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Investigation of the multiple anchors approach in oligonucleotide microarray preparation using linear and stem–loop structured probes

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ABSTRACT

Enzyme-mediated reactions are a useful tool in mutation detection when using a microarray format. Discriminating probes attached to the surface of a DNA chip have to be accessible to target DNA and to the enzyme (ligase or polymerase) that catalyses the formation of a new phosphodiester bond. This requires an appropriate chemical platform. Recently, an oligonucleotide hairpin architecture incorporating multiple phosphorothioate moieties along the loop has been proposed as an effective approach to solid-phase minisequencing. We have explored in depth several variables (stem length, number of phosphorothioates, stem–loop architecture versus linear structure) involved in this strategy by using a solid-phase ligation reaction. Microarrays were fabricated either from aminosilyl-modified glass or from aminated polymeric surfaces made of poly-lysine. Both platforms were bromoacetylated and reacted with thiophosphorylated oligonucleotides. The resulting microarrays were tested using either a synthetic template or a PCR-amplified 16S rRNA genomic region as the target sequence. Our results confirm the robustness of the proposed chemistry. We extend its range of application to solid-phase ligation, demonstrating the effectiveness of multiple anchors and suggest that linear oligonucleotides incorporating multiple phosphorothioates are equivalent to their hairpin-structured counterparts.

INTRODUCTION

The application of oligonucleotide microarray technology to gene mutation analysis is largely dependent on oligonucleotide (probe) availability in the molecular reactions adopted for mutation detection.

Since the advent of microarray technology, different chemical pathways have been proposed for oligonucleotide attachment to glass surfaces (1–4) requiring 5′ amino- or thiol-modified oligonucleotides. Several synthetic strategies have been explored, including those that incorporate polyfunctional linkers such as polyacrylamide gel pads (5), branched chains (6) and poly(acrylic acid-co-acrylamide) copolymers generated *in situ* (1). Such approaches overcome some of the difficulties arising from the poor loading capacity of the glass surface. Hybridisation is the conventional method for gene mutation analysis (7), but recently ligation (8), minisequencing (9) and primer extension (10) are gaining wide attention due to their excellent discriminating power of allele variants (11). These enzyme-mediated detection strategies involve a reaction occurring directly on the chip surface. Therefore, the development of coupling techniques that can maintain functional oligonucleotides and enzyme accessibility is crucial. Recently, a novel approach involving multiple anchoring points has been proposed for minisequencing applications (12). Such an approach is based on a chemistry (13) requiring the presence of phosphorothioates along the oligonucleotide chain to be bound to a bromoacetylated surface. Bromoacetyl moieties are prone to nucleophilic attack by sulphur atoms and yield phosphorothioesters. These bonds are created along the loop region of a stem–loop structure created at the 5′ end of the oligonucleotide to be attached. Zhao *et al.* (12) demonstrated how the number of anchoring points could influence the strength of the fluorescent signal after a minisequencing reaction. This approach is of interest for several reasons. Phosphorothioate modification on the oligonucleotide chain is simple to obtain and cheaper than the aminolinker required by most other chemistries. The reactive bromoacetylated surfaces are stable, do not require particular storage conditions and react quickly with phosphorothioates. The proposed stem–loop structure could create a proper lateral spacing among probes, increasing the accessibility to DNA polymerase. Being interested in robust and reliable chemical platforms for microarray-based mutation detection we studied the concepts previously reported (12,13). We used either the proposed aminosilyl-modified glass or a poly-amine (poly-L-lysine)-modified surface as a scaffold for oligonucleotide microarray preparation. We used ligation instead of minisequencing as the enzyme-mediated reaction. The previously described hairpin stem–loop structures were compared with others with more stable stems (8 bp compared with the original 5 bp). Moreover, linear- and hairpin-structured multiple anchored probes were studied in order to ascertain if stem–loops are mandatory in the oligonucleotide design. Our experiments

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were performed using either a synthetic template or a 16S rRNA gene PCR product as the target sequence.

MATERIALS AND METHODS

All chemicals and solvents were purchased from Sigma-Aldrich (Italy) and used without further purification. Oligonucleotides were purchased from Interactiva Biotechnologie GmbH (Germany) and MWG-BIOTECH AG (Germany). They were HPLC purified and checked by MALDI-MS. Precleaned non-derivatised microscope slides were used (Sigma-Aldrich). *Tth* DNA ligase was obtained from ABgene (UK). DynaZyme II DNA polymerase was purchased from FINNZYMES OY (Finland). Lambda exonuclease was from USB Corporation (USA). Bacterial genomic DNA was kindly provided by Biosearch Italia (Gerenzano, VA).

Preparation of slides

The 25 × 75 mm^2 glass slides were washed by soaking in 1 M NaOH for 2 h on a shaker followed by rinsing with distilled water, immersed in 1 N HCl solution overnight on a shaker and then rinsed again in distilled water. Silanisation was performed at room temperature according to one of the following protocols.

*GOPS/poly-*L*-lysine surface treatment*. Microscope slides were immersed in 96% ethanol for 10 min and then rinsed three times with distilled water. The slides were washed in acetone for 10 min, removed, dried and treated with 1% (v/v) 3-glycidoxypropylsilane (GOPS) in 95% ethanol for 1 h. Excess silane was removed by dipping the slides in 95% ethanol for 1 min. Finally, they were dried in an oven at 150°C for 20 min. GOPS-treated slides were immersed in 0.1× PBS containing 0.01% (w/v) poly-L-lysine and kept for 1 h on a shaker. Treated slides were washed five times with distilled water, centrifuged at 500 r.p.m. for 5 min and dried for 10 min at 45°C.

APMDES surface treatment. Microscope slides were washed in 95% ethanol for 10 min, immersed for 5 min in 95% ethanol containing 2% (v/v) 3-aminopropylmethyldiethoxysilane (APMDES) and rinsed with 95% ethanol. The slides were cured in an oven at 75°C for 4 h.

Surface activation by bromoacetylation. Surface activation was carried out in the dark at room temperature. Dried slides prepared by one of the two protocols described above were placed in a glass rack and immersed for 2 h in 160 ml of *N*,*N*-dimethylformamide containing 20 mM bromoacetic acid, 2 mM 4-(dimethylamino)-pyridine and 20 mM 1,3-dicyclohexylcarbodiimide. Activated slides were then washed with 95% ethanol for 10 min and dried in an oven at 75°C.

Oligonucleotide microarray preparation

Oligonucleotide sequences used in the present study are listed in Table 1. Oligonucleotides were dissolved in 1× TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.0) at a final concentration of 50 µM and spotted onto activated slides by a non-contact piezo-driven dispensing system (Nanoplotter, GeSiM, Germany). Slides were kept overnight in a humid chamber at room temperature. Afterwards they were washed with boiling MilliQ-filtered water.

Solid-phase ligation reactions using a synthetic template

Ligation reactions were performed onto the oligo microarray using a synthetic template (5′-GGGTGTTTCCGACTTTCCT-GACGTGACGGGCGGTGTGTACAA-3′) and a 5′-phosphate-3′-Bodipy-labelled oligonucleotide (5′-pho-CAGGAAAGTCG-GAAACACCC-Bodipy650/665-3′) (the so-called common probe complementary to the synthetic template), both at 1 µM final concentration. The ligation reaction buffer consisted of 100 mM Tris–HCl pH 8.3, 50 mM $MgCl_2$, 250 mM KCl, 5 mM EDTA, 5 mM NAD, 50 mM DTT, 0.5% (v/v) Triton X-100 and 0.25 U/µl of a *Tth* DNA ligase. The experiments were carried out at 55°C for 3 h in an automatic workstation (Gene TAC, Genomics Solutions, USA). After incubation, the slides were washed in MilliQ-filtered water at 75°C for 10 min and then spun at 800 r.p.m. for 3 min.

Fluorescent signals were acquired at 5 µm resolution using a ScanArray 4000 laser scanning system (Packard GSI Lumonics, MA). The Red laser was used for Bodipy650/665 dye (λ_{ex} 633 nm/λ_{em} 670 nm). Both the laser power and photomultiplier tube gain were set to 80%. To quantitate the fluorescent intensity of spots we used the QuantArray Quantitative Microarray Analysis software (Packard GSI Lumonics).

Solid-phase ligation reaction using a PCR fragment of the 16S rRNA gene

The PCR amplification of the 16S rRNA gene was performed with 5 ng of Actinomycetes or *Pseudomonas putida* genomic DNA at a final volume of 50 µl. The reaction mixture consisted of 500 nM F27 primer (5′-phosphate-AGAGTTTGATCMT-GGCTCAG-3′), 500 nM R1492 primer (5′-TACGGYTACCTT-GTTACGACTT-3′), DynaZyme buffer (10 mM Tris–HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM dNTPs, 1.5 mM $MgCl_2$ and 0.025 U/µl DynaZyme II DNA polymerase. The sample underwent a thermal cycling procedure consisting of a denaturation step (95°C for 5 min), 30 cycles of 94°C for 45 s, 61°C for 45 s, 72°C for 2 min and a final extension step (72°C for 10 min).

After an electrophoresis check, the PCR product (1450 bp) was purified using MicroSpin S-400 HR columns (Amersham Pharmacia Biotech, NJ) according to the manufacturer's protocol.

To obtain a single-stranded product, we performed a lambda exonuclease digestion. The enzymatic reaction was carried out in digestion buffer (67 mM glycine–KOH pH 9.3, 2.5 mM $MgCl_2$) with 0.1 U/µl lambda exonuclease at 37°C for 30 min, followed by enzyme inactivation at 75°C for 15 min. The resulting product (consisting of the reverse strand) was purified using MicroSpin S-400 HR columns. The purified single-stranded DNA was used as target sequence in the ligation reaction according to the procedure described above.

RESULTS

In this study we have investigated in detail an approach to oligonucleotide microarray preparation, recently proposed by Zhao *et al.* (12), based on multiple phosphorothioate moieties in the loop of a hairpin stem–loop structure as an anchorage point for the probe sequence on preactivated (bromoacetylated) glass slides. We investigated in depth this type of attachment procedure comparing hairpin- and linear-structured oligonucleotides.

Figure 1 is a schematic design of the diverse oligonucleotide backbones used in our experiments. Figure 1A shows the structure of the hairpin probes, incorporating from one to five phosphorothioate moieties within the polyA loop and including a 5 or 8 bp stem. Figure 1B summarises the structures of the linear probes incorporating from one to 10 phosphorothioate moieties within a 5′-polyA tail. Table 1 lists the probe sequences and the phosphorothioate moieties positions along the nucleotidic chain.

The ligation reaction in solid phase was employed as a testing method to ascertain the relevance of issues raised in the original work by using minisequencing. The ligation experiments were performed directly onto the bromoacetylated surface of glass slides (Fig. 2). A thermostable DNA ligase mediates the formation of a phosphodiester bond between the 5′ phosphate of a labelled common probe and 3′ hydroxyl termini of solid-phase anchored oligonucleotides in the presence of a complementary target sequence. The ligation reaction occurs only on perfectly paired DNA molecules: the presence of mismatches in the joining position prevents the ligation reaction.

In the meanwhile, we explored a new bromoacetylated surface based on GOPS (3-glycidoxypropyltrimethoxysilane)-poly-L-lysine compared with the original APMDES, as solid support for microarray preparation.

Comparison of hairpin- versus linear-structured oligonucleotides in solid-phase ligation reactions

We compared the performance of solid-phase ligation reactions using linear- and hairpin-structured oligonucleotides carrying a variable number of phosphorothioates. This comparison was performed on GOPS/poly-L-lysine- or on APMDES-treated surfaces using a 42 bp synthetic template as target. The fluorescent signals were collected and quantitated as explained in Materials and Methods. Forty spots (10 spots/array, four replicates) for each oligonucleotide were used to calculate signal average and standard deviation.

As shown in Figure 3, by increasing the number of thiophosphorylated adenines, the signal intensity increased both in the hairpin- and in the linear-structured oligonucleotides. Higher fluorescence intensity was found using oligonucleotides carrying five thiophosphorylated adenines positioned at either the 5′ end (linear probes) or in the loop (hairpin probes).

Figure 4 illustrates the ligation experiment results obtained by employing a 16S rRNA gene PCR fragment (from Actinomycetes genomic DNA, ~1450 bp, digested with an exonuclease to yield a single-stranded product) as template. Results are in qualitative agreement with those gained on the synthetic template.

A mismatching template was obtained using a PCR-amplified 16S rRNA gene from *P.putida*. No signals were collected from listed probes demonstrating the sequence specificity of the ligase-mediated reaction (data not shown).

DISCUSSION

In this study, we aimed at further exploring a novel chemical approach that has recently been proposed for oligonucleotide immobilisation to a preactivated glass surface (12). In order to explore the possibility of combining the proposed chemical platform and a ligase-mediated mutation detection strategy we tested several experimental variables. In the original paper, the synthetic target is positioned in a contiguous manner on the

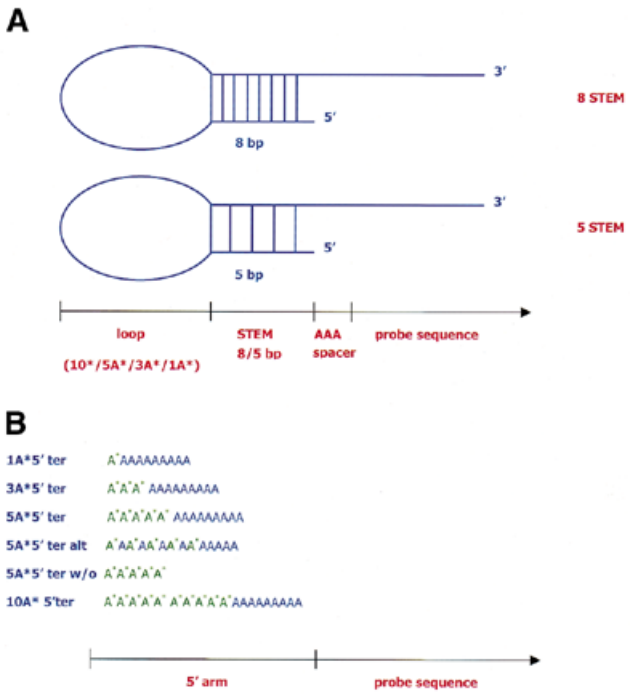

Figure 1. (**A**) The hairpin-structure oligonucleotide is composed of four elements: a loop comprising six adenosines with five, three or one phosphorothioate moieties, a double-strand stem 8 (5′-CCTGGCGC-3′) or 5 (5′-CCTGG-3′) bp long, three adenosines and the probe sequence (5′-TTGTACACAC-CGCCCGTCACGT-3′). (**B**) The linear structure oligonucleotide composed of a 5′ arm with different number and position of phosphorothioate modifications (A*) and the same probe sequence (5′-TTGTACACACCGCCCGTCACGT-3′).

probe sequence, therefore yielding a very stable couple due to an extended stacking effect (figure 2 in ref. 12). However, this molecular situation is unlikely to occur in a real mutation detection experiment. Therefore, as shown in Figure 1A and in Table 1, we added three bases (AAA) between the stem–loop structure and the probe sequence complementary to the chosen targets, avoiding any steric interference between the stem and the complementary targets.

Furthermore, we noted that the originally proposed loops, consisting of nothing but the phosphorothioated nucleotides were too constrained when a single base or two were used. Therefore, we decided to use a 6 bp loop including one, three or five phosphorothioate moieties.

This probe architecture was employed in combination with the proposed 5 bp stem as reference and compared with similar probes having a longer stem (8 bp resulting from a simple extension of the original 5 bp sequence). As shown in Figures 3 and 4, a side by side comparison between 8 bp stem and 5 bp stem probes was performed. Using the synthetic template on either the original chemical platform (Fig. 3A) or the GOPS/poly-L-lysine chemistry (Fig. 3B) no significant differences were found among probes carrying the same number of phosphorothioate moieties. Similar results were obtained with the single-stranded PCR fragment (Fig. 4).

Linear probes without stem–loop structures were designed incorporating one, three or five phosphorothioate moieties placed 5′ to a 9 bp polyA tail (1A* 5′ ter, 3A* 5′ ter, 5A* 5′ ter, Table 1). As shown in Figures 3 and 4 these linear oligonucleotides compared similarly or even favourably with

Table 1. The oligonucleotide sequences used in this study

N°	OLIGONUCLEOTIDE	LENGTH	SEQUENCE FROM 5' TO 3'
1	5A*-STEM8	47 bp	CCTGGCGC(A*)5-AGCGCCAGGAAAttgtacacaccgcccgtcacgt
2	3A*-STEM8	47 bp	CCTGGCGCA(A*)3-AAGCGCCAGGAAAttgtacacaccgcccgtcacgt
3	1A*-STEM8	47 bp	CCTGGCGCAAA*AAAGCGCCAGGAAAttgtacacaccgcccgtcacgt
4	5A*-STEM5	41 bp	CCTGG(A*)5-ACCAGGAAAttgtacacaccgcccgtcacgt
5	3A*-STEM5	41 bp	CCTGGA(A*)3-AACCAGGAAAttgtacacaccgcccgtcacgt
6	1A*-STEM5	41 bp	CCTGGAAA*AAACCAGGAAAttgtacacaccgcccgtcacgt
7	1A*-5'ter	32 bp	(A*)1-AAAAAAAAAttgtacacaccgcccgtcacgt
8	3A*-5'ter	34 bp	(A*)3-AAAAAAAAAttgtacacaccgcccgtcacgt
9	5A*-5'ter	36 bp	(A*)5-AAAAAAAAAttgtacacaccgcccgtcacgt
10	5A*-5'ter-alternate	36 bp	A*AA*AA*AA*AA*-AAAAAttgtacacaccgcccgtcacgt
11	5A*-5'ter-w/o spacer	27 bp	(A*)5-ttgtacacaccgcccgtcacgt
12	10A*-5'ter	41 bp	(A*)10-AAAAAAAAAttgtacacaccgcccgtcacgt
13	10A*-STEM5	52 bp	CCTGG(A*)10-ACCAGGAAAttgtacacaccgcccgtcacgt
	synthetic template	42 bp	GGGTGTTTCCGACTTTCCTGACGTGACGGGCGGTGTGTACAA
	common probe for ligation	20 bp	Pho-CAIGAAAGTCGGIAACACCC-Bodipy650/630

A* is an adenosine with phosphorothioate modification. The stem motif is in red, the loop bases are in green, the spacer is in blue and the probe sequence is in lower case.

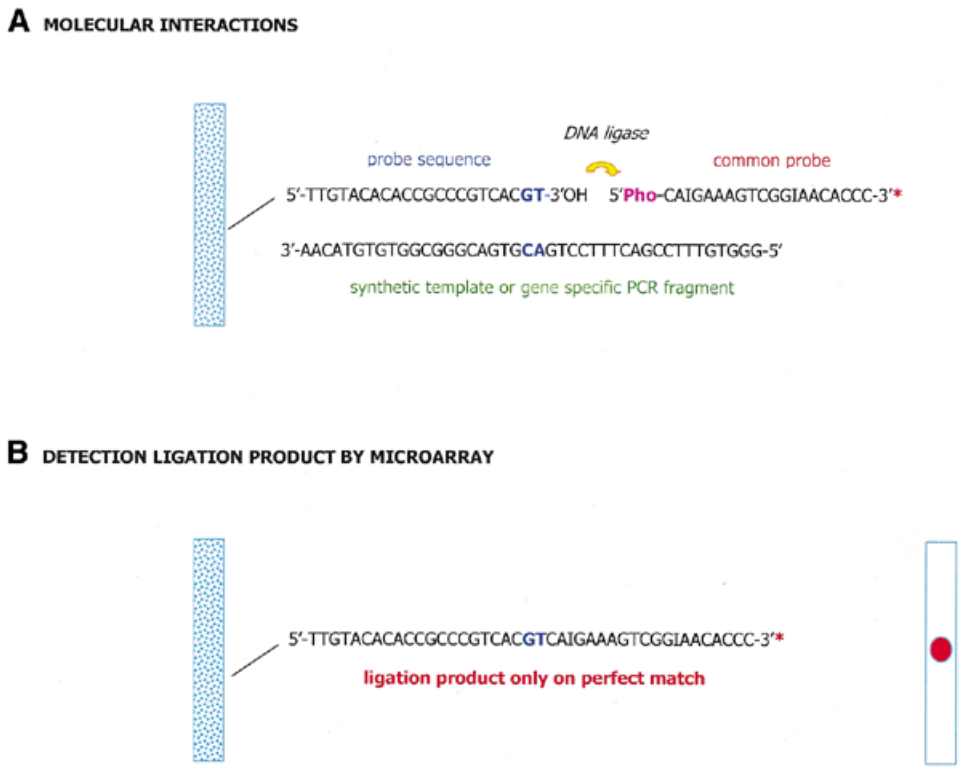

Figure 2. Enzymatic reaction scheme of solid-phase ligation reaction. (**A**) Molecular interactions; (**B**) detection ligation product by microarray.

respect to their hairpin-structured counterparts. These results point out that the stem–loop structure (generating longer and more expensive probes) does not represent a significant improvement over traditional linear probes. On the contrary, a direct relationship between the number of phosphorothioate moieties and signal intensity was consistently found (confirming observations made in the previous report) whatever the structure of the probes. We further investigated this aspect by testing a linear probe (10A* 5′ ter) and a hairpin probe (10A*-STEM5), both including 10 phosphorothioate moieties and two linear probes with five phosphorothioate moieties placed contiguously at the 5′ end without any AAA spacer (5A* 5′ w/o) or alternate to phosphate moieties along the 5′ polyA tail (5A* 5′ ter-alternate). As shown in Figures 3 and 4

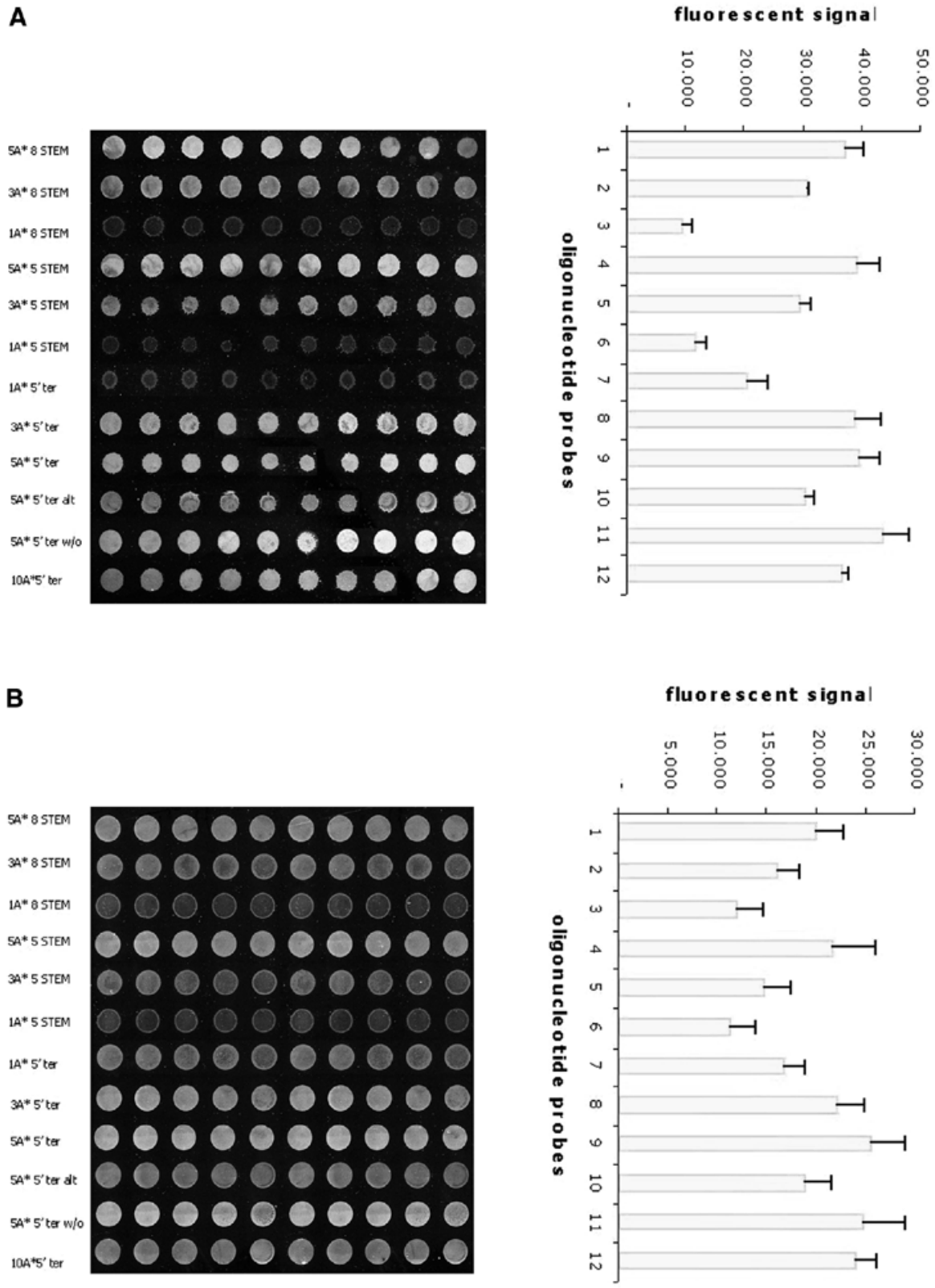

Figure 3. Solid-phase ligation reaction onto the APMDES (**A**) and GOPS (**B**) surfaces. Spots image (on the left) and average and standard deviation graph (on the right) of 40 replicates.

and summarised in Figure 5, the fluorescence signal strength does not increase linearly with the number of phosphorothioate moieties included within the probe sequence. Probes with 10 phosphorothioates (either linear or within a loop) perform similarly

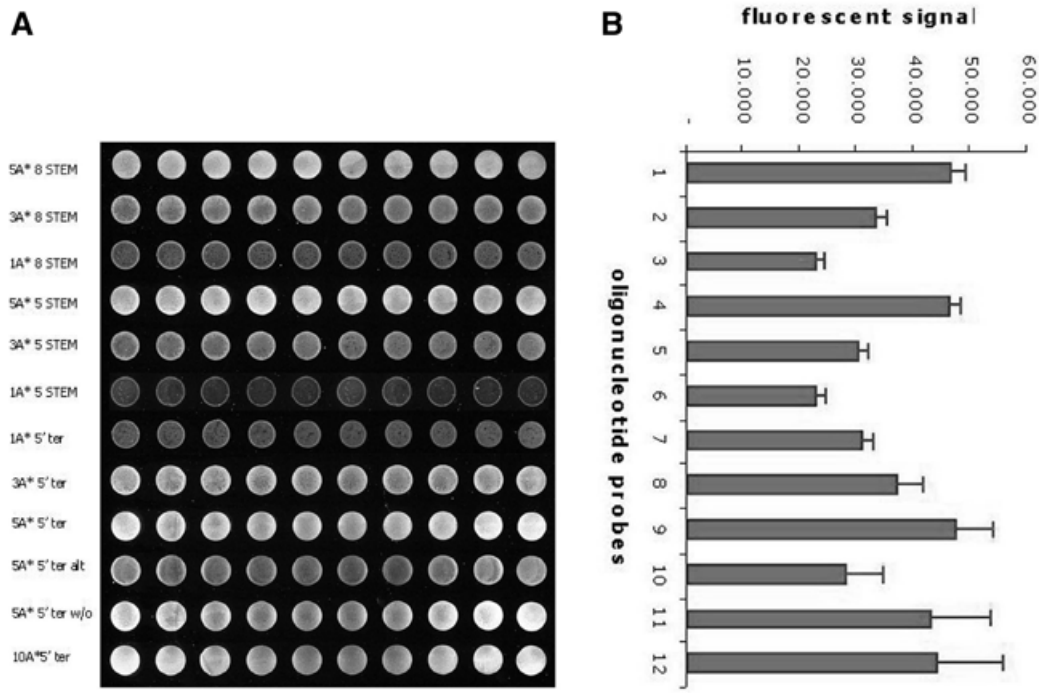

Figure 4. Solid-phase ligation reactions onto the GOPS surface with the biological sample such as 16S rRNA gene PCR product. (**A**) Spots image; (**B**) average and standard deviation graph of 40 replicates.

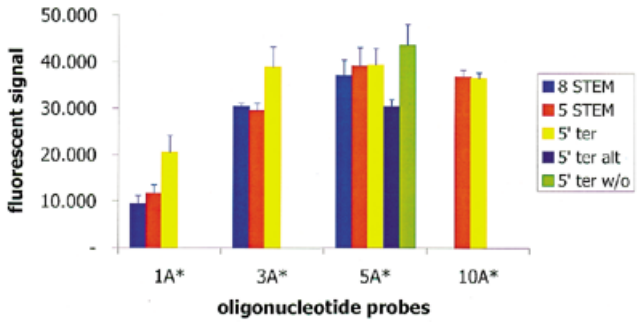

Figure 5. Comparison between oligonucleotide structures with a variable number of phosphorothioate moieties. 1A*, 3A*, 5A* and 10A* represent oligonucleotides with one, three, five and 10 phosphorothioate moieties, respectively.

to the corresponding probes with only five phosphorothioates (Fig. 5). This could be due to saturation of the bromoacetylated reactive sites on the surface, excessive crowding of probes (inhibiting the hybridisation or the ligation step) or to fluorescence quenching. Whatever the reason, five phosphorothioate moieties seem to yield a reasonable compromise between probe performance and probe cost. Alternate phosphate/phosphorothioate moieties (5A* 5′ ter-alternate) yield poorer results with respect to contiguous stretches of phosphorothioates (5A* 5′ ter) while the linear probe without polyA spacer (5A* 5′ w/o) was equivalent to it. This suggests a further simplification in oligonucleotide design and a corresponding decrease in probe cost.

As a general remark we found that the behaviour of the whole set of probes was similar on APMDES (Fig. 3A) and GOPS/poly-L-lysine (Fig. 3B) and different templates [synthetic template (Fig. 3B) or PCR-amplified 16SrRNA gene (Fig. 4)].

In conclusion, we have demonstrated the robustness of a recently proposed chemical platform for oligonucleotide microarray, extending its application to mutation detection by ligation. Furthermore, we propose some valid alternatives to the proposed strategy: a modified (GOPS/poly-L-lysine based) platform for thiophosphorylated oligonucleotide attachment and, more importantly, a simplification in oligonucleotide structure design in order to avoid expensive and unnecessary hairpin-structured probes.

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