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DNA microarray-based solid-phase PCR on copoly (DMA–NAS–MAPS) silicon coated slides: An example of relevant clinical application

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ABSTRACT

In a previous study we developed a highly sensitive DNA microarray for the detection of common KRAS oncogenic mutations, which has been proven to be highly specific in assigning the correct genotype without any enrichment strategy even in the presence of minority mutated alleles. However, in this approach, the need of a spotter for the deposition of the purified PCR products on the substrates and the purification step of the conventional PCR are serious drawbacks. To overcome these limitations we have introduced the solid-phase polymerase chain reaction (SP-PCR) to form the array of PCR products starting from the oligonucleotide primers. This work was possible thanks to the great thermal stability of the copoly (DMA–NAS–MAPS) coating which withstands PCR thermal cycling temperatures. As an example of the application of this platform we performed the analysis of six common mutations in the codon 12 of KRAS gene (G12A, G12C, G12D, G12R, G12S, and G12V). In conclusion solid-phase PCR, combined with dual-color hybridization, allows mutation analysis in a shorter time span and is more suitable for automation.

1. Introduction

DNA microarray technology represents an ideal platform for rapid, inexpensive and high-throughput clinical identification of relevant Single Nucleotide Polymorphisms (SNPs) or mutations. In a previous paper, we introduced a sensitive silicon substrate for microarrays, functionalized with a polymer, named copoly (DMA–NAS–MAPS). The method was based on the immobilization of amino modified PCR products onto crystalline silicon slides, coated by a thermally grown silicon dioxide (SiO_2) layer that promotes the intensification of the fluorescence signals on the surface as a result of optical constructive interference between the incident and reflected lights of the fluorescent radiation (Cretich et al., 2009; Volle et al., 2003). This kind of substrate is functionalized by adsorption of a copolymer of dimethylacrylamide (DMA), N-acryloyloxysuccinimide (NAS) and meta-acryloyl propyl trimethoxy silane (MAPS), copoly (DMA–NAS–MAPS), which allows for the covalent binding between the amino-modified amplicons and the surface (Pirri et al., 2004). This coating procedure is simple and reproducible, when compared to organo-silanization, a process that requires highly controlled conditions and suffers from poor reproducibility. This functional polymer has been widely applied in the biosensor field for the bio-functionalization of polystyrene nanobeads (Marquette et al., 2007), silicon microcantilevers (Oliviero et al., 2008), polydimethylsiloxane (Cretich et al., 2008), and nitrocellulose substrates (Cretich et al., 2010).

One of the drawbacks of our previous mutation detection platform was that the PCR products required a purification step before spotting to enhance the concentration of the PCR products and to replace the amplification buffer with a suitable spotting buffer. Moreover, in the amplicon down approach, the purified PCR amplicons had to be spotted by an expensive commercial arrayer not available in most clinical chemistry laboratories. To overcome these disadvantages we tested the feasibility of introducing the solid-phase PCR (SP-PCR) in order to directly bind the PCR products onto the substrates. The PCR reaction takes place on the surface from immobilized primers (Adessi et al., 2000; Cheng et al., 2010; Kranaster et al., 2008). In perspective the chips with the immobilized primers could be sold to the user already printed as a part of a diagnostic kit, so as to make the printer quite unnecessary. In literature there are very few papers dealing with the analysis of relevant human mutations with platforms that perform

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SP-PCR in combination with microarrays. In this work we have applied this methodology to improve the KRAS mutation genotyping.

A key requirement for generating PCR products onto substrates is the grafting of the primers in a SP-PCR compatible manner. In this case the chemical bond between oligonucleotide primer and the array-substrate should withstand high temperatures and thermo cycling between 50 °C and 95 °C. Furthermore, the binding chemistry must ensure that the free 3′-OH end is accessible and extendable by a DNA polymerase; thus the substrate and immobilization chemistry to be used in SP-PCR have special requirements. To this aim, different immobilization protocols on glass (Ham et al., 2011; Khodakov et al., 2008; Sun et al., 2011; Von Nickisch-Rosenegk et al., 2008) and polymers (Carmon et al., 2002; Kinoshita et al., 2007) have been published. Most of the methods report on covalent immobilization of oligonucleotides through EDC chemistry, where 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) mediates the linkage of 5′-NH_2 modified DNA to hydroxylated substrates (Adessi et al., 2000). Another method is based on the UV-crosslink poly-dT modified DNA directly to glass (Sun et al., 2011) or to a 3-D hydrogel matrix on plastic substrates (Brandstetter et al., 2010). Besides that, acrydite-modified oligonucleotides in an acrylamide gel can be spotted on a substrate for oriented co-polymerization (Cheng et al., 2010). Indirect immobilization methods utilize homobifunctional linking molecules like glutaraldehyde (Khodakov et al., 2008) and 1,4-phenylene diisothiocyanate (PDITC) (Kranaster et al., 2008) for attaching oligonucleotides to activated or modified (mostly aminosilanized) surfaces. Another requirement for the PCR on a surface is a high surface density of spotted primers. To this aim a three-dimensional (3-D) hydrophilic-gel (e.g., polyacrylamide) has been employed as a substrate to improve the surface area in SP-PCR (Strizhkov et al., 2000). The DNA attachment by this approach requires a number of tedious steps. Therefore the whole process besides being complicated and time consuming, it has the drawback of producing arrays with a high background due to non-specific binding of unbound labeled probes which are difficult to remove from the 3-D matrix.

A need exists for a simple, robust, and versatile immobilization strategy compatible with SP-PCR. In this work for the first time we demonstrate the thermal stability of copoly (DMA–NAS–MAPS). Thanks to the stability of this polymer in thermal cycling conditions which entails the use of a temperature 95 °C, we were able to successfully use SP-PCR to overcome the spotting of different PCR products. Only the common reverse primer is spotted on the surface. During thermal cycling the amplification reaction occurs and the PCR products are subsequently grafted to the substrate. All the reaction components (Taq polymerase, liquid primers, etc.) are removed by a simple washing step at the end of the amplification cycles. Due to the fact that all the mutations under study are in the same PCR fragment, the spotting of only one primer enables the identification of the genotype after hybridization with dual color fluorescent probes of all the mutations of interest. The first part of this article focused on experiments that demonstrate the thermal stability of the polymer coating whereas in the second part, after the optimization of the protocol for the solid-phase amplification reaction, we genotyped the six most common KRAS mutations in codon 12 and we evaluated the sensitivity of the system by means of a dilution curve.

2. Experimental section

2.1. Materials and reagents

Tris(hydroxymethyl)aminomethane (Tris), ethanolamine, ammonium sulfate, sodium dodecyl sulfate (SDS), Triton X100, bovine serum albumin (BSA), Phosphate-buffered saline (PBS) tablets, DMA, MAPS, and 20X standard saline sodium citrate (SSC) solution (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0), were purchased from Sigma (St. Louis, MO, USA). NAS was obtained from Polysciences (Warrington, PA, USA). Oligonucleotides and primers were synthesized by MWG-Biotech AG (Ebersberg, Germany). DNA polymerase was purchased from Roche Applied Science (Mannheim, Germany).

GeneFrames were purchased from Sigma (St. Louis, MO, USA).

Untreated silicon 1000 Å Thermal Oxide slides (75×25 and 14×14 mm^2) were supplied by SVM, Silicon Valley Microelectronics Inc. (Santa Clara, CA USA).

2.2. Samples

Mutant-bearing plasmids were generated through the cloning of specific mutagenized PCR products harboring the six mutations tested in the assay and the corresponding wild-type fragment (Stenirri et al., 2004). On the contrary, genomic DNA extracted from whole blood of healthy individuals was used as wild-type control samples.

2.3. Procedure to evaluate the thermal stability of the polymer coating

See the online Electronic supplementary information for a detailed description of the preparation of the silicon chip coating.

Synthetic 23-mer 5′-amine-modified oligonucleotides, OLIGO1 (5′-GCC CAC CTA TAA GGT AAA AGT GA-3′), and 3′-amino-modified oligonucleotides labeled with 5′-Cy3, OLIGO2 (5′-TCA CTT TTA CCT TAT AGG TGG GC-3′), 100 μM stock solutions, were dissolved in the printing buffer (sodium phosphate 150 mM, pH 8.5, 0,01% Triton X100) to a concentration of 10 μM. The amino-modifications are necessary to bind the oligonucleotides covalently to the substrate through a reaction between the amino groups and the active esters of the polymer coating. These solutions of oligonucleotides were printed on four copoly (DMA–NAS–MAPS) coated silicon chips (14×14 mm^2) to form a pattern of 2×2 subarrays (a row with 2 subarrays of OLIGO1 and the second row with 2 subarrays of OLIGO2) using a piezoelectric spotter, SciFLEXARRAYER S5 (Scienion Germany). Spotting was carried out at +20 °C and 50% humidity. After the spotting the chips were placed in an uncovered storage box, laid in a sealed chamber, saturated with sodium chloride (40 g/100 mL H_2O), and incubated overnight. After incubation, all residual reactive groups of the coated silicon surfaces were blocked by dipping the slides in 50 mM ethanolamine, 0.1 M Tris pH 9.0 at 50 °C for 15 min. Then, the chips were washed with water and dipped for 15 min in 4X SSC, 0.1% SDS buffer, pre-warmed at 50 °C and rinsed with water and dried. After these steps the silicon chips were scanned with ProScanArray (Perkin Elmer, MA, USA) to determine the start fluorescence of the Cy3 labeled oligonucleotide (OLIGO2). Then, on the spotted chips a reaction chamber was created by GeneFrames. To evaluate the thermal stability of the polymer coating, 35 μL of the PCR reaction mixture was spread into each GeneFrame. After sealing the silicon chips with the provided plastic lid three chips (the fourth one is used as room temperature, RT, control) were placed in the slide thermocycler (Mastercycler Eppendorf) and incubated at 90 °C for 1, 2 and 3 h respectively. After the thermal incubation, GeneFrames were detached and chips were washed for 20 min at room temperature in 0.1X SSC, 0.1% SDS, and for 10 min in 0.1X SSC, and finally nitrogen jet blown dry. Then the three chips and the RT control chip were scanned again to check the difference of the fluorescence signal before and after the thermal incubation. Subsequently a 23-mer oligonucleotide, complementary to OLIGO1, labeled at the 5′ terminus with Cy3, OLIGO1C (5′-TCACTTTTACCT-TATAGGTGGGC-3′), was dissolved in the hybridization buffer (2X