and precise diagnosis of different forms of SCD, and, on the basis of underlying genotype, for supporting the most appropriate treatment options. In this context, we describe a simple and reproducible protocol for the molecular identification of the β^S mutation based on surface plasmon resonance (SPR) using the Biacore™ X100 affinity biosensor. This technology has already demonstrated its diagnostic suitability for the identification of point mutations responsible for genetic diseases such as cystic fibrosis and β thalassemia, using a protocol based on immobilization of PCR products on the sensor chip. On the contrary, in this work we applied a SPR strategy based on an innovative interaction format, recently developed in our group also for β thalassemia mutations. In particular, we correctly detected the β^S mutation responsible for SCD, both in homozygous and heterozygous states, after hybridization of two oligonucleotide probes (normal and mutated) for the β^S mutation, immobilized on sensor chip, with unbalanced PCR products obtained from 53 genomic DNAs carrying different β^S allele combinations.

1. Introduction

The human β globin gene (*HBB*) is located on chromosome 11 and encodes the β globin protein, which combines with α globin protein, generating in red blood cells the adult hemoglobin (HbA) tetramer ($\alpha_2\beta_2$), the molecule responsible for the oxygen transport throughout the body. More than 900 different mutant alleles for the *HBB* gene have been identified which either produce different protein variants, or cause a reduced or absent production of the protein product, leading to several forms of hemoglobinopathies, the most common recessive diseases worldwide [1]. Hemoglobinopathies can be classified in two main categories: those resulting from structural hemoglobin (Hb) variants and thalassemias [2], but also their combinations can occur. Among the structural Hb variants, the most significant, from a clinical point of view, is the Hb variant causing sickle cell diseases (SCD) [3,4]. People with these disorders present an atypical hemoglobin, called sickle

hemoglobin (HbS), at abnormal levels (proportion of HbS > 40%) due to the presence of at least one β globin S allele (β^S) [5,6]. In fact the SCD include homozygous sickle-cell disease (HbSS) and a range of mixed heterozygous hemoglobinopathies (HbS/ β -thalassemia, HbSC disease, and other combinations) [7], but in all cases the β^S allele results from substitution of nucleotide A with T at codon 6 of the *HBB* gene, replacing the normal glutamic acid with a valine residue [7,8]. This variant hemoglobin is more rigid and less soluble compared to the normal hemoglobin and can distort red blood cells into sickle-shaped cells causing anemia, vaso-occlusion, pain and chronic organ dysfunction in homozygous patients with elevated HbS [9,10].

The identification of the β^S allele is an important issue, especially in those areas showing high frequencies of this Hb variant like Africa, Mediterranean region, Southeast Asia, Spanish-speaking regions in South America, Central America, and parts of the Caribbean [3, 11–13]. In this respect, early and fast diagnosis might be very important in

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preventing SCD crisis in newborns, that are among the most common (and in some cases fatal) complications in the early life.

At present isoelectric focusing and/or high-performance liquid chromatography (HPLC) analysis of peripheral blood are being used for the HbS quantification during the premarital and newborn screening [14–16]. In certain cases genetic testing is also performed [6], based mainly on polymerase chain reaction (PCR) and requiring in most cases time-consuming and/or relatively expensive methods, such as amplification refractory mutation system (ARMS) [17], high resolution melting (HRM) [18], restriction fragment length polymorphism (RFLP) [19], DNA sequencing [20].

In this article we describe the real-time and label-free detection of β^S single point mutation using a low-cost and highly sensitive protocol based on Biacore™ X100, a Surface Plasmon Resonance (SPR)-based biosensor.

Several articles report SPR-based biomolecular investigations, but only a limited number of them describe methods to identify single point mutations causing genetic diseases, including single point mutations of the *HBB* gene responsible for β thalassemia or SCD. In all these articles, the SPR biosensor technology was applied to mutation analysis of the human *HBB* gene using large PCR products as targets [21–23]. In details, the PCR-amplified regions of genomic DNAs from analyzed subjects were immobilized on the sensor chips and oligonucleotide probes for β thalassemia or β^S single point mutations were sequentially injected in solution onto the sensor chip surface, in order to determine possible hybridization with the target. The described interaction format was able to discriminate single point mutations, preventing however the reuse of the same flow cell for further diagnostic analyses, with high costs and limited number of subjects analyzed per sensor chip, depending on the number of control samples used.

In order to overcome all these drawbacks, we have recently published the application of an SPR approach for the detection of four β thalassemia single point mutations using a novel format based on the immobilization of allele-specific probes on flow cells of the sensor chip and injection of asymmetric PCR products obtained from analyzed donors [24,25]. Thanks to this format, high signal enhancement was obtained in order to improve the sensitivity and to detect low levels of the target mutation, as in prenatal diagnosis of genetic diseases [24].

The novelty of this work is that the β^S single point mutation detection using the SPR-based approach, is based on two oligonucleotide probes immobilized on the sensor chip (one complementary to the normal sequence, the other complementary to the mutated sequence) and on the injection of asymmetric PCR products (as target molecule), covering the β^S region of genomic DNA isolated from the analyzed subjects. In order to discover the best probe for β^S detection, we have comparatively evaluated the hybridization between five pairs of oligonucleotide probes having different lengths and two complementary oligonucleotide targets, normal or mutated, immobilized on sensor chip (Fig. 1A). In this way, the 12-mer oligonucleotide pairs were selected as normal and mutated probes and immobilized on two different sensor chips to validate their interaction with single-stranded target PCR products obtained using genomic DNAs isolated from 53 donors carrying or not a β^S allele (Fig. 1B).

2. Materials and methods

2.1. Samples collection

Blood samples were obtained using Vacutainer LH Treated tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) containing either lithium heparin or EDTA as anticoagulants. Recruitments and sampling were conducted after approval of the Ethical Committee of S. Anna Hospital Ferrara (Italy) and Thalassemia and Hemoglobinopathies Center, Laiko General Hospital, Athens, Greece. In all cases informed consent was obtained and the experiments performed in agreement with the Declaration of Helsinki. A progressive number was assigned to

each specimen to ensure the anonymity.

2.2. Extraction of genomic DNA using QIAamp® DNA blood mini kit

Genomic DNA, collected at Ferrara University (Italy), was extracted from 500 μ l of blood using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and as reported in Breveglieri et al. [25] and **Supplementary materials**.

2.3. Synthetic oligonucleotides

Synthetic oligonucleotides were purchased from IDT (Integrated DNA Technologies, Coralville, Iowa, USA) and used for SPR experiments or PCR reactions. The relative nucleotide sequences are reported in **Table 1**.

2.4. Unbalanced polymerase chain reaction (PCR)

For PCR reactions performed for SPR-based detection of the β^S mutation, different pairs of primers were designed having different melting temperatures and producing distinct amplicon lengths (**Table 1**). After a balanced amplification reaction using 25 ng of genomic DNA obtained from blood donors, unbalanced amplification was performed using only the forward primer in order to obtain single-stranded PCR products as reported by Breveglieri et al. [25] and in **Supplementary materials**.

2.5. Biospecific interaction analysis with Biacore™ X100

The Biacore™ X100 analytical system (GE Healthcare, Chicago, Illinois, USA) and the Biacore™ X100 Control Software (GE Healthcare) were used in all SPR-based analyses as reported by Breveglieri et al. [25] and in **Supplementary materials**.

2.6. Statistical analysis

Statistical analysis was performed using the Student's t-test, selecting a 95% confidence level and a statistical significance at $p < 0.05$.

3. Results and discussion

3.1. Identification of the optimal probes for HbS detection

The first part of our experimental plan was the identification of the optimal probes for the detection of the β^S mutation (Fig. 1A). This phase consisted in the immobilization on sensor chip of two 19-mer biotinylated target oligonucleotides, one normal and the other β^S mutated, simulating the unknown DNA to be analyzed and less prone to secondary structure formation compared to PCR products, and in the subsequent injection of complementary probe pairs with different lengths.

First of all, the normal 19-mer biotinylated oligonucleotide (β^N) was immobilized, obtaining a RU increase of 1866 units; in order to produce sensor chips with comparable microfluidic cells, the second immobilization, relative to the β^S mutated oligonucleotide (β^M), was carried out by setting the instrument software to reach more or less the same RU value.

Then we injected five complementary probe pairs of different lengths (9-mer, 10-mer, 11-mer, 12-mer, 13-mer) in order to evaluate their hybridization efficiency with the normal or mutated oligonucleotide immobilized on sensor chip. Each pair consisted of a normal probe (β^N) and a β^S mutated probe (β^M) of the same length. The probe sequences are reported in **Table 1**. This first part of the experimental plan was undertaken since, as described in literature [21], the probe length is a key point to be considered in order to perform a correct and

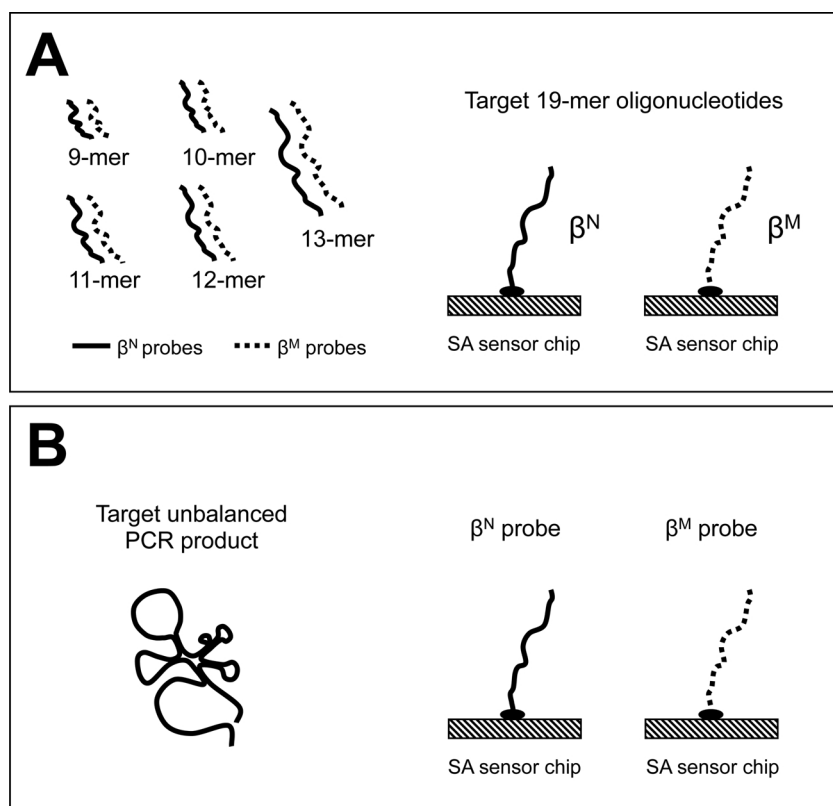


Fig. 1. Experimental strategy for detecting the β^S single point mutation by the affinity biosensor Biacore™ X100. (A) Immobilization of normal (β^N , solid line) and mutated (β^M , dotted line) 19-mer biotinylated oligonucleotides, containing the sequence of interest, on different SA sensor chips, pre-coated with streptavidin, and injection of couples of DNA probes of different length (9-mer, 10-mer, 11-mer, 12-mer, 13-mer), complementary to the targets and containing the normal (β^N , solid lines) or β^S mutated (β^M , dotted lines) sequence. (B) Immobilization of the selected PCR probes on sensor chip and injection of single-stranded PCR products obtained from genomic DNA of different genotypes for the β^S mutation.

Table 1

List of normal (β^N) and mutated (β^M) oligonucleotides employed as probes or targets in Biacore™ analyses, and primer pairs used to obtain the 70 bp, 60 bp and 62 bp PCR products used as targets. For each target and probe oligonucleotide, the name, the nucleotide sequence and the length in nucleotides (nt) are reported. The mutation position is indicated in bold. For each primer, the name, the nucleotide sequence and the length in base pairs (bp) of the respective PCR product are indicated.

Target oligonucleotide name	Nucleotide sequence	Length (nt)
Bio-HbS19N	5'-biot-TGACTCCTGAGGAGAAGTC-3' (β^N)	19
Bio-HbS19M	5'-biot-TGACTCCTGTGGAGAAGTC-3' (β^M)	19
Probe oligonucleotide name	Nucleotide sequence	Length (nt)
HbS-9N	5'-TTCTCCTCA-3' (β^N)	9
HbS-9M	5'-TTCTCCACA-3' (β^M)	9
HbS-10N	5'-CTTCTCCTCA-3' (β^N)	10
HbS-10M	5'-CTTCTCCACA-3' (β^M)	10
HbS-11N	5'-ACTTCTCCTCA-3' (β^N)	11
HbS-11M	5'-ACTTCTCCACA-3' (β^M)	11
HbS-12N	5'-ACTTCTCCTCAG-3' (β^N)	12
HbS-12M	5'-ACTTCTCCACAG-3' (β^M)	12
HbS-13N	5'-GACTTCTCCTCAG-3' (β^N)	13
HbS-13M	5'-GACTTCTCCACAG-3' (β^M)	13
Primer name	Nucleotide sequence	PCR product length (bp)
HbS-F1	5'-AGCAACCTCAAACAGACACCCAT-3'	70
HbS-R1	5'-CCCCACAGGGCAGTAACG-3'	
HbS-F2	5'-GCAACCTCAAACAGACACCA-3'	60
HbS-R2	5'-GCAGTAACGGCAGACTTCTC-3'	
HbS-F3	5'-CATGGTGACCTGACTCCTG-3'	62
HbS-R3	5'-CGTTACACCTGCCCCACA-3'	

efficient detection of single point mutations.

Fig. 2 shows the sensorgrams obtained after injection of 9-mer (A, B), 10-mer (C, D), 11-mer (E, F), 12-mer (G, H) or 13-mer (I, L) normal (β^N , solid lines) or mutated (β^M , dotted lines) pair probes onto flow cells containing target 19-mer oligonucleotides carrying the normal (β^N) (A, C, E, G, I) or β^S mutated (β^M) (B, D, F, H, L). The graphs show the mean values of the final (RU_{fin}) and residual (RU_{res}) RUs, measured respectively at the end of the injection and after the washing step. The values derive from three different injections of the analyte. The RU

values are reported in **Table 1s, Supplementary materials**.

As for the 9-mer probes (Fig. 2A, B), there were no interactions with the target oligonucleotides for both the used probes (RU_{fin} and RU_{res} values equal to zero): this lack of hybridization can be explained by the reduced length of the probes, which could prevent efficient interaction even between complementary sequences.

For the 10-mer (Fig. 2C, D) and 11-mer (Fig. 2E, F) probe pairs, each probe was able to bind its complementary target sequence with low RU_{res} values, demonstrating the generation of an unstable analyte-

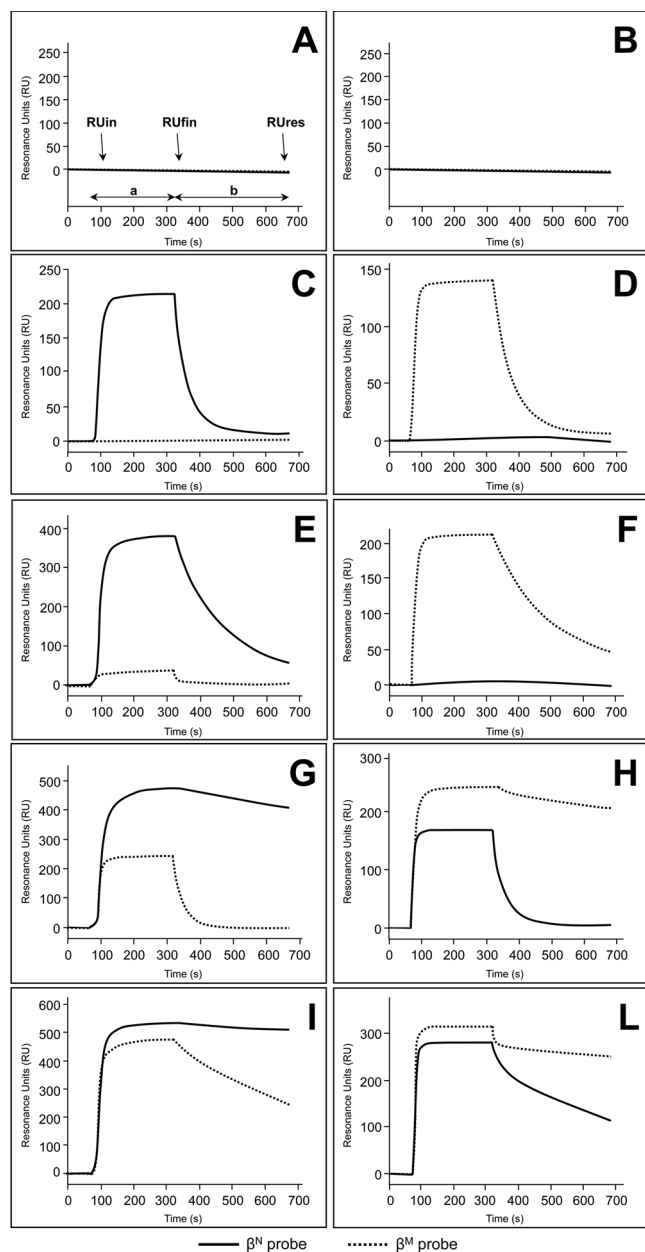


Fig. 2. Sensorgrams obtained after injection of 9-mer (A, B), 10-mer (C, D), 11-mer (E, F), 12-mer (G, H) or 13-mer (I, L) couples of normal (β^N , solid lines) or mutated (β^M , dotted lines) oligonucleotide probes onto flow cells containing target 19-mer oligonucleotides carrying the normal (β^N) (A, C, E, G, I) or mutated (β^M) (B, D, F, H, L) sequence with respect to the β^S mutation. a, analyte injection; b, washing with HBS-EP+. RUin, RUfin, RUres, resonance unit (RU) values measured before the analyte injection, after the injection and after the washing, respectively. The assays were performed by using the Biacore™ X100 instrument, at 25 °C and 5 μ l/min flow rate; the running buffer was HBS-EP+. The resulting plots were obtained after subtracting the sensorgrams produced by both analyte injection onto an empty flow cell and the running buffer alone injection. In order to compare the sensorgrams, the initial RU signal was arbitrarily set to zero.

ligand complex due to the reduced probe length. As expected, neither mutated (Fig. 2C, E) nor normal probes (Fig. 2D, F) bound the mismatch oligonucleotides.

As for the 12-mer probes (Fig. 2G, H), the affinity and stability of hybridization with the complementary sequences were much higher as compared with the 10-mer probes. In fact the β^N probe (Fig. 2G) bound

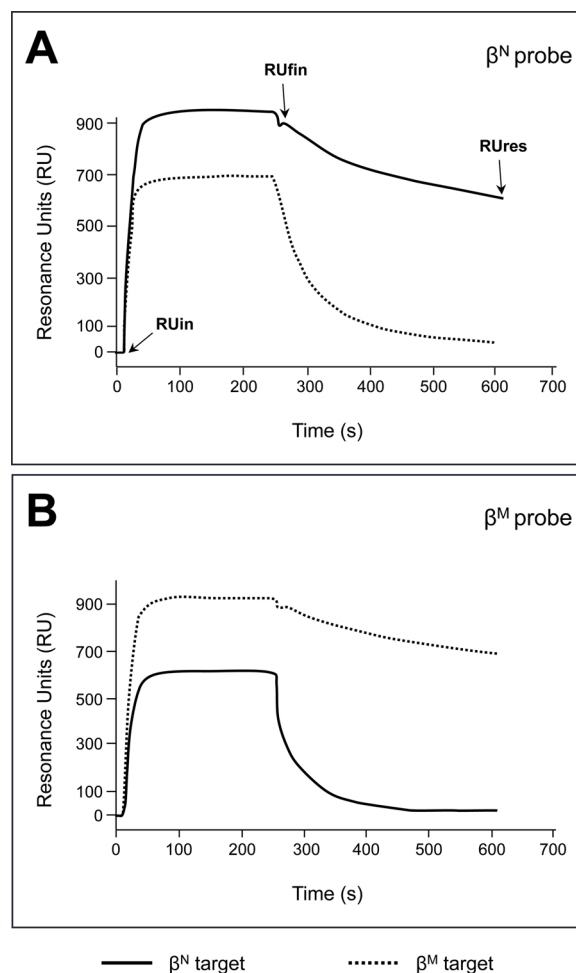


Fig. 3. Sensorgrams obtained after injection, on sensor chips carrying 12-mer normal (β^N) (A) or β^S mutated (β^M) (B) probes, of single-stranded complementary normal (β^N , solid lines) or mutated (β^M , dotted lines) target oligonucleotides. The assays were performed by using the Biacore™ X100 instrument, at 25 °C and 5 μ l/min flow rate; the running buffer was HBS-EP+. The resulting plots were obtained after subtracting the sensorgrams produced by both analyte injection onto an empty flow cell and the running buffer alone injection.

the normal target oligonucleotide efficiently, reaching RUfin and RUres values of 480 and 406 respectively, indicating a high specific and stable interaction. The β^M probe also recognized the same target (RUfin = 234), but after washing it was completely dissociated (RUres = 9). Similarly, the 12-mer β^M probe (Fig. 2H) hybridized with high efficiency with the complementary oligonucleotide (RUfin = 255), and the interaction remained stable over time (RUres = 209); again the β^N probe bound the target containing the mutation (RUfin = 161), but the presence of the mismatch caused the total detachment during the washing phase (RUres = 4). Finally, the 13-mer probes (Fig. 2I, L) showed an even higher affinity for the targets, reaching greater RUfin values (540 for β^N probe and 315 for β^M probe); however, the interaction occurred both in the presence of the complementary and mismatch sequences with a minor dissociation phase during the washing step, reporting a very reduced discrimination capability.

In conclusion, we selected the 12-mer probe pairs for the immobilization on the sensor chip, because of their ability to better discriminate between normal and mutated target sequence, during both association (see the RUfin values) and dissociation (see the RUres values) steps.

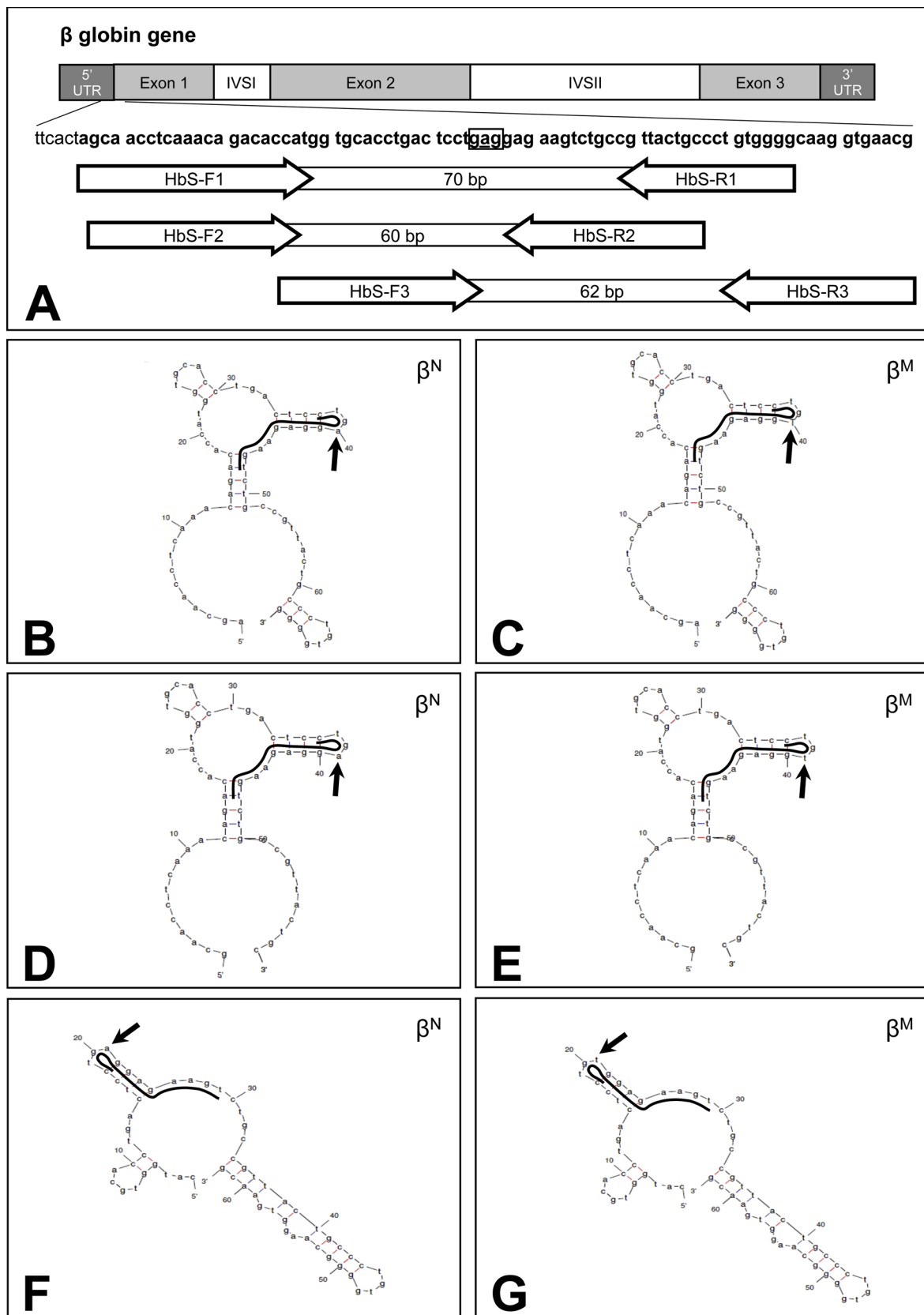


Fig. 4. (A) Map of the β globin gene, where the region of the nucleotide sequence containing the β^S mutation is shown. The exonic portion of the sequence is in bold, while the codon and the nucleotide involved in the mutation are boxed and underlined, respectively. The positions of the 70 bp, 60 bp, 62 bp amplification products used in Biacore™ analyses, together with the respective HbS-F1/HbS-R1, HbS-F2/HbS-R2, HbS-F3/HbS-R3 primer pairs, are reported. (B–G) Secondary structures, calculated by using *The mfold Web Server* (<http://mfold.rut.albany.edu/?q=mfold/>) software [26], of normal (β^N) (B, D, F) and mutated (β^M) (C, E, G) single-stranded PCR products obtained using HbS-F1/HbS-R1 (B, C), HbS-F2/HbS-R2 (D, E) and HbS-F3/HbS-R3 (F, G) primer pairs, respectively. For each structure, the sequence involved in binding with the complementary probe is shown by a black line, while the arrow indicates the position of the β^S mutation.

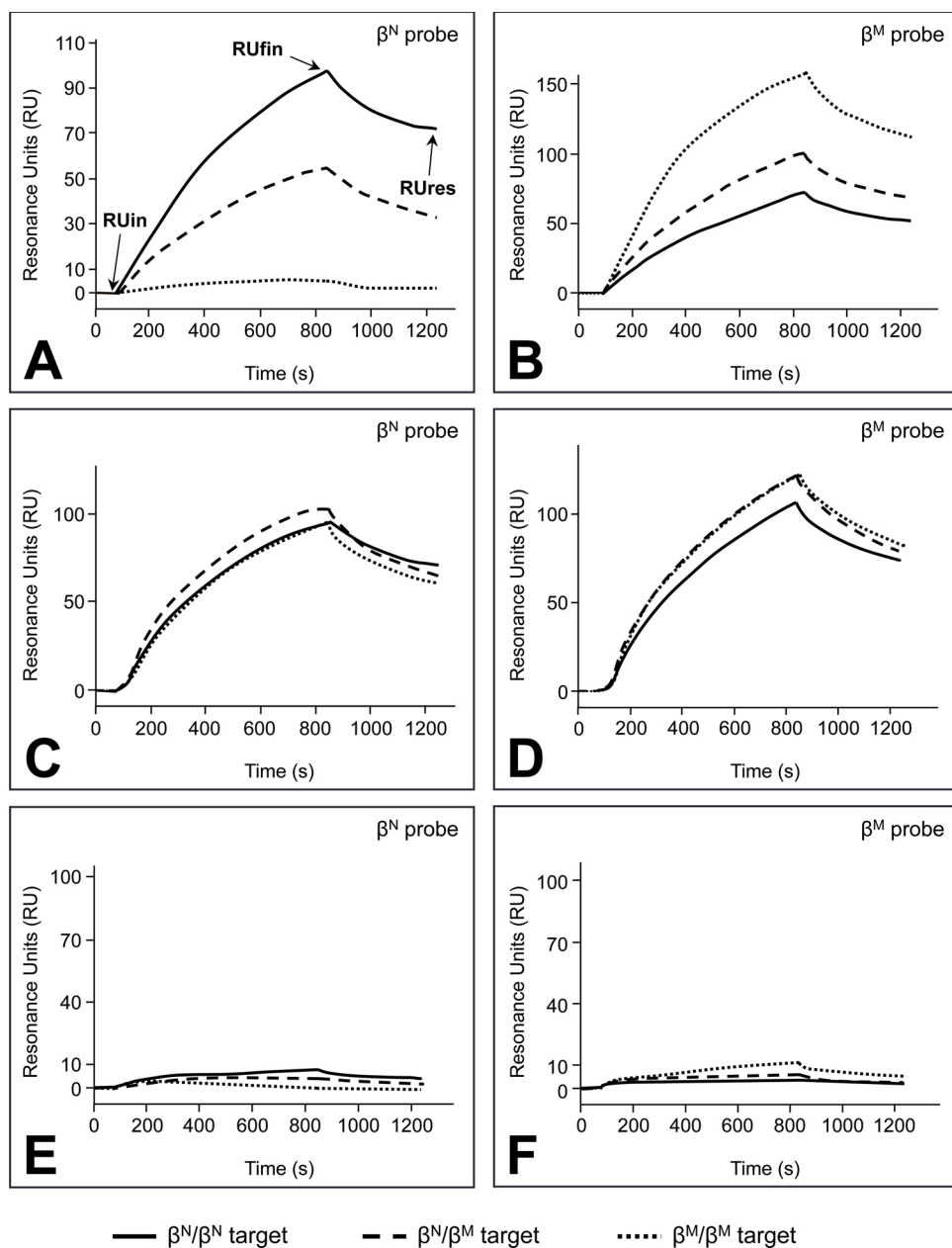


Fig. 5. Sensorgrams obtained after injection, on sensor chips carrying 12-mer normal (β^N) (A, C, E) or β^S mutated (β^M) (B, D, F) probes, of single-stranded PCR products generated by normal (β^N/β^N , solid lines), heterozygous (β^N/β^M , dashed lines) or homozygous (β^M/β^M , dotted lines) genomic DNAs for β^S mutation, produced by using HbS-F1/HbS-R1 (A, B), HbS-F2/HbS-R2 (C, D) or HbS-F3/HbS-R3 (E, F) primer pairs, respectively. The assays were performed by using the Biacore™ X100 instrument, at 25 °C and 5 μ l/min flow rate; the running buffer was HBS-EP+. The resulting plots were obtained after subtracting the sensorgrams produced by both analyte injection onto an empty flow cell and the running buffer alone injection. RUin, RUfin, RUres, resonance unit (RU) values measured before the analyte injection, after the injection and after the washing, respectively. In order to compare the sensorgrams, the initial RU signal was arbitrarily set to zero.

3.2. Validation of 12-mer probes immobilized on sensor chip for β^S detection

The identified 12-mer allele-specific probes were biotinylated and linked to the surface of the sensor chips in order to investigate their ability to discriminate between two DNA sequences differing by only a single nucleotide, testing the hybridization capacity with single-stranded complementary normal (β^N) or mutated (β^M) oligonucleotides (Fig. 3 and Table II). As expected, the β^N probe showed a greater and more stable hybridization with the normal oligonucleotide (Fig. 3A, solid line) than with the mutated one (Fig. 3A, dotted line): in fact the RU values, after the injection of the mutated oligonucleotide, decreased considerably during the washing phase, showing an unstable interaction. Similarly, the β^M probe was able to bind stably and specifically the mutated complementary oligonucleotide (Fig. 3B, dotted line), while the normal one (Fig. 3B, solid line) initially hybridized, but then quickly dissociated from the generated complex after the washing step. Therefore, the 12-mer normal and mutated probes were able to efficiently discriminate between the two complementary target sequences

and were proposed for the detection of the β^S mutation using this SPR-based protocol and clinical samples.

3.3. Set-up of unbalanced PCR conditions as target for β^S detection

After the identification of the β^S probes, the experimental strategy, developed for a possible diagnostic application (Fig. 1B) in clinical relevant samples, was based on single-stranded PCR products generated using genomic DNA obtained from SCD patients and healthy donors.

First of all, we set-up the optimal amplification conditions using genomic DNA, in order to allow an efficient and specific hybridization between the injected single-stranded PCR product and the allele-specific probes immobilized on sensor chip.

A possible drawback of this procedure is that long single-stranded PCR products are expected to produce secondary structures, negatively affecting their hybridization efficiency with the probes. For this reason we designed and comparatively tested 3 primer pairs (HbS-F1/HbS-R1, HbS-F2/HbS-R2, HbS-F3/HbS-R3), able to amplify small different regions (60–70 bp) of the β globin gene, all of them containing the

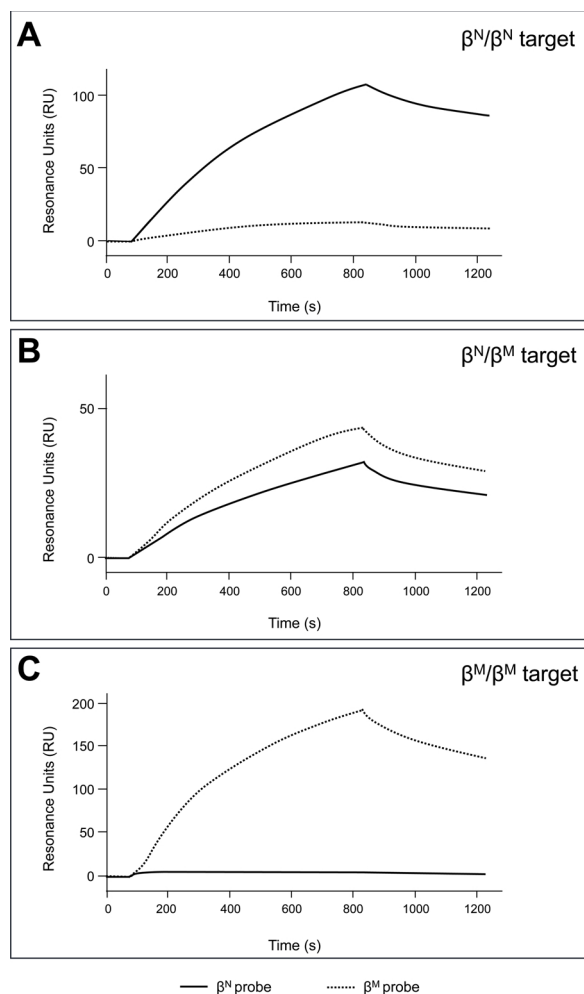


Fig. 6. Representative examples of sensorgrams obtained after injection, on sensor chips carrying normal β^N (solid lines) or Hbs mutated β^M (dotted lines) probes, of unbalanced PCR products from genomic DNA of different genotypes: normal (β^N/β^N) (A), heterozygous (β^N/β^M) (B) or homozygous (β^M/β^M) (C) for the β^S mutation, respectively. The assays were performed by using the Biacore™ X100 instrument, at 25 °C and 5 μ l/min flow rate; the running buffer was HBS-EP +; the resulting plots were obtained after subtracting the sensorgrams produced by both analyte injection onto an empty flow cell and the running buffer alone injection. In order to compare the sensorgrams, the initial RU signal was arbitrarily set to zero.

nucleotides corresponding to the β^S mutation (Fig. 4A and Table 1).

For each PCR product, we determined the possible secondary structure preferentially generated in the experimental conditions employed during the hybridization step (i.e. 25 °C, $[\text{Na}^+]$ 150 mM, $[\text{Mg}^{2+}]$ 1.8 mM) using *The mfold Web Server software* (<http://mfold.rutgers.edu/?q=mfold/>) [26] (Fig. 4B–G).

Both the normal (β^N) (Fig. 4B, D, F) and mutated (β^M) (Fig. 4C, E, G) single stranded PCR products obtained using HbS-F1/HbS-R1 (Fig. 4B, C), HbS-F2/HbS-R2 (Fig. 4D, E), HbS-F3/HbS-R3 (Fig. 4F, G) primers were found able to generate secondary structures involving the nucleotide of the β^S allele, causing possible difficulties in hybridization with the probes in biospecific interaction analyses.

Therefore, in order to select the more suitable primer pairs, it was important for the hybridization to also predict the overall conformation of the PCR products, since their three-dimensional structure and steric hindrance could interfere with probe accessibility. To evaluate this aspect, for each PCR structure we determined the ratio between the number of nucleotides not involved in hydrogen bonds and the total number of nucleotides. For lower ratio values, a greater level of

structured PCR molecule was expected. In our cases, the resulting values for the single-stranded PCR products using HbS-F1/HbS-R1, HbS-F2/HbS-R2 and HbS-F3/HbS-R3 primer pairs were 0.60, 0.63, 0.45, respectively.

According with these data, it is possible to hypothesize that the HbS-F3/HbS-R3 primers were responsible for the generation of a highly structured PCR product (exhibiting the lowest value), negatively affecting the hybridization efficiency with the probes. On the contrary, for the other two primer pairs, showing greater values, the respective PCR products could show better accessibility and interaction efficiency with the probes.

Therefore the next step was testing each unbalanced PCR product for hybridization with the 12-mer normal or β^S mutated probes using the SPR-based biosensor (Fig. 5).

The single-stranded PCR products employed for interaction with complementary antisense probes immobilized on the sensor chips were obtained by two main steps, as previously published [25] and reported in **Supplementary materials**.

With the aim of verifying the primer pair that could give the best results in terms of efficiency, stability and specificity of the molecular interaction with the immobilized probes, DNA samples of different genotype for the β^S mutation were PCR amplified: not mutated homozygous (N/N), heterozygous (N/M) and β^S mutated homozygous (M/M). Then the unbalanced PCR products were injected to the surface of the sensor chips with the immobilized normal 12-mer (Fig. 5A, C, E) or β^S mutated (Fig. 5B, D, F) probes. The injection was performed in HBS-EP + buffer for 12 min, which is a much longer time compared to the one used when oligonucleotide targets were employed (4 min, Fig. 3). This protocol was followed in order to allow possible interactions even in the presence of unfavorable experimental conditions, due to the secondary structures and the steric hindrance of the asymmetric PCR products employed.

Fig. 5 reports a representative example of the sensorgrams generated by injecting target PCR products obtained using (a) each pair of primers (HbS-F1/HbS-R1, Fig. 5A, B; HbS-F2/HbS-R2, Fig. 5C, D; HbS-F3/HbS-R3, Fig. 5E, F) and (b) three different genotypes for β^S mutation (not mutated, β^N/β^N , solid lines; heterozygous, β^N/β^M , dashed lines; homozygous, β^M/β^M , dotted lines). The probes were the 12-mer normal (β^N , Fig. 5A, C, E), or the β^S mutated (β^M , Fig. 5B, D, F) probes, immobilized on two different flow cells of the sensor chip. The relative RU values are reported in **Table III**, **Supplementary materials**.

As for the HbS-F1/HbS-R1 primers, the normal probe (Fig. 5A) generated hybridization complexes with the PCR target obtained from a healthy subject showing high efficiency and fair stability, while it did not bind the PCR product from a β^S homozygous subject and reported, as expected, an intermediate trend of the generated RU values of the complex after interaction with a target PCR product amplified from a β^S heterozygous subject, due to the presence of a single β^S allele. An opposite performance was observed for the mutated probe (Fig. 5B) with the complementary homozygous and heterozygous β^S mutated PCR products. When the PCR target obtained from a healthy subject was employed some cross-hybridization was observed; this exhibited, however, much lower hybridization efficiency and stability compared to the other samples. In conclusion, the PCR products for the three different genotypes, obtained using this primer pair, were efficiently and specifically discriminated by the 12-mer probes immobilized on the sensor chip, suggesting it could be a good candidate for the further amplification reactions.

As far as the HbS-F2/HbS-R2 primer pair, all PCR products interacted with both immobilized probes (normal and mutated) with the same high efficiency and fair stability, resulting in no discrimination for the three different genotypes (Fig. 5C, D).

As previously hypothesized, for the PCR products amplified by HbS-F3/HbS-R3 primers, no interactions probes/PCR targets were described (Fig. 5E, F), demonstrating that their secondary structure could inhibit this binding.

Target genotype	No. analyzed samples	SCD-index (mean \pm SD)
β^N/β^N	20	6.109 \pm 2.398
β^N/β^M	14	0.593 \pm 0.096
β^M/β^M	19	0.092 \pm 0.052

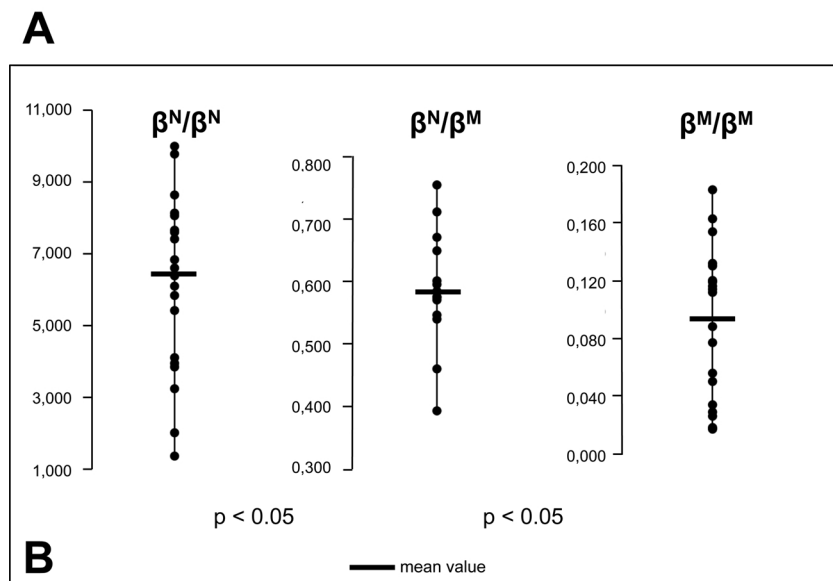


Fig. 7. (A) SCD-index values calculated after SPR biosensor analysis of normal (β^N/β^N), heterozygous (β^N/β^M) or homozygous (β^M/β^M) genomic DNAs for the β^S mutation, respectively, expressed as mean values \pm standard deviations. The number of analyzed samples for each genotype is also indicated. (B) Distribution of SCD-index values obtained after SPR-based BIA of β^S normal (β^N/β^N), heterozygous (β^N/β^M), or homozygous (β^M/β^M) genomic DNAs extracted from blood samples. The mean values of the distributions and the statistical significance of the analysis are also indicated. Statistical differences between groups were compared by the Student's t-test, selecting a 95% confidence level.

3.4. Detection of β^S mutation in genomic DNA from blood clinical samples

After the optimization of amplification conditions using the HbS-F1/HbS-R1 primer pair, we validated our SPR-based protocol for SCD detection using the genomic DNAs extracted from blood samples of subjects carrying different β^S allele genotypes. We analyzed in total 53 DNA samples, previously characterized for their genotypes by DNA sequencing: 20 without β^S allele (not mutated, N/N), 14 with one β^S allele (heterozygous, N/M), 19 with both β^S alleles (homozygous, M/M).

Three representative sensorgrams for β^S mutation discrimination are shown in Fig. 6. They were obtained by a not mutated subject (Fig. 6A), a heterozygous carrier (Fig. 6B) and a homozygous SCD patient (Fig. 6C). The asymmetric HbS-F1/HbS-R1 PCR products from the healthy subject (β^N/β^N target), specifically hybridized with the normal probe (β^N , solid line) but not with the mutated probe (β^M , dotted line) (Fig. 6A); on the contrary, asymmetric PCR products from genomic DNA from homozygous patients (β^M/β^M target) efficiently interacted with the mutated probe (β^M , dotted line), while a low RU signal was observed with the normal probe (β^N , solid line) (Fig. 6C); finally, PCR products from heterozygous genotypes hybridized with both probes (Fig. 6B).

In order to quantify the SPR biosensor results obtained through the analysis of 53 samples, we calculated the SCD-index [21,25], according to the following formula: $[\text{RU}_{\text{fin}} - \text{RU}_{\text{in}}]_{\beta^N} / [\text{RU}_{\text{fin}} - \text{RU}_{\text{in}}]_{\beta^M}$, where RU_{in} and RU_{fin} are the resonance unit (RU) values measured at the beginning of analysis and at the end of analyte injection, respectively, relative to the hybridization with normal (β^N) and mutated (β^M) probes immobilized on sensor chips. As previously reported for the Thal-Index applied to SPR-based protocol to identify thalassemia mutations [21,25], the SCD-index values, calculated for each genotype, were found greater than 1 in the case of a not mutated target, or between 0 and 1 in the case of at least one β^S allele (Fig. 7).

In detail, Fig. 7A reports the number of analyzed samples including healthy subjects (β^N/β^N), heterozygous carriers (β^N/β^M) and homozygous SCD patients (β^M/β^M) and the corresponding mean values of

SCD-index, confirming the conclusions relative to representative sensorgrams reported in Fig. 6. In fact, high significant values of SCD-index were obtained for all not mutated DNAs; while for the homozygous mutated DNAs, the SCD-indexes were always lower than 1; finally, in the case of heterozygous DNAs intermediate values were obtained. In theory for the β^S heterozygous sample (β^N/β^M), the number of normal DNA molecules would be equivalent to the mutated ones, producing a similar hybridization with both probes (normal and mutated) and a SCD-index close to 1.

From the experimental point of view, it is highly unlikely that the hybridization efficiency of the β^N and β^M probes is exactly the same; therefore we expect that the experimental SCD-index values might slightly deviate from the predicted 1; in the case we expect however a limited level of variability. In agreement with these considerations, in our analysis we obtained a SCD-index value of 0.593 ± 0.096 . However, the most important aspect of the procedure, exhibiting a high diagnostic impact, is that the ranges of the SCD-index values found from the samples belonging to different genotypes are clearly distinct from each other, independently from the absolute values obtained.

In Fig. 7B, we can observe the SCD-index distribution obtained for each genotype: the three populations of values, in fact, are clearly distinct and the differences are statistically significant (p value < 0.05), allowing a correct genotypic discrimination.

4. Conclusions

The SPR biosensor technology has been already applied to mutation analysis of the human *HBB* gene, by immobilization on sensor chip of large PCR products, obtained from donor genomic DNAs, followed by injection of specific probes for the identification of single point mutations. This interaction format efficiently discriminates single point mutations, but is not compatible with the reuse of the same sensor chip for other diagnostic determinations. In fact this protocol requires PCR immobilization for each subject to be analyzed and for each analysis. In this article, we have described a novel interaction format, recently applied also to β thalassemia mutations by our group, and based on two

oligonucleotide probes (normal and mutated) specific for β^S mutation immobilized on sensor chip. These probes were able to discriminate normal alleles from the mutated ones, after hybridization with selected target PCR products obtained using the genomic DNAs extracted from blood of 53 donors with different genotypes for β^S allele. Contrary to be already proposed protocols, this strategy is low-cost, requiring only two flow cells for the analysis of several samples from several subjects, and it permits the real-time detection of single point mutations at an efficiency suitable for prenatal diagnosis. Of course, both probes and PCR primers should be carefully characterized to obtain the best performances. In conclusion, we have developed a protocol based on SPR-biosensor allowing to correctly detect the β^S mutation responsible for SCD, both in homozygous and heterozygous conditions: this is a good prerequisite as a potential diagnostic tool.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.snb.2019.05.081>.

References

- [1] J.M. Old, Screening and genetic diagnosis of haemoglobinopathies, *Scand. J. Clin. Lab. Invest.* 67 (2007) 71–86.
- [2] B.G. Forget, H.F. Bunn, Classification of the disorders of hemoglobin, *Cold Spring Harb. Perspect. Med.* 3 (2) (2013) a011684.
- [3] A.D. Khattab, B. Rawlings, I.S. Ali, Care of patients with haemoglobin abnormalities: history and biology, *Br. J. Nurs.* 15 (2006) 994–998.
- [4] C.S. Thom, C.F. Dickson, D.A. Gell, M.J. Weiss, Hemoglobin variants: biochemical properties and clinical correlates, *Cold Spring Harb. Perspect. Med.* 3 (3) (2013) a011858.
- [5] A.J. Marengo-Rowe, Structure-function relations of human hemoglobins, *Proc. (Bayl Univ. Med. Cent)* 19 (2006) 239–245.
- [6] M.A. Bender, Sickle cell disease, gene reviews, in: M.P. Adam, H.H. Ardinger, R.A. Pagon, S.E. Wallace, L.J.H. Bean, K. Stephens, A. Amemiya (Eds.), *GeneReviews*[®][Internet], University of Washington, Seattle (WA), 2017, pp. 1993–2018.
- [7] M.H. Steinberg, Sickle cell anemia, the first molecular disease: overview of molecular etiology, pathophysiology, and therapeutic approaches, *Sci. world J.* 8 (2008) 1295–1324.
- [8] E. Kohne, Hemoglobinopathies: clinical manifestations, diagnosis, and treatment, *Arztebl. Int.* 108 (2011) 532–540.
- [9] D.C. Rees, T.N. Williams, M.T. Gladwin, Sickle-cell disease, *Lancet* 376 (2013) 2018–2026.
- [10] M. Husain, A.D. Hartman, P. Desai, Pharmacogenomics of sickle cell disease: steps toward personalized medicine, *Pharmgenomics Pers. Med.* 10 (2017) 261–265.
- [11] C. Oner, A.J. Dimovski, N.F. Olivieri, G. Schiliro, J.F. Codrington, S. Fattoum, A.D. Adekile, R. Oner, G.T. Yüregir, C. Altay, et al., Beta S haplotypes in various world populations, *Hum. Genet.* 89 (1992) 99–104.
- [12] F.B. Piel, T.V. Adamkiewicz, D. Amendah, T.N. Williams, S. Gupta, S.D. Grosse, Observed and expected frequencies of structural hemoglobin variants in newborn screening surveys in Africa and the Middle East: deviations from Hardy-Weinberg equilibrium, *Genet. Med.* 18 (2016) 265–274.
- [13] V.J. Bitoungui, G.D. Pule, N. Hanchard, J. Ngogang, A. Wonkam, Beta-globin gene haplotypes among cameroonians and review of the global distribution: is there a case for a single sickle mutation origin in Africa? *OMICS* 19 (2015) 171–179.
- [14] K. Ryan, B.J. Bain, D. Worthington, J. James, D. Plews, A. Mason, D. Roper, D.C. Rees, B. de la Salle, A. Streetly, British Committee for Standards in Haematology. Significant haemoglobinopathies: guidelines for screening and diagnosis, *Br. J. Haematol.* 149 (2010) 35–49.
- [15] S.A. Mousa, M.H. Qari, Diagnosis and management of sickle cell disorders, *Methods Mol. Biol.* 633 (2010) 291–307.
- [16] N. Uaprasert, R. Settapiboon, S. Amornsiriwat, P. Sarnthammakul, T. Thanapat, P. Rojnuckarin, P. Sutcharithchan, Diagnostic utility of isoelectric focusing and high performance liquid chromatography in neonatal cord blood screening for thalassemia and non-sickling hemoglobinopathies, *Clin. Chim. Acta* 427 (2014) 23–26.
- [17] P.J. Singh, A.C. Shrivastava, A.V. Shrikhande, Prenatal diagnosis of sickle cell disease by the technique of PCR, *Indian J. Hematol. Blood Transfus.* 31 (2015) 233–241.
- [18] L. Yue, M. Lin, J.T. Chen, X.F. Zhan, D.S. Zhong, S.M. Monte-Nguba, P.F. Liu, X.F. Pan, J.H. Huang, X. Wang, J.C. Ehapo, U.M. Eyi, H.T. Yang, L.Y. Yang, Rapid screening for sickle cell disease by polymerase chain reaction-high resolution melting analysis, *Mol. Med. Rep.* 9 (2014) 2479–2484.
- [19] R.K. Saiki, S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, N. Arnheim, Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, *Science* 230 (1985) 1350–1354.
- [20] C.L. Harteveld, State of the art and new developments in molecular diagnostics for hemoglobinopathies in multiethnic societies, *Int. J. Lab. Hematol.* 36 (2014) 1–12.
- [21] G. Feriotto, G. Breveglieri, A. Finotti, S. Gardenghi, R. Gambari, Real-time multiplex analysis of four β thalassemia mutations employing surface plasmon resonance and biosensor technology, *Lab. Invest.* 84 (2004) 796–803.
- [22] G. Feriotto, R. Gambari, Surface plasmon resonance based biosensor technology for real-time detection of PCR products, in: T. Weissensteiner, H.G. Griffin, A. Griffin (Eds.), *PCR Technology: Current Innovations*, CRC Press, Boca Raton, FL, 2003, pp. 141–154.
- [23] E.O. Atala, E. Ustel, S. Yildiz, A. Atalay, Surface plasmon resonance-based molecular detection of Hb S [β (A3)Glu-&Val, GAG->G] at the gene level, *Hemoglobin* 30 (2006) 385–391.
- [24] G. Breveglieri, T.E. Gallo, A. Travan, P. Pellegatti, G. Guerra, R. Gambari, M. Borgatti, Surface plasmon resonance analysis to detect the $\beta(+)$ IVSI-110 thalassemia mutation in circulating cell-free fetal DNA, *Clin. Chim. Acta* 462 (2016) 133–134.
- [25] G. Breveglieri, E. D'Aversa, T.E. Gallo, P. Pellegatti, G. Guerra, L.C. Cosenza, A. Finotti, R. Gambari, M. Borgatti, A novel and efficient protocol for Surface Plasmon Resonance based detection of four B-thalassemia point mutations in blood samples and salivary swabs, *Sensors and Actuators, B: Chemical* 260 (2018) 710–718.
- [26] M. Zuker, Mfold web server for nucleic acid folding and hybridization prediction, *Nucleic Acids Res.* 31 (2003) 3406–3415.