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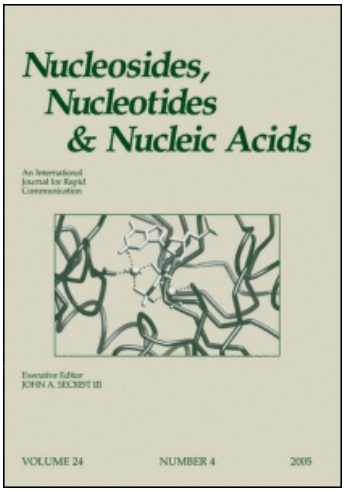

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TWO EFFICIENT POLYMERIC CHEMICAL PLATFORMS FOR OLIGONUCLEOTIDE MICROARRAY PREPARATION

Clarissa Consolandi[a]; Bianca Castiglioni[b]; Roberta Bordoni[b]; Elena Busti[a]; Cristina Battaglia[a]; Luigi Rossi Bernardi[a]; Gianluca De Bellis[b]
[a] Dipartimento di Scienze e Tecnologie Biomediche, Università degli Studi di Milano, LITA, Segrate (Mi), Italy [b] Istituto di Tecnologie Biomediche, Consiglio Nazionale delle Ricerche, Segrate (Mi), Italy

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TWO EFFICIENT POLYMERIC CHEMICAL PLATFORMS FOR OLIGONUCLEOTIDE MICROARRAY PREPARATION

Clarissa Consolandi,[1] Bianca Castiglioni,[2] Roberta Bordoni,[2] Elena Busti,[1] Cristina Battaglia,[1] Luigi Rossi Bernardi,[1] and Gianluca De Bellis[2,*]

[1]Dipartimento di Scienze e Tecnologie Biomediche, Universita' degli Studi di Milano, LITA, Via Fratelli Cervi 93, 20090 Segrate (Mi), Italy
[2]Consiglio Nazionale delle Ricerche, Istituto di Tecnologie Biomediche

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ABSTRACT

In this report we describe two robust procedures for oligonucleotide microarray preparation based on polymeric coatings. The proposed chemical approaches include: 1) a glass functionalisation step with appropriate silanes (γ-aminopropyltriethoxysilane-APTES or 3-glycidoxypropyltrimethoxysilane-GOPS), 2) a coating step using polymers (poly-L-Lysine or poly(acrylic acid-co-acrylamide) copolymer) covalently bound to the modified glass and 3) a surface activation step to allow for the attachment of amino-modified oligonucleotides. Results obtained using these chemistries in oligo microarray preparation show: 1) an overall high loading capacity and availability to hybridisation against targets, 2) a good uniformity, 3) resistance to consecutive probing/stripping cycles, 4) stability to thermal cycles, 5) effectiveness in hybridisation-mediated mutation detection procedures and 6) the possibility to perform enzymatic reactions, such as ligation.

*Corresponding author. Fax: +39-0226422770; E-mail: debellis@itba.mi.cnr.it

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Key Words: Amino-modified oligonucleotides; Oligo-microarrays; Mutation detection

INTRODUCTION

Oligonucleotide microarrays are becoming a valuable tool in modern molecular biology,[1] opening a way for large-scale screening of mutations and studies of gene polymorphisms.

They allow for massive DNA analyses in parallel with unprecedented speed. Although they can be mass produced by photolithographic techniques in situ,[2] many users prefer home-made microarrays prepared by the spotting of modified oligonucleotides.[3] This widely adopted option includes direct attachment of presynthesised oligonucleotides to an activated surface.

This approach permits a very quick set up of new microarrays and their upgrading incorporating new genetic information. This method is acceptable for low and medium density microarrays. With modern high throughput robotics, large-scale chip production by this method is well within the reach of many research laboratories.

A key step in this approach is the preparation of surfaces modified with appropriate functional groups allowing for the attachment of oligonucleotides to the solid support. Glass is the favourite support in microarray technology and bifunctional silanes of the general structure $(RO)_3Si(CH_2)_nX$ have been extensively used to modify and activate it.[4,5] Two commonly used silanes of this structure are γ-aminopropyltriethoxysilane (APTES) and 3-glycidoxypropyltrimethoxysilane (GOPS). Many other chemicals have been proposed including those compatible with 5′ thiolated oligonucleotides,[6] although in general amino-modified oligonucleotides are preferred. It has been noted that planar glass surfaces, although excellent for their chemical inertness and low fluorescent background, have a limited loading capacity, due to their surface structure.[3] Gel pads have been proposed to overcome this problem[7] as well as the use of branched linkers.[3] Similarly, chemically pre-activated microscope slides have been commercially proposed offering 3D reactive structures. Analogously, in the present study we have investigated a method for covalent attachment of amino-modified oligonucleotides to polymeric coatings acting as 3D spacers and insulators with respect to glass. Polymers are covalently bound to glass slides activated with either γ-aminopropyltriethoxysilane (APTES) and 3-glycidoxypropyltrimethoxysilane (GOPS). Silanised slides are reacted with Poly-L-Lysine (GOPS-modified glass) or with poly(Acrylic acid-co-Acrylamide) copolymer (APTES-modified glass). Polymer-coated slides are then activated by 1,4 phenylenediisothiocyanate (PDITC) for Poly-L-Lysine and ethylcarbodiimide/N-hydroxysuccinimide for poly(Acrylic acid-co-Acrylamide). In this way the attachment of amino-modified oligonucleotides is extremely stable

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and can even withstand PCR cycling conditions. These features make the method useful for many applications, among these the development of mutation detection systems based on hybridisation or ligase-mediated reactions.

MATERIALS AND METHODS

All chemicals and solvents were purchased from Sigma-Aldrich (Milan, Italy) and, unless otherwise stated, used without further purification. Pre-cleaned non-derivatised microscope slides ($25 \times 75 \times 1$ mm) were used (Sigma-Aldrich). Oligonucleotides were purchased from Interactiva Biotechnologie GmbH (Ulm, Germany) and MWG-BIOTECH AG (Ebersberg, Germany).

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Cleaning and Silanisation of Microscope Slides

Glass slides were cleaned by soaking in 1 M NaOH for two hours 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 surface treatment*. Microscope slides were immersed in 96% ethanol for 10 min and then washed three times with distilled water. Slides were immersed in acetone for 10 min, removed and dried. Then they were treated with 1% (v/v) GOPS (3-glycidoxypropylsilane) in 95% ethanol for 1 h. Excess silane was removed by dipping the slides in 95% ethanol for 1 min. Finally they were dried at 150°C for 20 min.
- *APTES surface treatment*. Microscope slides were washed in acetone for 10 min, immersed in 2% γ-methacryloyloxypropyltrimethoxysilane, 0.2% triethylamine in $CHCl_3$ for 30 min at 25°C and then washed two times with $CHCl_3$ for 15 min. The slides were immersed for 15 min in 1% 3-aminopropyltrimethoxysilane (APTES) solution in 95% acetone/distilled water. Afterwards, the slides were washed five times (5 min each) with acetone and dried.

Polymeric Coating Synthesis and Surface Activation

Poly-L-lysine Coating and Surface Activation

GOPS-treated slides were left in a PBS based solution containing 10% Poly-L-Lysine for 1 h on a shaker. Treated slides were washed five times with

distilled water, centrifuged at 500 rpm for 5 min and dried for 10 min at 45°C. The slides were then activated by immersion into a 0.2% solution of 1,4-phenilene diisothiocyanate in 90% N,N-dimethylformamide and 10% pyridine. The activation reaction was carried out at room temperature for two hours. After washing with methanol and acetone (2 min each), the activated slides were dried and stored, until use, in a dark closed box under vacuum. Derivatised slides were stable for at least two months. During this period no decrease of performance was noted.

Poly(Acrylic Acid-co-acrylamide) Coating and Surface Activation

The coating solution was prepared by copolimerisation of 0.8% acrylamide and 0.2% acrylic acid in bidistilled H_2O by heating this solution to 70°C for 30 min with 0.8% ammonium persulphate. This solution can be stored at 4°C for later use. The coating solution was activated by mixing it in a 1:1 ratio with a solution consisting of 0,1 M 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride, 20 mM N-hydroxysuccinimide in 0,1 M K_2HPO_4/KH_2PO_4 buffer (pH 6.0).

Twenty μL of this activated mixture were distributed onto a APTES-treated glass surfaces by using cover slips (24 mm × 50 mm) that had been previously silanised with Sigma-Cote (Sigma-Aldrich). The use of cover slips decreases the reagent consumption. The coating step was carried out at RT for 3 h; removal of the cover slips was performed by rinsing slides with deionized water. Treated slides were then dried and stored under vacuum until used. Derivatised slides were stable for at least two months. During this period no decrease of performance was noted.

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Covalent Attachment of Oligonucleotides onto the Activated Surfaces

Amino-modified oligonucleotides were diluted in Printing Buffer (150 mM sodium phosphate, pH 8.5). Spotting was performed using a non-contact piezo-driven dispensing system (Nanoplotter, GeSiM, Groberkmannsdorf, Germany). Alternatively, an in-house modified Hamilton 2200 robotic workstation equipped with Stealth 2 pins (Telechem International Inc., Sunnyvale, CA, USA) was used. Covalent attachment to both types of activated supports and the deactivation process were performed according to one of the following protocols:

GOPS/Poly-L-Lysine

Printed slides were placed in a saturated NaCl humidification chamber. After overnight incubation at RT, the deactivation step was carried

out by treatment with a solution consisting of 50 mM ethanolamine, 0,1 M Tris (pH 9), 0, 1% SDS at 50°C for 15 min. After rinsing twice with distilled water, the modified surfaces were immersed into a 4X SSC/0,1% SDS solution (pre-warmed to 50°C) for 15 min on a shaker. Finally, printed surfaces were washed twice with distilled water and spinned at 800 RPM for 3 min.

APTES/Poly(Acrylic Acid-co-acrylamide)

After printing, slides were incubated for 1 h at 65°C in a humidified chamber, then immersed in a solution composed of 300 mM bicine (pH 8.0), 300 mM NaCl, 0.1% SDS for 30 min at 65°C. After rinsing in distilled water deactivated slides were dried by spinning.

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Commercial Slides

The commercial slides used were 3D-Link™ (SurModics Inc., Eden Prairie, MN, USA), designed to covalently immobilise NH_2-modified oligonucleotides. They are prepared using a hydrophilic, polymeric, amine reactive coating. Amino-modified oligonucleotides were immobilised to 3D-Link™ activated slides according to the protocol given by the manufacturer, similar to the treatment employed in the GOPS/Poly-L-Lysine chemistry.

Coupling Efficiencies (Loading Capacity) of Activated Support Media

The loading capacity of the two chemically modified surfaces and of the commercial slides was tested by spotting different concentrations (10–100 μM) of a 29 mer oligonucleotide (5′-AAA AAA AAA ATT GTG GTA GTT GGA GCT GC-3′) with a 5′-amino modification and a 3′-Bodipy 650–665 fluorescent label.

Hybridisation to Oligo-array

The two novel chemistries and the commercial slides were tested in hybridisation experiments. These were performed using a 5′ Cy3-labelled oligonucleotide with a sequence complementary (5′-GCA GCT CCA ACT ACC ACA A-3′) to the covalently attached amino oligos (see above). Thirty μL of hybridisation solution consisting of the Cy3-labelled oligonucleotide (1 μM or 10 μM), 5X SSC, 0.1% SDS and 0.1 mg/mL denatured salmon sperm DNA was used to perform hybridisation under a cover slip in a

humidified chamber. The hybridisation solution was heated in a boiling water bath for 2 min and then cooled in ice before the application onto the array. Hybridisation experiments were carried out at room temperature for 3–16 h. The hybridisation time is variable depending on the length and the molecular complexity of the probes.

After incubation, slides underwent four washing steps at room temperature by using the following solutions: 4X SSC to remove the cover slip, 2X SSC/0.1% SDS for 10 min, 0.2X SSC for 1 min and 0.1X SSC for 1 min. Finally, hybridised oligo-arrays were spinned at 800 rpm for 3 min.

Ligation Reaction onto Oligo-arrays

Microarrays, prepared by spotting of four oligonucleotides (LIG 1, 2, 3, 4 each spotted 10 times in a row), were used for enzymatic ligation reactions performed in two separate steps as follows:

1) Hybridisation step: microarrays were incubated as described above with a hybridisation mixture including all synthetic templates (compl LIG 1, 2, 3, 4) and a common probe (both at 1 μM, relative sequences are shown in Table 1); then slides were washed with 2X SSC/0.1% SDS to remove the cover slips.
2) Ligation step: hybridized microarrays, were incubated at 45°C for 1 h with a ligation solution consisting of reaction buffer (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 thermostable Ligase (*Tth* DNA Ligase, Abgene, Surrey, United Kingdom). Thereafter, the microarrays were washed in boiled water for 20 min in order to remove the unreacted oligonucleotides, spinned at 800 rpm for 3 min and scanned.

Stripping Procedure

After each hybridisation, slides were stripped by incubation in boiled water for 15 min and then spinned at 800 rpm for 3 min. Each slide was checked by fluorescent scanning for complete probe removal and background detection levels were quantitated prior to the next hybridisation.

Thermal Cycling Procedure

Printed surfaces underwent a thermal cycling procedure consisting in 25 cycles of 94°C for 1 min, followed by 50°C for 1 min and 72°C for 2 min in a PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl). Microarrays were

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washed with deionized water, dried, hybridised as explained above and scanned.

Quality Control Using Cy3 Labelled $p(dT)_{10}$

Quality control of printed surfaces (on microarrays prepared using oligonucleotides bearing a 5′ $p(dA)_{10}$ tail) was performed by hybridising at RT for 3 h a solution containing 1 μM 5′ Cy3 labelled $p(dT)_{10}$. After scanning the labelled probe was stripped as described.

Signal Detection and Data Analysis

Fluorescent signals were acquired at 5-μm resolution using a ScanArray 4000 laser scanning system (Packard GSI Lumonics, Meriden, CT, USA). The Green laser was used for CY3 dye (λ_{ex} 543 nm/λ_{em} 570 nm) and the Red laser for CY5 dye and BODIPY dye (λ_{ex} 633 nm/λ_{em} 670 nm). Both the laser and photomultiplier tube (PMT) power were set to 70%.

To quantitate the fluorescent intensity of spots we used the QuantArray Quantitative Microarray Analysis software (Packard GSI Lumonics).

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RESULTS

In this study, we devised an approach to prepare glass surfaces suitable for oligonucleotide microarray preparation involving the creation of a polymeric layer, such as Poly-L-Lysine or Poly(Acrylamide-co-acrylic acid). These polymeric coatings are covalently bound to the glass surface and activated with phenyldiisothiocyanate (PDITC) or with ethylcarbodiimide/N-hydroxysuccinimide, acting as crosslinkers that allow the attachment of amino modified oligonucleotides to the solid support. The reaction schemes for the preparation of surfaces are summarised in Fig. 1 (a and b). These chemical approaches include four steps summarized as follows: 1) a glass functionalisation step with appropriate silanes, 2) a coating step using polymers covalently bound to the modified glass, 3) a surface activation step and 4) the attachment of amino-modified oligonucleotides onto the activated surface.

Overall Performance Results

The two modified surfaces and the commercial one (used as reference) were analysed before oligonucleotide spotting by laser scanning at 543 nm

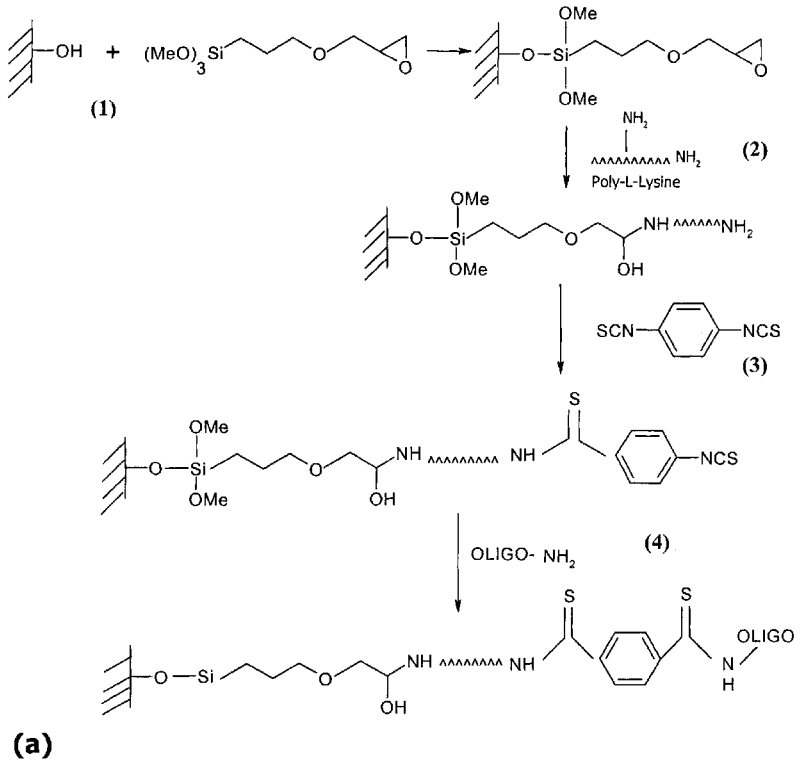

Figure 1. Chemical reaction schemes of the chemistries for covalent attachment of amino-modified oligonucleotides onto glass supports by their 5′-terminal position. a) GOPS/Poly-L-Lysine chemistry; b) APTES/Poly(Acrylamide-co-acrylic acid). The surface derivatisation requires several steps: 1) glass support silanisation, 2) three dimensional coating preparation, 3) surface activation, 4) amino-modified oligonucleotides covalent attachment.

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(suitable for Cyanine 3 dye excitation) and at 633 nm (suitable for BODIPY 630/650 dye excitation) to determine their overall fluorescent background, which was found in all cases to be very low at both wavelengths (data not shown).

For DNA microarray production, a piezo-driven dispensing system (Nanoplotter, GeSiM, Germany) was used. Typically, the spotted volume is 15–60 nL, the resulting spots being approximately 300–500 μm in diameter. We also tested a contact pin spotter with similar results (apart from the spotted volume and the spot diameter, which decreased to 150 μm). Both contact and non-contact systems performed equally well, demonstrating the mechanical stability of the polymeric coatings we propose.

We have then verified the coupling specificity of the proposed chemical platforms, their loading capacity, their efficiency in hybridization

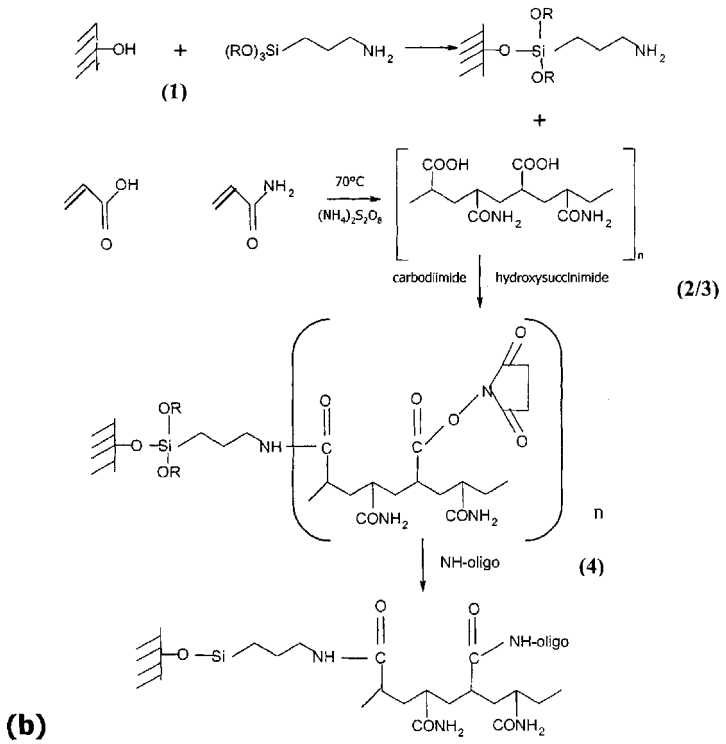

Figure 1. Continued.

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experiments, the reusability, thermal stability and finally their performance in mutation detection experiments by either hybridization and ligation assays.

For each issue, fluorescence intensities and standard deviation were calculated by average of 40 spots (10 spots in 4 different arrays on 2 separate microscope slides).

Coupling Specificity

The coupling specificity was tested by spotting 5′-amino modified and unmodified oligonucleotides bearing a 3′ fluorescent label. Either modified and unmodified probes were found to be attached onto the activated supports as well as on the 3D-Link slides, but only 5′-amino oligonucleotides were resistant to two hybridisation and stripping cycles and still accessible for probe hybridisation (data not shown).

Array Loading Capacity

We employed a 5′-amino, 3′-BODIPY 650–655 modified 29 mer oligonucleotide to ascertain the loading capacity of our surfaces, an important

issue in the microarray field. As previously demonstrated by Southern,[8] excessive crowding of oligonucleotides on surfaces can be detrimental to the successful hybridisation process for reasons related to steric hindrance and electrostatic repulsion.

To explore this issue, we compared our derivatised surfaces with the commercial slides by spotting the doubly modified oligonucleotide at different concentrations (ranging from 10 μM to 100 μM in 9 different concentrations) yielding a 10×12 array. The results shown in Fig. 2(a) show the relationship between the oligonucleotide concentration and the surface loading capacity measured as fluorescent intensity collected at 665 nm.

Efficiency of Hybridisation to Immobilised Oligonucleotides

Hybridisation experiments were performed to determine the relationship between loaded oligonucleotide concentration and hybridisation efficiency. Oligo microarrays, consisting of a 10×12 matrix obtained as described above, were hybridised with a 1 μM solution of a Cy3 labelled oligonucleotide complementary to the spotted one. Figure 2(b) depicts the relationship between the loaded oligonucleotide concentration and the hybridisation signal intensity measured as fluorescence collected at 570 nm. The same results were obtained when we performed a hybridisation reaction with a 10 μM solution of the Cy3 labelled oligonucleotide described above (data not shown).

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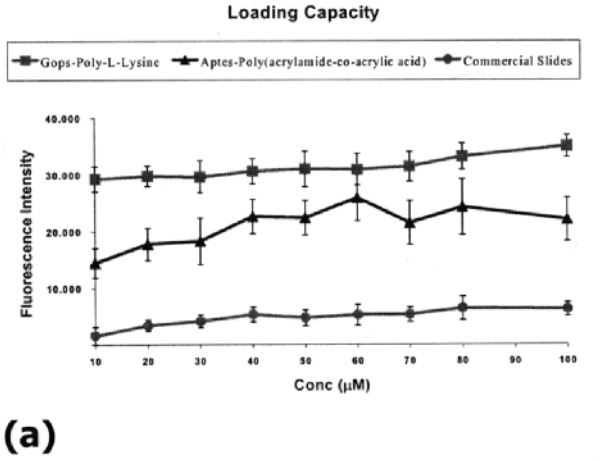
Re-usability

An important feature of a microarray is its re-usability after hybridisation. Our oligo microarrays underwent hybridisation/stripping cycles twice. Figure 2(c) shows the corresponding results.

Thermal Stability

The thermal stability of the covalent bond between the surface and oligonucleotides is an interesting feature that should be checked when a thermal cycling reaction has to be performed. To verify the chemical stability to cycling conditions, our microarrays (prepared using the usual 5′-amino, 3′-Bodipy 650–655 modified oligonucleotide) were incubated in a PCR buffer for 25 cycles. After cycling, microarrays were hybridised with the Cy3 labelled complementary oligonucleotide; the hybridisation results are shown in Fig. 2(d).



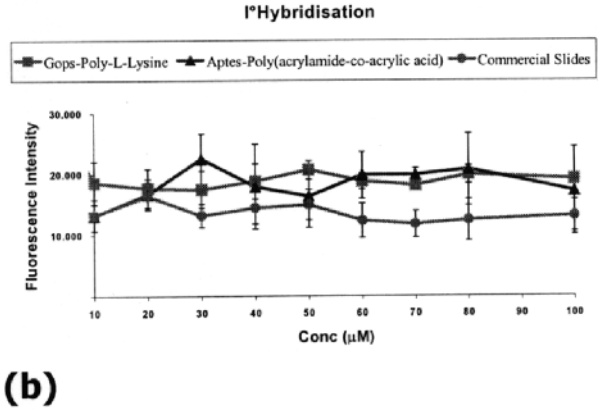


Figure 2. (a): Array loading capacity. GOPS/Poly-L-Lysine, APTES/Poly(Acrylamide-co-Acrylic acid) modified surfaces and commercial slides were spotted by using increasing concentrations from 10 to 100 μM of an 5′-aminomodified 29 mer oligonucleotide labelled with BODIPY-650 at 3′ terminal position. The sequence is 5′-AAA AAA AAA ATT GTG GTA GTT GGA GCT GC-3′. The bars represent the standard deviations. (b) and (c): Comparison of hybridisation experiments. Slides printed with different concentration of oligonucleotide (19 mer, 5′-GCA GCT CCA ACT ACC ACA A-3′) were hybridised twice with 1 μM of a Cy3 labelled oligonucleotide complementary to the spotted one. Panel (b): first hybridisation experiment; Panel (c): second round of hybridisation after stripping. Panel (d): Hybridisation after thermal cycling. Printed slides with different concentration of covalently attached oligonucleotides were hybridised with a Cy3 labelled oligonucleotide (1 μM, see Fig. 4) after 25 thermal cycles of three steps (94°C for 1 min, 50°C for 1 min and 72°C for 2 min). Fluorescence intensities and standard deviation were calculated by average of 40 spots (10 spots in 4 different arrays on 2 separate microscope slides). The bars represent the standard deviations.

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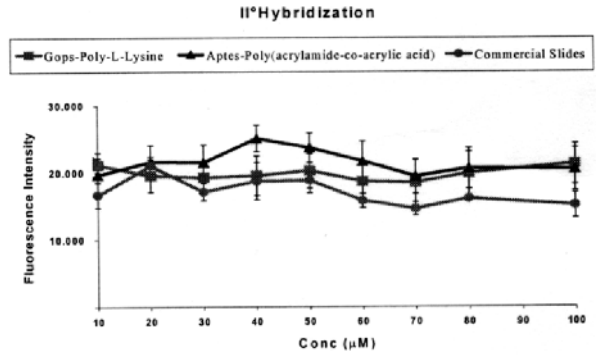
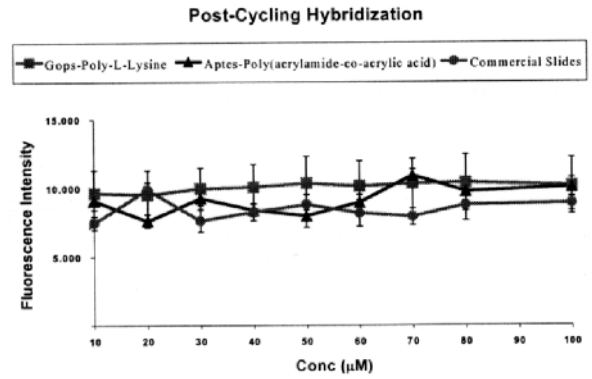


(c)



(d)

Figure 2. Continued.

Mutation Detection by Hybridisation

To explore the potential of the new surfaces in mutation detection applications we designed 4 probes defined as 2 wild-type and 2 mutated, representing two different mutations: a C insertion and a C/A point mutation (both corresponding to known BRCA-1 gene mutations).[9] These sequences are described in Table 1. Their length is variable (14–16 bp) in order to maintain homogeneous melting temperatures.

After spotting, oligonucleotide microarrays were controlled as described in methods (Fig. 3, panels a, b) to ascertain their quality. Then, in a second round of hybridization, we hybridised the same microarrays with two cy3-labelled oligonucleotides complementary to the mutated sequences

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Table 1. Oligonucleotide Sequences Used in Hybridisation and in Ligation Experiments

Oligonucleotide	Length	Sequence from 5' TO 3'	5' Modification	3' Modification
BRCA1* Hybridisation Experiments				
(1): wild type 1	25 bp	$(A)_{10}$-AGA ATC CCA GGA CAG	NH2	none
(2): mut 1	24 bp	$(A)_{10}$-AGA ATC CCC AGG AC	NH2	none
(4): wild type 2	25 bp	$(A)_{10}$-AGC TAC CCT TCC ATC	NH2	none
(3): mut 2	26 bp	$(A)_{10}$-CAG CTA ACC TTC CAT C	NH2	none
compl wild type 1	15 bp	CTG TCC TGG GAT TCT	none	Cy3
compl mut 1	14 bp	GTC CTG GGG ATT CT	none	Cy3
compl wild type 2	15 bp	GAT GGA AGG GTA GCT	none	Cy3
compl mut 2	16 bp	GAT GGA AGG TTA GCT G	none	Cy3
Ligation Experiments				
LIG 1	28 bp	$(T)_{10}$-GTG GTA GTT GGA GCT GGT	NH2	none
LIG 2	28 bp	$(T)_{10}$-TGT GGT AGT TGG AGC TGC	NH2	none
LIG 3	27 bp	$(T)_{10}$-TAG TTG GAG CTG GTG GC	NH2	none
LIG 4	28 bp	$(T)_{10}$-TCG ACA ACA GCA GGT CAA	NH2	none
compl LIG 1	32 bp	*"CAG TAG CGT AGT" ACC AGC TCC AAC TAC CAC AA	none	none
compl LIG 2	31 bp	*"CAG TAG CGT AGT" GCA GCT CCA ACT ACC ACA A	none	none
compl LIG 3	29 bp	*"CAG TAG CGT AGT" GCC ACC AGC TCC AAC TA	none	none
compl LIG 4	30 bp	*"CAG TAG CGT AGT" TTG ACC TGC TGT TGT CGA	none	none
Common probe	12 bp	ACT ACG CTA CTG	phos	Cy3

*sequence complementary to the Common probe.
BRCA1*: human breast cancer 1 gene, Accession number XM_017567.

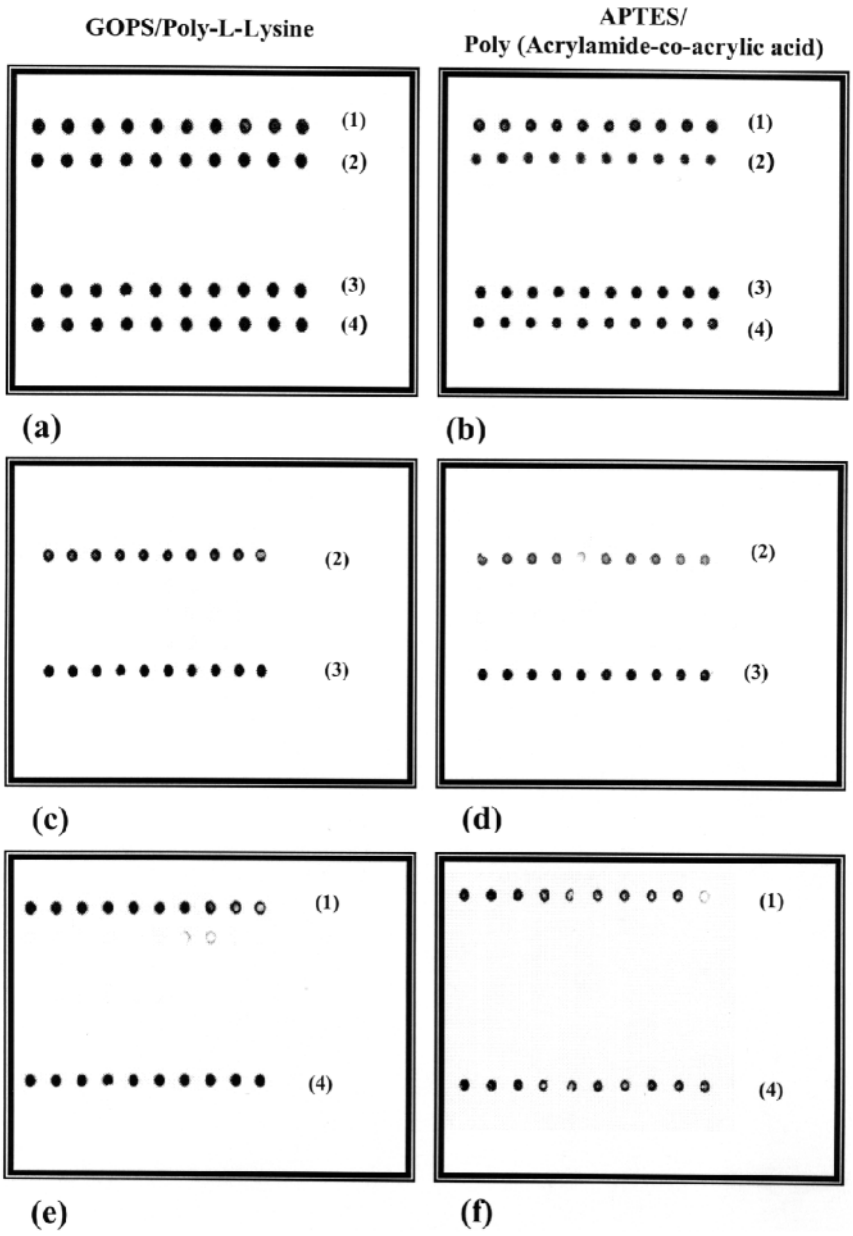

Figure 3. Mutation detection by hybridisation. Oligonucleotides sequences are described in Table 1. Each oligonucleotide was spotted ten times in a row. Panels (a), (c), (e) GOPS/Poly-L-Lysine modified surface: a) Microarray quality control by means of Cy3 labelled poly-T oligonucleotide; c) hybridisation with oligonucleotides complementary to the mutated sequences; e) hybridisation of oligonucleotides complementary to the wild-type sequences. Panels (b), (d), (f) APTES/Poly(Acrylamide-co-acrylic acid) modified surface: b) Microarray quality control by means of poly-T oligonucleotide; d) hybridisation of oligonucleotide complementary to the mutated sequences f) hybridisation of oligonucleotides complementary to the wild-type sequences.

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(Fig. 3, panels c, d). After stripping, these microarrays were hybridised with two oligonucleotides complementary to the wild-type sequences (Fig. 3, panels e, f). Figure 3 shows this successful multiple mutation detection experiment with both our chemistries. Figure 4 compares the signal intensities from the quality controlled spots and the mutation detection.

Mutation Detection by Enzymatic Reaction: Efficiency of Ligation Reaction onto the Surfaces

An alternative approach to mutation detection involves the ligation reaction. A thermostable DNA ligase catalyses the formation of a phosphodiester bond between juxtaposed 5′ phosphate of a labelled probe (common probe) and 3′ hydroxyl termini of the oligonucleotide covalently anchored to the support media (discriminating oligo), on the base of the hybridisation of both oligonucleotides to a complementary template. The ligation occurs when the common probe and the discriminating oligo are perfectly paired to a complementary template. The oligonucleotides involved in this reaction are: LIG 1, 2, 3 and 4 which are covalently attached onto the solid support media; a "common probe", bearing a phosphate group in 5′-terminal position and a fluorescent label (Cy3) in 3′-terminal position; Compl Lig 1, 2, 3, 4 which are the oligonucleotides acting as templates and complementary either to the anchored oligonucleotides and to the common probe. Their sequences are listed in Table 1. Successful results of the overall solid phase ligation procedure for both surfaces are shown in Fig. 5 (panels a, b).

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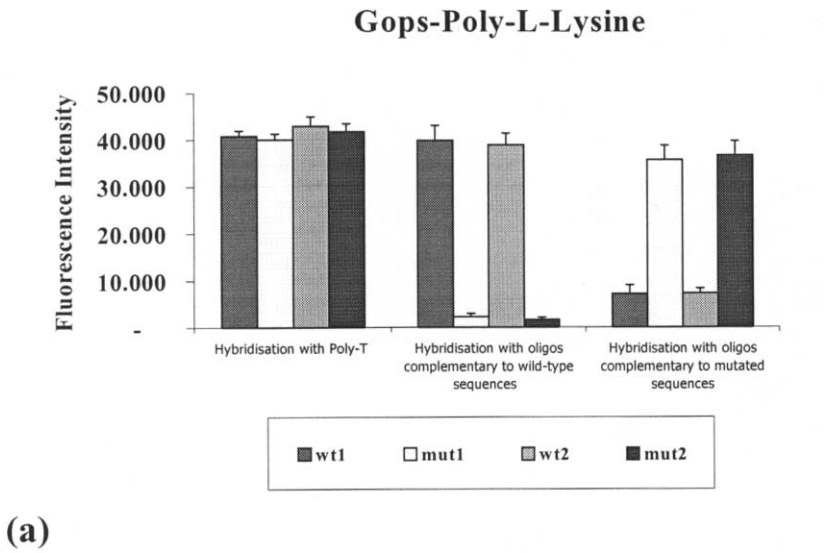
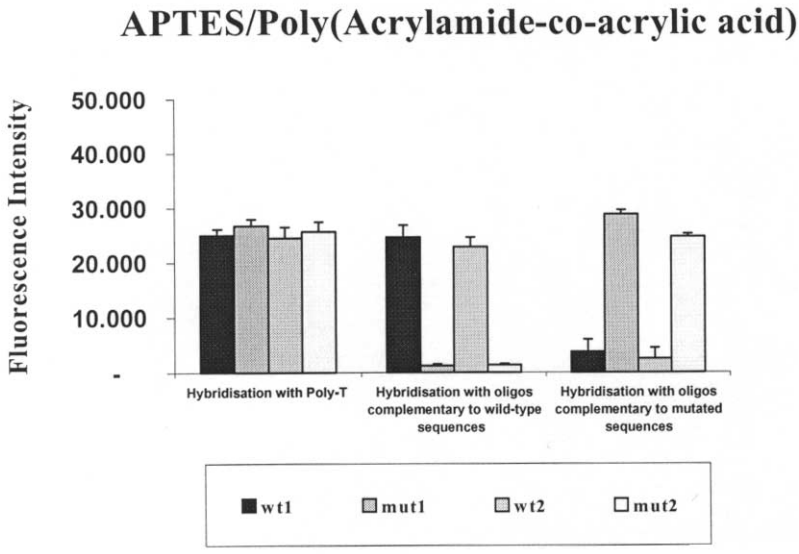
DISCUSSION

Since the advent of microarray technology several chemistries have been proposed for oligonucleotide attachment to glass surfaces.[10–13, 15] We have tested many of the simplest strategies to attach amino-modified oligonucleotides, but in our experience such chemistries are not suited for oligonucleotide microarray preparation because of their low reproducibility and selectivity for polymorphism or mutation detection. More complex strategies have been proposed, including those that incorporate polyfunctional linkers such as polyacrylamide gel pads,[7] branched chains[3] and poly(acrylic acid-co-acrylamide) copolymers generated in situ.[10] Such approaches overcome some difficulties arising from poor loading capacity of the planar glass surface due to the three-dimensional structure of the swollen polymers. Following such an approach we selected two polymers: Poly-L-Lysine and Poly(Acrylamide-co-acrylic acid) for creating a chemical platform suitable for oligonucleotide microarray application in polymorphism detection. The



(a)



(b)

Figure 4. Quantification of signal intensity after hybridisation for mutation detection. Panel (a): Quantification of hybridisation signals onto GOPS/Poly-L-Lysine modified surface. Panel (b): Quantification of hybridisation signals onto APTES/Poly(Acrylamide-co-acrylic acid) modified surface. Both graphs show on the left the results of hybridisation with poly-T, in the middle the results of hybridisation with oligonucleotides complementary to wild-type sequences and on the right the results of hybridisation with oligonucleotides complementary to mutated sequences. (See Fig. 3, Table 1). Quantification protocols are described in ''Signal detection and data analysis'' session.

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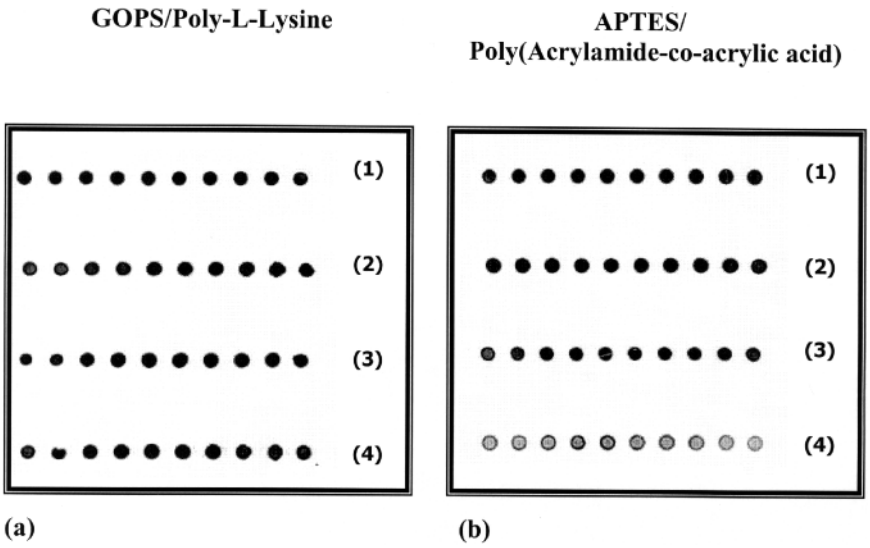

Figure 5. Mutation detection by ligation enzymatic reaction. Oligonucleotides sequences are described in Table 1. Each oligonucleotide was spotted ten times in a row. Panel (a): Results of Ligase mediated Enzymatic Reaction onto GOPS/Poly-L-Lysine modified surface. Panel (b): Results of Ligase mediated Enzymatic Reaction onto APTES/Poly(Acrylamide-co-acrylic acid) modified surface. Ligation templates and the common probe (see Table 1) were used as explained in Materials and Methods.

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polymers should have the capability of anchoring amino-modified oligonucleotides on multiple reactive sites along the chains firmly attached to the functionalized glass surface.

The amino and carboxyl moieties (in Poly-L-Lysine and Poly-(Acrylamide-co-acrylic acid) respectively) request alternative strategies for their chemical binding to the surface and for the activation toward reactive amino groups on modified oligonucleotides. These two approaches are depicted in Fig. 1.

Poly-L-Lysine has been successfully used in Pat Brown's laboratory at Stanford for cDNA microarray fabrication (http://cmgm.stanford.edu/pbrown/index.html). This widely adopted method involves the grafting of poly-L-lysine onto the glass slides. DNA fragments (usually PCR amplified material) are immobilized by UV-irradiation to form covalent bonds between the thymidine residues and the positively charged amine groups on the polylysinated slides. In our approach Poly-L-Lysine is covalently bound to a silanised glass surface (by GOPS). Then the polymer is activated with PDITC, which is acting as cross linkers that allow the specific attachment of amino modified oligonucleotides to the solid support (for further chemical details, see Fig. 1). Our experiments indicate that slides simply prepared by grafting poly-L-Lysine and subsequently activated by PDITC are not suited for the attachment of the amino-modified oligonucleotides (data not shown).

In the case of Poly(Acrylamide-co-acrylic acid), our approach is an improvement over the method developed by Gerry and co-workers.[10] In fact the copolymer described by these authors is generated in situ onto every single presilanized (methacryloyloxypropyltrimethoxysilane) glass slide. Every polymerisation reaction is performed separately. Our procedure requires the preparation of a linear poly(acrylic acid-co-acrylamide) copolymer which is stable for at least 6 months and can be used to prepare thousands of glass slides using the same starting material, thus resulting in a very robust procedure. We varied the total concentration of acrylamide monomers from 10 to 1% in order to prepare copolymers with different concentrations and molecular weights. We found that the suggested concentration (1% total acrylamide monomers, consisting of 0.8% acrylamide, 0.2% acrylic acid) is crucial for the property of the polymeric coating and should be used for microarrays fabrication (data not shown). Interestingly, the glass silanization with APTES (acting as anchoring point for the ethylcarbodiimide/N-hydroxysuccinimide activated copolymer) was less effective than the slide treatment with γ-methacryloyloxypropyltrimethoxysilane followed by APTES. Silanization of γ-methacryloyloxypropyltrimethoxysilane alone was uneffective (data not shown). This could be explained by presuming that silanized surfaces overloaded with amino groups could react with most of the activated carboxyl moieties along the copolymer.

As reported, we compared the performance of these two chemical platforms with respect to commercially available chemically activated slides (3D-Link, recently ranked among the best available supports for oligonucleotide microarray)[14] in order to characterise their behaviour and ascertain their potential in oligonucleotide microarray preparation. As shown in Fig. 2(a), the loading capacity of our anchoring chemistries is higher than the commercial one, whilst the availability to hybridisation is comparable (Fig. 2b). Hybridization efficiency is related not only to surface loading but also to probe accessibility and therefore we can hypothesize that accessibility is somehow limited. Loading capacity doesn't increase steadily with probe concentration thus suggesting that loading limits are attained within the range of probe concentrations tested. It should be noted that, although our slides are not produced in an industrial setting, we obtained similar standard deviations (Fig. 2a, 2b and 2c, calculated on 40 spots on 4 arrays and 2 slides), thus demonstrating a general surface homogeneity and the robustness of the procedure.

Moreover our surfaces demonstrated their stability after repeated cycles of probing and stripping (Fig. 2c) and even withstood thermal cycling procedures (Fig. 2d) although a 50% decrease of the signal after hybridization was found. This effect could be due to loss of probes during the cycles. This feature can be of interest to those wishing to experiment PCR and enzymatic reactions such as ligation chain reaction, cycled primer extension or minisequencing in a microarray format. We measured also the level of

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background after hybridisation and stripping (data not shown). Although this was found to be higher than the one measured on 3D-Link slides after the first round of hybridisation, we obtained similar results in term of signal-to-noise ratio after the second round of hybridization and after PCR cycling.

To assess the potential of these surfaces, we tested a mutation detection scheme based on hybridisation to detect two mutations, a C insertion and C/A point mutation. As shown in Figs. 3 and 4, we were able to discriminate both the insertion and the C/A mutation on both chemistries. Signal levels after microarray quality control using poly(dT_{10}) and after stripping and probing with properly labelled oligonucleotides further demonstrated the robustness of these platforms to repeated use (see Fig. 4a and b). Ligation reaction on anchored probes was successful as well, demonstrating the accessibility of paired nucleic acids onto these surfaces to enzymes (Fig. 5) and thus holding promise for Ligation Chain Reaction (LCR) protocols.

In conclusion, in this study we describe two robust chemical methods for covalent immobilisation of amino-modified oligonucleotides on glass surfaces based on polymeric coatings. We found comparable performances between the two proposed oligonucleotide microarray platforms in comparison to a commercially available product in terms of coupling specificity, loading capacity, and availability to hybridisation. Furthermore we demonstrated the chemical and thermal stability for our chemistries and the accessibility to ligase for enzyme-mediated reactions in solid phase showing applications in mutation detection. Our procedures are at disposal of any laboratory wishing to set-up their own DNA microarrays.

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