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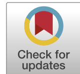

Surface coupling of oligo-functionalized dendrimers to detect DNA mutations after blocked isothermal amplification

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A R T I C L E I N F O

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A B S T R A C T

This study presents a useful strategy for the bioconjugation of probes for developing high-performance versatile chips applied to fast-response, low-cost DNA biosensing. We herein demonstrate that the high-density immobilization of dendrimer-oligonucleotide hybrids promotes the reliable sensitive sensing of single-nucleotide variants despite their low concentration.

Carboxyl-terminated poly(amidoamine) dendrimers directly coupled to amine-derivatized oligonucleotides were anchored to the activated surfaces of thermoplastics (polycarbonate and cycloolefin polymer). Surface characterization techniques and hybridization assays reported that the multiple functional sites of the oligo-functionalized dendrimer facilitated the efficient immobilization of probes and the sensitive capture of DNA targets (5 pM detection limit). Array performance was better than that of the surfaces functionalized with linear crosslinkers, such as vinyltriethoxysilane or 3-(triethoxysilyl)propyl isocyanate, as well films with unconjugated dendrimer molecules.

Based on oligo-dendrimers hybrids, disposable DNA-based biosensing platforms were developed for point-of-care diagnostics. A selective high-sensitive hybridization assay was performed that included amplification products of blocked PCR and recombinase polymerase amplification (RPA) as an isothermal reaction. The chip results indicated that the single-nucleotide mutant variant of the *BRAF* oncogene was correctly discriminated in colorectal tissues from cancer patients.

1. Introduction

Scientific advances have allowed biosensors to play an important role for *in vitro* diagnostic purposes based on nucleic acids. The integration of novel sensing components has promoted the development of reliable devices to fulfill the smart, simple and inexpensive detection goal of clinical targets [1]. Of these, hybridization-based sensors occupy a relevant position because they enable the high-throughput identification of specific sequences associated with disease diagnostics and prognostics [2]. They generally involve the hybridization of the target sequence to an immobilized probe on the sensor substrate.

The most widely used substrates are mainly solid inorganic materials, such as glass slides, metal electrodes, silica chips/fibers and plastic substrates [3]. Several strategies to modify the physico-chemical properties of surfaces for immobilizing specific probes have been reported [4]. Nevertheless, these reactions are single-site systems or linear approaches for oligonucleotide coupling (i.e. 2D immobilization).

Nanotechnology offers novel issues for the biosensing of nucleic acids, such as smart materials [5]. For instance, three-dimensional supramolecular architectures based on highly branched macromolecules have been successfully proposed [6]. In particular, poly(amidoamine) dendrimers (PAMAM) show a well-defined 3D-globular backbone, as well as higher versatile moieties to be further modified [7]. Different end-group functionalized dendrimers are available like thiol, phosphate, aldehyde, amino and carboxylic acid, among others. They are an excellent entity to link oligonucleotide probes with a solid surface in

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electrochemical [8] and optical sensors [9]. As a result, the larger active area of dendrimers provides more sensitive devices than those based on linear molecules [10].

The general approach is a dendrimer-mediated immobilization method, i.e. the oligonucleotide is coupled to the surface coated with a dendrimer film. Thus several surface modification techniques for inorganic materials have been reported, including both covalent and non covalent binding [7,10–13]. Some examples are the attachment of DNA probes to dendrimer-modified thermoplastics, such as SU-8 and PDMS for *E. coli* adsorption in a microfluidic chip [14–16] and PMMA for immunoassays on conventional plates [17]. However, the aggregation of dendrimer molecules has been described during the functionalization of surfaces [18]. This cluster formation reduces the later immobilization of DNA probes and hybridization assays, but worsens sensing performance results. Our proposal is to directly anchor oligo-functionalized dendrimers to planar chips to minimize chain interactions and to improve chip fabrication and assay sensitivity. We hypothesized that dendrimers conjugated with oligonucleotides in solution would provide flexible orientation and higher reaction yields compared to other surface modification chemistries, including dendrimer-mediated approaches.

In this work, we study the potential biosensing properties of DNA-dendrimer-functionalized surfaces based on plastic polymer substrates, such as polycarbonate (PC) and cycloolefin polymer (COP). Both substrates have the potential of being integrated into disposable chips, miniaturized biosensors or wearable devices for personalized health monitoring purposes [1,3,19]. As proof-of-concept for clinical diagnostics, this research was addressed to discriminate the amplification products obtained by polymerase chain reaction (PCR) and a novel variant of recombinase polymerase amplification (RPA) called blocked RPA, which was recently developed by our research group [20]. The former was the commonest DNA amplification reaction and the latter was a relevant isothermal alternative with a high potential for accessible sensitive nucleic acid detection outside laboratories, and even for self-testing [21,22]. The approach is based on the selective blocking of reactions to detect single nucleotide variations under isothermal conditions (37 °C for 40 min). The reaction mixture contains a blocker oligonucleotide that matches the wild-type sequence in the target locus by inhibiting its amplification. Here the studied application of the oligo-functionalized dendrimer-based chips was the sensitive identification of single-point mutations for genotyping in tumor tissues required for the correct selection of therapies, such as lung cancer or colorectal cancer [23].

2. Materials and methods

2.1. Materials

Unmodified slides of COP (cycloolefin polymer, Zeonor® 1060R) and PC (polycarbonate, Makrolon®) were used as substrates (dimensions 75 mm × 25 mm). Carboxyl terminated PAMAM dendrimers (generation 3.5), ethylendiamine (EDA), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), 2-(N-morpholino)ethanesulfonic acid (MES) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The reagents used for genomic DNA extraction formed part of the MagMax Cell-free DNA Isolation Kit (Applied Biosystems, Austin, TX). The DNA amplification kits for PCR and RPA were the DNA Amplitool kit (Biotools, Madrid, Spain) and the TwistAmp Basic RPA kit (TwistDx, Cambridge, UK), respectively. The array printing solution was EDC at 50 mM and NHS 50 mM in MES 0.1 M. The hybridization buffer was saline-sodium citrate (SSC) 1×: sodium chloride at 150 mM, sodium citrate at 15 mM, formamide 25% (pH 7.0). The hybridization washing solution contained NaCl 15 mM and trisodium citrate 1.5 mM. The developing buffer (pH 7.4) was a Tween 20 (0.05%) solution in phosphate-buffered saline (PBS) containing 137 mM NaCl, 12 mM phosphate and 2.7 mM KCl. Abcam (Cambridge, UK) supplied the sheep monoclonal anti-digoxigenin and monoclonal antisheep-HRP antibodies. The HRP-substrate was 3,3′,5,5′-tetramethylbenzidine solution (ep(HS) TMB-mA, SDT Reagents, Baesweiler, Germany). DNA oligomers were purchased from Eurofins genomics (Ebersberg, Germany) (Table S1).

2.2. Chip fabrication

Activation of substrates PC and COP was achieved by UV/ozone oxidation as previously described [20]. Amine functionalization was performed by incubating in crosslinker solution (1% EDA, 50 mM EDC) for 30 min at room temperature with gentle stirring. Later chips were immersed in 70% ethanol solution and air-dried. The mixtures of dendrimer (10 nM), NH_2-oligonucleotide probes (100 nM) and EDC (50 mM) were incubated in printing solution at room temperature for 30 min with end-over rotation. After DNA-dendrimer coupling, solutions were arrayed on EDA-functionalized surfaces by a non contact spotter (CE 1500 BioDot Inc., Irvine, CA, USA) at room temperature, 40 nL drop volume and 70% humidity. The end-NH_2 groups were coupled with the free COOH of the oligo-functionalized dendrimer by a carbodiimide reaction. After incubation at room temperature for 1 h, excess dendrimer was removed by washing the chip twice with PBST, followed by air drying. Finally, BSA (30 mg mL^{-1}) was dispensed for surface blocking. DNA biochips were stored at 4 °C.

2.3. Hybridization assay and detection

Chips enabled DNA products to be detected by selective hybridization assays. Briefly, amplification products (5 μL) were mixed with hybridization buffer (45 μL) and heated (95 °C, 5 min) to unwind the double strand in a laboratory heater (VWR, Leuven, Belgium). Solutions were dispensed over sensing areas and incubated at 37 °C for 60 min in an oven (Memmert, Schwabach, Germany). Arrays were rinsed with progressive dilutions of hybridization washing buffer. An immunoreaction was performed for chip staining. For digoxigenin labeling, the reagents were the anti-digoxigenin antibody (1:2500) and the HRP-labeled secondary antibody (1:400) in developing buffer. After substrate dispensation, a dark blue solid deposit formed on positive spots. Plastic-dendrichips were directly scanned (Epson Perfection 1640SU office scanner, Los Alamitos, CA, USA) to give monochromatic images (Tagged Image File Format, color depth 16-bit, scale 0–65535). The optical intensities of each spot and the local background were quantified.

2.4. Application 1: Generic detection of oncogenes based on PCR and RPA

The *KRAS* (codon 12), *NRAS* (codon 61) and *BRAF* (codon 600) genes were the target regions. The genomic DNA from both the human cell line *SK-N-AS* (ATCC CRL-2137) and patient tumor tissues was isolated as described in [20]. Six arrays of specific probes were printed per chip for the simultaneous analysis of six samples, including six replicates per probe.

<u>PCR-based method</u>. Each amplification mixture (50 μL) contained 1 × DNA polymerase buffer, 3 mM of $MgCl_2$, 200 μM of deoxynucleotide triphosphate, 300 nM of upstream primer, 300 nM of downstream digoxigenin-labeled primer, 4 ng of genomic DNA and 1 unit of DNA polymerase. The reaction was performed by an initial denaturation cycle of 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s and elongation at 72 °C for 60 s. The thermal cycler was a Mastercycler Gradient (Eppendorf, Westbury, NY, USA).

<u>RPA-based method</u>. Each reaction mixture (50 μL) was prepared by mixing rehydrated buffer, 14 mM of magnesium acetate, 480 nM of upstream primer, 480 nM of downstream digoxigenin-labeled primer, 4 ng of genomic DNA and the enzyme pellet. Vials were incubated at 37 °C for 40 min in a block heater (VWR, Leuven, Belgium). Measurements were taken by ESEquant TS2 for optimization purposes (Qiagen,

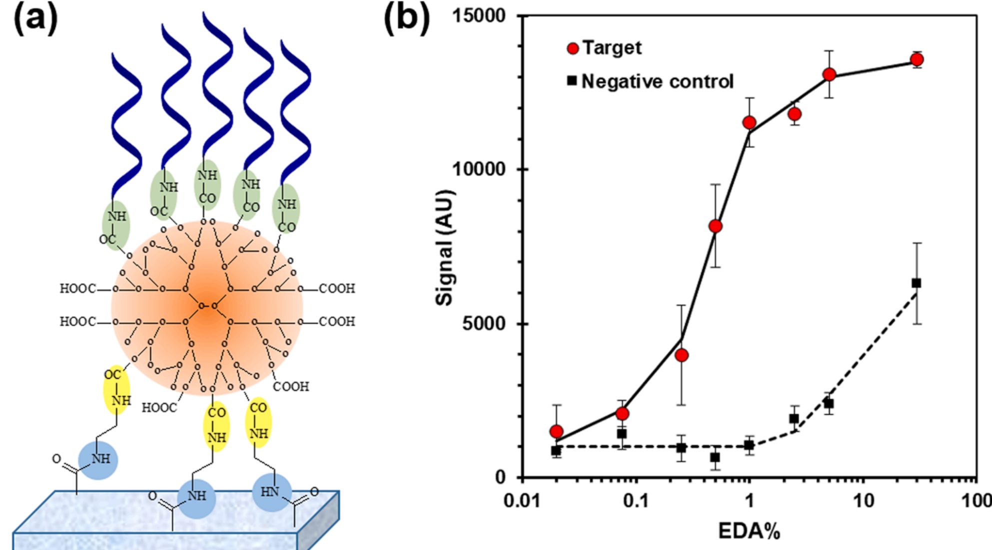


Fig. 1. Immobilization of the oligo-functionalized dendrimers on thermoplastic substrates: (a) scheme of the EDA-mediated reaction. (b) Spot signal of the hybridization assay at different EDA crosslinker concentrations (0.0075–30%). Substrate: PC.

Stockach, Germany).

The hybridization-detection assays were performed on the chip as described above.

2.5. Application 2: Detection of a single-nucleotide mutation based on blocked RPA

The studied hotspot was the *V600E* mutation located in the *BRAF* gene, in which thiamine (T) was substituted for adenine (A) at nucleotide 1799. The genomic DNA from patients' tumor tissues was isolated as described in [20]. Six arrays of allele-specific probes were printed per chip, including three replicates per target probe and controls (the chip layout was 3 × 3).

Each reaction mixture (50 μL) was prepared by mixing rehydrated buffer, 14 mM of magnesium acetate, 480 nM of upstream primer, 480 nM of downstream digoxigenin-labeled primer, 50 nM of blocker, 4 ng of genomic DNA and the enzyme pellet. The blocking oligonucleotide was complementary to the wild-type variant, designed as previously described [24]. Solutions were incubated at 3 °C for 40 min. The hybridization-detection assays were performed on the chip as described above.

3. Results and discussion

3.1. Surface activation and immobilization based on an amine-end crosslinker

In the present study, the dendrimers of generation 3.5 were selected because these polymers have enough terminal groups (64 end-groups) for effective multi-point coupling to nucleic acids [11,17]. The initial experiments were performed using the hybrids between the COOH-functionalized dendrimers (COOH-PAMAM) and amino-end oligonucleotides, prepared following the carbodiimide reaction. As the initial challenge was high-yield immobilization, stoichiometric relation 1:34 was chosen to ensure that enough free COOH-groups were available (at least 50%).

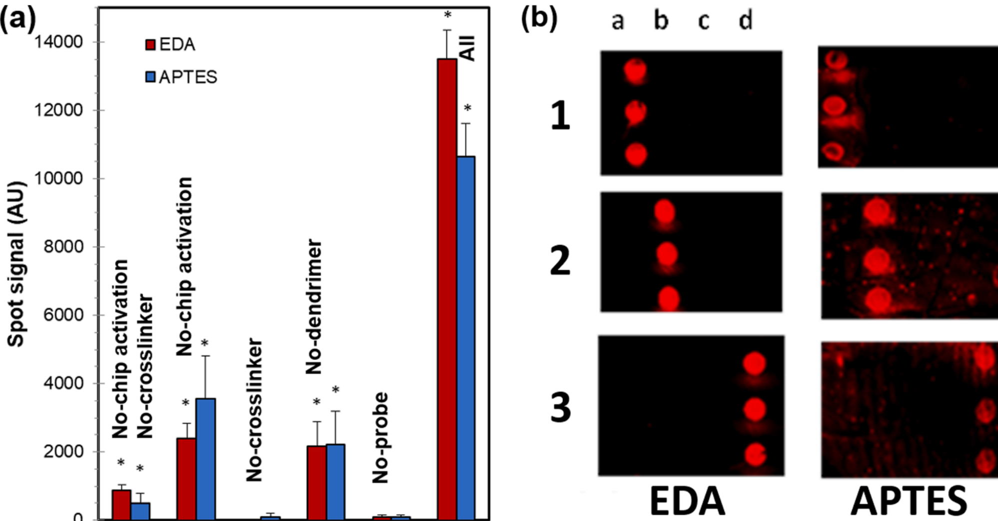
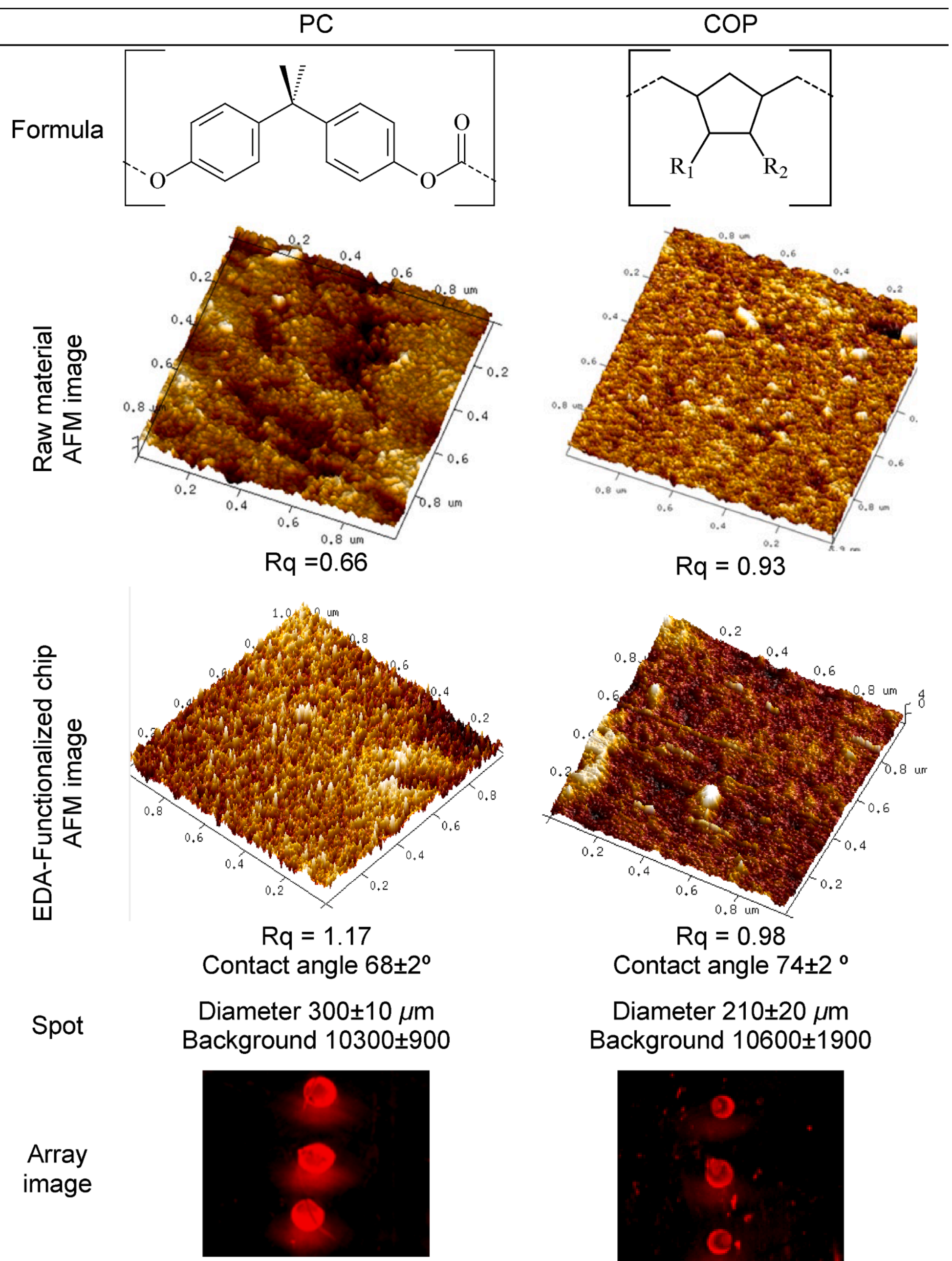


Fig. 2. (a) Spot signals recorded for the manufactured dendrimer chips compared to the control chips built with some preparation step missing. Assay: Recognition of perfect-match oligonucleotide. (b) Capture array image for the EDA and APTES dendrimer-plastic chips for target discrimination. Assay: Multiplex hybridization; samples (1–3) are perfect-match oligonucleotides to probes a-b-d, respectively (c is a negative control).

Table 1
Comparison of chips PC and COP based on the physico-chemical data. Immobilization chemistry: the EDA/COOH-dendrimer/NH_2-probe.



	PC	COP
Formula		
Raw material AFM image	Rq =0.66	Rq = 0.93
EDA-Functionalized chip AFM image	Rq = 1.17 Contact angle 68±2°	Rq = 0.98 Contact angle 74±2 °
Spot	Diameter 300±10 μm Background 10300±900	Diameter 210±20 μm Background 10600±1900
Array image		

The coupling chemistry of these oligo-functionalized dendrimers on chips was explored (Fig. S1). In all the studied approaches, the first step was the activation of thermoplastics (PC and COP) based on plasma UV/ozone oxidation by generating carboxylic-activated surfaces. Water contact angle measurements (WCA) showed that the activation method yielded a high coverage of oxygenated groups (WCA 20°-50°) compared to the raw material (WCA 80°-90°).

The second step involved attaching the amine-end crosslinkers for generating amine-functionalized chips. APTES as a crosslinker was evaluated in the presence of FPTS as a horizontal spacer molecule (Fig. S2). By controlling the silanization conditions (APTES 1%, PFTS 0.1%), the surface was quite hydrophobic (WCA 90 ± 0.5°) and the spot shape was regular. Residual non specific binding was associated with excess free amino groups on the active surface, which induced electrostatic interactions. EDA-mediated coupling was studied. The spot signal showed a linear dependence from its concentration and immobilization yield of the DNA-dendrimer hybrid (Fig. 1). This short-length crosslinker at the 1% concentration provided low background signals, short reaction times and very sensitive responses.

Binding specificity was measured by running simple recognition assays on chips that lacked one anchoring procedure step (or more), as reported in Fig. 2a. The direct immobilization of DNA probes in raw substrates (no activation, no dendrimer) led to low signals associated with residual non specific binding. Positive responses were found in the chips without photo-activation, which were slightly higher in the APTES than in the EDA approach. These results showed the formation of an

adsorption film of the crosslinker on the raw plastic chip (hydrophilic interactions). With no crosslinker, the recorded signals were comparable to the background of the activated chips because the electrostatic repulsion of the surface COOH groups completely prevented the non specific retention of the carboxylic terminated-dendrimers. In the absence of a dendrimer, a residual signal was measured and associated with the non covalent binding between the free carboxylic reactive groups on the surface and amino probes (electrostatic forces). As expected, the experiment performed without a probe confirmed the null signal contribution of the immobilization reagents. Finally, the chips fabricated with all the reagents gave the highest responses, which demonstrated the multisite covalent bonding of the oligo-functionalized dendrimers. Their flexible structure allowed correct coupling of multi-branched dendrimers to the surface despite its partial oligo-functionalization.

An evaluation of biosensing capabilities was assessed by performing multiplexed hybridization assays on both amino-modified plastic surfaces (Fig. 2b). Responses were selective and quantitative because positive spots were detected only for the perfect-match probe, and the recorded intensities were proportional against the target concentration (Table S2). In short, these results confirmed adequate oligo-functionalized dendrimer immobilization by maintaining probe activity and enabling hybridization assays. Nevertheless, the chips prepared by EDA as the crosslinker provided higher spot intensities and lower backgrounds than those functionalized by APTES.

3.2. Nature of the dendrimer

Having demonstrated the principle, the following challenge was to improve oligo- immobilization by modifying dendrimer properties. The carboxyl-end (COOH-PAMAM) and amino-end (NH_2-PAMAM) dendrimers were examined because those nanomaterials provide excellent performance for DNA biosensing [5,9,11]. For the NH_2-PAMAM dendrimers, coupling chemistry also involved glutaraldehyde as a secondary crosslinker (bi-aldehyde molecule).

After coupling to the oligonucleotide probes, the hybrids of NH_2-PAMAM/probe and COOH-PAMAM/probe were coupled to the EDA-functionalized surface by a carbodiimide reaction. The concentration of both the oligonucleotide and dendrimer was examined to generate PAMAM/probe hybrids. Adequate signals were obtained for the dendrimer at $10^{-8\ mol}$ and the probe at $10^{-7} - 10^{-6\ mol}$, which is the equivalent to a stochiometric ratio of 1:10/1:100 (Fig. S3). The AFM measurements reported two well-defined surface regions associated with quite densely packed spots (350 μm diameter) and a background surface, respectively. Higher hybrid concentrations led to signal saturation and irregular spots.

The biosensing performances of both hybrids (NH_2-PAMAM/probe and COOH-PAMAM/probe) were compared (Fig. S4). The amine dendrimers yielded a better spot morphology and homogeneity (positive), but a lower response (about 40%) and a higher (2-fold) degree of non specific adsorption (negative). These results agree with those reported by a previous study based on the DNA immobilization on dendrimer-activated surfaces that compared carboxylic and amine dendrimers on glass slides and silicon wafers [11]. In their study, the researchers observed lower background signals (up to 100-fold). Therefore, the COOH-PAMAM/probe hybrids were chosen for further experiments.

3.3. Thermoplastic substrate selection

The studied materials were PC and COP, which were selected for their high resistance, transparency and flexibility for biochips fabrication. In order to test activation and dendrimer binding, parallel experiments were run for the physico-chemical characterizations. The AFM measurements showed a homogeneous surface structure of active chips (Table 1). The WCA values varied according to the expected surface group; i.e., lowering for polar groups (Table S3). The results confirmed

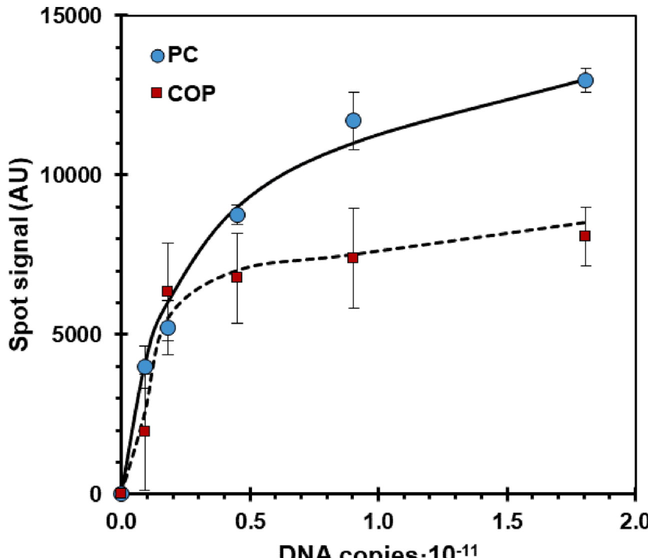


Fig. 3. Comparison of chips PC and COP based on the DNA hybridization assay. Immobilization chemistry: the DNA/COOH-dendrimer/NH_2-probe.

that each functionalization step was correctly performed on both thermoplastic substrates and enabled the DNA-dendrimer solutions to be confined at the anchored site.

Regarding the DNA biosensing features, immobilization capability was examined by varying the amount of probe to be immobilized (Fig. 3). The observed behavior matched the Langmuir model and the standard base-to-base pairing in the solid-phase format [25,26]. However, the saturation signal was lower for COP (8000 AU) than for PC (13000 AU), which indicates fewer immobilized probes. The maximum immobilization density was around 13 pmol cm^{-2} according to the measurements taken of the double functionalized probes (amine and Cy5-fluorophore). The estimated immobilization yield was 55–60% at the 100 nM probe concentration. Compared to previous studies, these values are adequate for DNA applications [2,14–21].

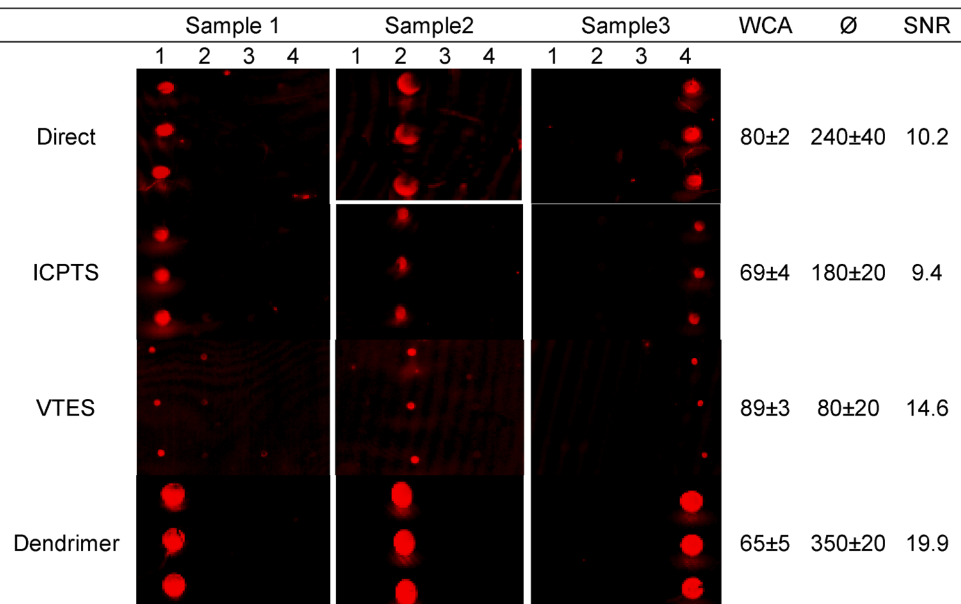
The optical response after a recognition assay was also evaluated (Table 1). On both platforms, the proposed reaction succeeded to chemically modify these low-reactivity materials, which led to site-specific immobilization. High response ratios were observed for the fully complementary probes compared to those for the non complementary ones, which proved the multiplexing capabilities. Nevertheless, homogeneity, spot intensities and the standard deviation of background signals were better for PC than for COP (around 2-fold).

In conclusion, both substrates showed specific oligo-dendrimer binding, excellent compatibility with the hybridization assays and recognition selectivity. Nevertheless, the PC substrate was selected because it exhibited low non specific absorption and higher responses and, thus, better biosensing capabilities were expected.

3.4. Comparison to other coupling chemistries

The performance of the performed coupling reaction was examined and compared to other described surface reactions to engraft the DNA probe to plastic or glass substrates. These chips were fabricated as reported in the Supplementary Information.

The conventional dendrimer-mediated approach was applied (Table S4). This immobilization method is based on a two-step sequence, including the film formation of non conjugated dendrimers, followed by the binding of oligonucleotide probes [8]. A controlled film formation of dendrimers COOH-PAMAM was achieved by spin coating, particularly at low concentrations (Table S5). The AFM images and roughness data



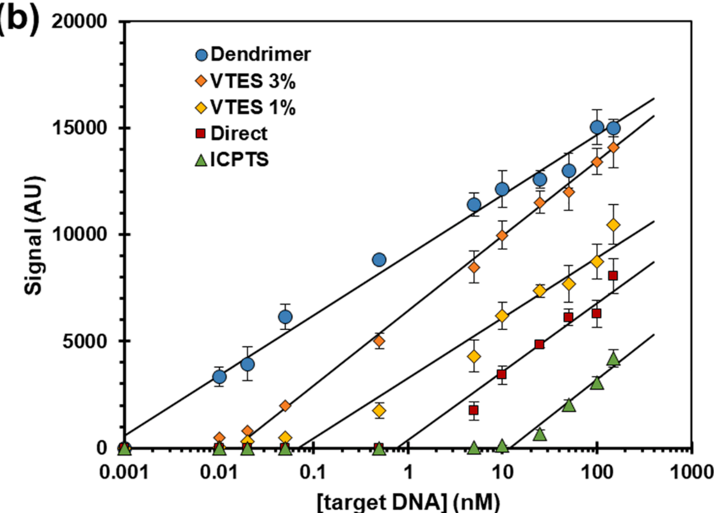


Fig. 4. Comparison of dendrimer-based immobilization to other coupling chemistries. a Array features. b Spot signal according to the target concentration. Array layout: 4 probes × 3 replicates. Probes: target 1 (1); target 2 (2); negative control (3); target 3 (4). Targets: Perfectly matched oligonucleotides at 100 nM.

suggested that surfaces were quite homogeneous, and the cluster or dendrimer aggregation observed in other substrates did not form [18]. In addition, the mean depth variation agreed well with the EDA-dendrimer dimensions (approx. 5 nm). Then the amino-ended oligonucleotides were attached to the dendrimer film by a carbodiimide reaction in a heterogeneous format.

The dendrimer-mediated approach displayed several disadvantages for biosensing purposes compared to our method for reliable DNA quantitative assays [2,4] (Table S4). These chips yielded high background hybridization signals (12000 ± 1000 au), low reproducibility and no uniformity of spot profiles. These features suggested incomplete oligonucleotide probes coupling, which would render free COOH groups and the consequent physisorption of non specific molecules. On the contrary, our synthesis procedure was partially performed in the solution phase, which allowed a more effective process and a better control of the repulsion of the adjacent oligonucleotides (anionic DNA backbone), which improved probe immobilization [25]. Likewise based on our approach, probe attachment was faster (40 min less) and reagent use was lower (2500-fold, calculated from the volume ratio).

Three non dendrimer-mediated systems were also analyzed, including the non crosslinker, ICPTS and VTES functionalized chips (Fig. S5). The first involved the amino-end probes directly coupled to the carboxyl groups (–COOH) of the activated substrates. In the second, the ICPTS reagent worked as an organosilane crosslinker. Then the surface was covered by isocyanate groups (-C = N = O) capable of binding amino-end probes. The third chemistry also required the silanization of platforms by VTES, an organosilane reagent that contains a terminal vinyl group (–CH = CH_2). The attachment of thiol-end probes was easily achieved via the photoclick-chemistry mechanism.

For the required resources, our functionalization offered advantages over other linear crosslinkers or previously published immobilization chemistries for plastics [2,19]. The activation procedure, based on

Table 2
Comparison of oncogene detection using the oligo-functionalized dendrimer chips after PCR and RPA amplification.

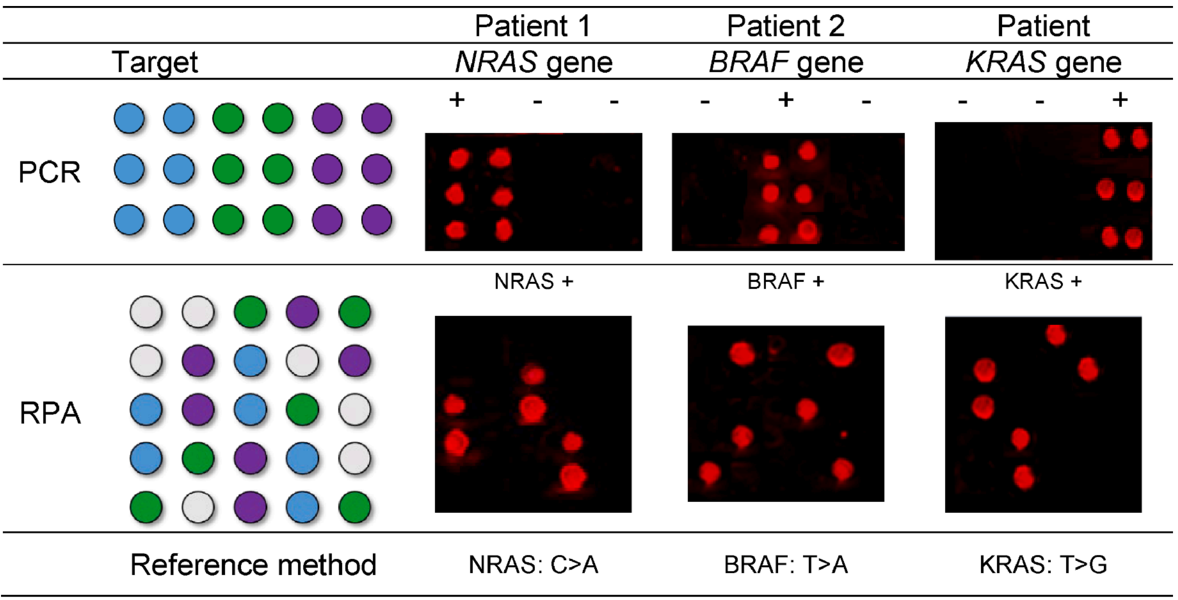


photo-oxidation by UV-ozone, is feasible in complexity, cost-effectiveness and the required instrumentation terms if alternative techniques are considered, such as physical vapor deposition, chemical vapor deposition, physical laser deposition or sputtering. For instance, times were 45 min for the novel strategy, 90 min for the silane-based approaches and several hours for the other techniques. For the covalent binding of amino probes, our method was also fast (1 h), and was overcome only by thiol-ene click reactions like VTES-based chemistry. All the platforms were suitable when considering DNA biosensing requirements [1,3]. Our immobilization scheme was considerably cheaper when comparing the cost and reagent concentrations.

Wettability features were also estimated from the spot diameter measurements (Fig. 4a). As expected, the smallest spots were obtained for the VTES-functionalized chips (80 µm) and the largest ones for the dendrimer-mediated chips (350 µm). These results indicated that silane modification exhibited a more hydrophobic nature due to the terminal vinyl groups ($-CH=CH_2$) than the other active surfaces, including the novel approach. All the chips displayed sharp spots with excellent signal distribution. Intraspot homogeneity was slightly better for the novel method, with a 15 ± 5% relative standard deviation for direct activation, 9 ± 7% for ICTPS, 15 ± 5% for VTES and 7 ± 3% for the EDA-dendrimer-chip.

The multiplexed hybridization assay in the array format was studied. First, no relevant surface effect was observed because the recognition kinetics of the DNA target and probe on the sensing surface was comparable to other coupling chemistries (Fig. S6). Second, an excellent recognition process between the probe and target DNA was achieved, as all the array images show (Fig. 4a). Assay selectivity, expressed as the signal percentage for the complementary probe compared to other probes, was 94% for the dendrimer-based coupling and 92–96% for the other bindings. Reproducibility, expressed as the relative standard variation of replicated experiments, was 4% and 4–7%, respectively.

The most relevant difference for DNA biosensing was assay sensitivity (Fig. 4b). The spot intensities in the dendrimer chips were 2- to 100-fold higher. In particular, the dynamic range for dendrimer coupling significantly improved compared to direct functionalization [20]. These better results can be explained mainly by the structure of the highly branched macromolecules (3D immobilization) compared to the brush-like structure of single-site attachment (2D immobilization). The flexible probe configuration should facilitate the displacement of the target molecule from the solution to the surface (interface movement), as well as further binding. These hyperbranched molecules reduce the interaction between the adjacent probe molecules compared to the typical vertical alignment of linear functionalization [25,26]. The hybridization event to the template is promoted to obtain better detection limits, with values of 0.005 nM (dendrimer), 0.05 nM (VTES 3%), 0.5 nM (VTES 1%), 5 nM (direct) and 50 nM (ICTPS). These results correlate with the number of immobilized probes with adequate orientation and accessibility. Compared to other DNA sensing systems, the detection limit is lower than, or of the same order as, previous studies based on dendrimer-surfaces [11,16] and linear crosslinkers [13,19]. Thus the excellent analytical performances display a clear advantage for our multipoint attachment over all other oligonucleotide immobilization systems for POC detection.

3.5. *Generic detection of oncogenes based on PCR and RPA*

One important application of DNA-based chips and biosensors is the multiplexed detection of amplification products [1,2]. This study examined two amplification reactions: standard PCR and an isothermal alternative.

Our experiments intended to test platform capabilities for the specific detection of clinically relevant DNA regions. As proof of concept, the hotspots in oncogenes *KRAS, NRAS* and *BRAF* were amplified from cell line cultures and human samples (biopsied tissues). PCR and RPA products were hybridized in the PC/EDA/COOH-PAMAM/probe chips, but RPA products led to high background signals. These results were interpreted as the non specific binding of certain RPA mix components, such as high-molecular-weight polyethylene glycol and crowding agents. After including a BSA blocking treatment of chip surfaces, background signals were comparable to PCR products (test t, p-value 0.27).

Table 2 provides examples of the recorded array images. Selective and sensitive responses were found for both amplification reactions, which proved that amplicons were hybridized with gene-specific probes, while no responses were obtained for the others. The same human tissues were analyzed by the chips with probes directly immobilized on photo-activated plastics, as described in [20]. The signals recorded for

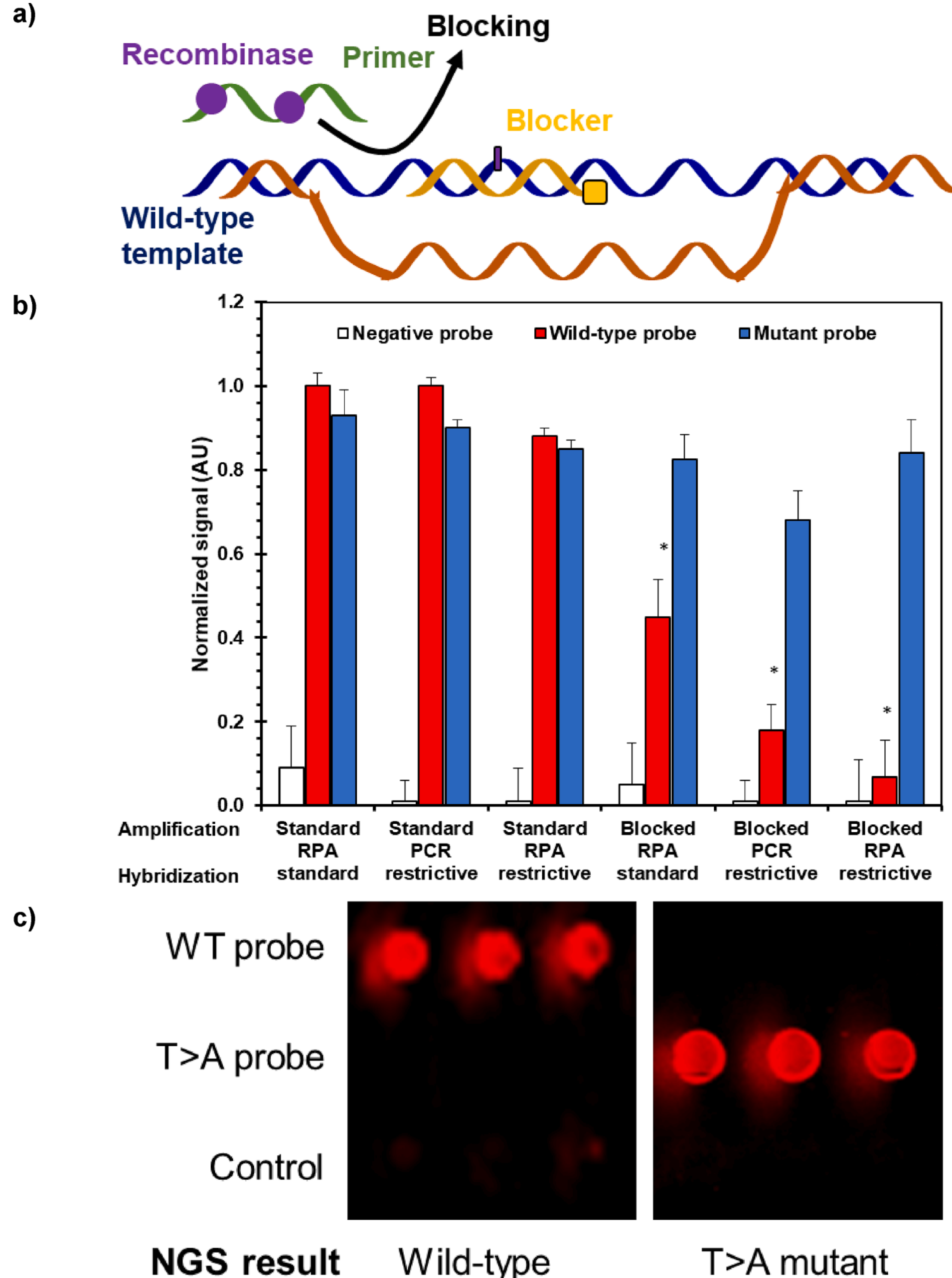


Fig. 5. (a) Scheme of the blocked RPA inhibiting the wild-type amplification. (b) Mutant detection capability on the dendrimer-mediated chips in accordance with the amplification method and hybridization conditions (standard or restrictive). * p-value < 0.01 (*t* test). (c) Array image for the developed method based on blocked RPA and the sequencing result (reference method) from oncology patients' tumor tissue (wild-type and *V600E BRAF* mutants).

the dendrimer-based coupling increased 40% *vs.* the direct coupling between the amino-end probes and the carboxyl groups of the activated chips.

These results demonstrated that our immobilization method can attach specific DNA probes independently of the amplification reaction. Nevertheless, RPA was faster and offered better operational conditions to develop integrated devices, such as lower temperature and fewer bubbles, and can avoid thermal cycling systems [1–3,20,21].

3.6. Specific detection of mutations based on blocked RPA

The performance of dendrimer-based DNA microarrays in point-mutation discrimination was investigated for its clinical relevance [27]. As proof of concept, variant p.V600E in gene *BRAF* (c.1799 T > A) was identified from human samples (biopsied tissues). The analytical challenge lies in the discrimination between the A-mutant and the wild type (T) because the probability of a human genome having cytosine (C) or guanidine (G) in that locus is extremely low.

The suppression of the wild-type variant was studied by blocked RPA [20]. The discrimination mechanism of this isothermal technique, operating at 37 °C, is based on decreased polymerase action due to the addition of blocking agent, complementary to the wild-type template (Fig. S7). To enhance this effect, the blocker was designed to partially overlap the upstream primer because the primer annealing step was also controlled. First, the designed oligonucleotides (primers and blocker) were confirmed by the conventional blocked PCR approach in a thermocycler (Fig. S8). Then the blocked RPA combined to the array hybridization assay was optimized by varying the reaction time, blocker concentration and relative concentrations of primers (Fig. S9). The responses associated with the wild-type variant significantly decreased in

the presence of 50 nM of blocker, a stoichiometric ratio between primers and a reaction time of 40 min.

The aim of the following experiments was to detect the mutant variant (c.1799 T > A) in clinical samples (biopsied tissues). A reduction in the wild-type variant was obtained by blocked PCR and blocked RPA, although detectable signals were observed in association with the wild-type probe (Fig. 5). The remaining signal was related to non specific recognition during hybridization because the difference between the wild-type and mutant variants is a single nucleotide. To improve performance, a restrictive hybridization buffer (2 × SSC, 25% formamide) was tested. The results confirmed our hypothesis and a clear spot pattern was achieved in accordance with the patient's genotype (Fig. S10).

For the quantitative analysis, discrimination ratios were determined as the ratio between the mutant and wild-type signals. The values were 0.4–0.5 for the wild-type samples (genotype: T) and 3.8–12.4 for the mutant samples (genotype: A). These values were similar to those obtained by the blocked RPA approach and the restrictive hybridization assay for the *KRAS* gene [24]. For this mutation, high spot signals were recorded in association with the perfect-match probes, and the discrimination ratios exceeded 1 (Fig. S11). The ratios were lower than 1 for the wild-type and the other mutants in the same locus (single-nucleotide change). So the experiments proved the discrimination capability applied to real clinical samples and endorsed that this method can be a useful tool for supporting the correlation of genetic variations and individual phenotypes [27–29].

4. Conclusions

We demonstrate that the multipoint attachment of oligonucleotide probes based on dendrimers is an excellent approach for the functionalization of polycarbonate and cycloolefin thermoplastics, which are widely used materials in commercial microfluidic chips. Our novel approach comprises the formation of dendrimer-probe hybrids and their later effective immobilization, which are precise at the surface site and robust in surface wettability or roughness terms. The branched flexible structure nature of hybrids should particularly favor higher recognition of DNA target yields by providing up to 100-fold lower detection limits. These values are better than those achieved with surfaces functionalized with either linear crosslinkers or other dendrimer-based approaches.

Apart from these chips being able to detect several PCR products, we herein report for the first time the selective hybridization of RPA products on dendrimer-mediated probes. This isothermal amplification combines constant low reaction temperature with high sensitivity, specificity, fast reaction speed, portability and capability for clinical diagnostics outside laboratories. The dendronized-thermoplastic chip enhancement of sensitivity allowed us to address a challenging need: the discrimination of low-frequent specific DNA biomarkers, such as single-nucleotide mutations in clinical samples. Having been proved for the selective rapid detection of oncogene mutations in human tissues, the results will contribute to better diagnose diseases and toward reliable prognoses based on the use of disposable devices.

CRediT authorship contribution statement

Sara Martorell: Methodology, Investigation. **Luis Antonio Tortajada-Genaro:** Conceptualization, Data curation, Writing - review & editing. **Miguel Angel González-Martínez:** Conceptualization, Writing - review & editing. **Angel Maquieira:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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