

The effects of glass surfaces and probe GC content on signal intensities of a 60-mer diagnostic microarray

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Abstract - The effects of glass surfaces and probe GC content on signal intensities of a 60-mer diagnostic microarray were studied. Twelve virus-specific oligonucleotide probes for severe acute respiratory syndrome coronavirus (SARS-CoV) were divided into a high GC content group ($\geq 50\%$) and a low GC content group ($< 50\%$), and spotted onto four different chemically-modified glass surfaces: a poly-amine coating activated by 1,4-phenylene diisothiocyanate (Poly-Amine surface), an acrylic acid-co-acrylamide copolymer coating activated by 1-(3-dimethylamino propyl)-3-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide (AACA-Copolymer surface), a commercial Corning CMT-GAPS amino surface, and a Telechem SuperAmine amino surface. RNA samples from cultured SARS-CoV strain were labelled using direct cDNA labelling with restriction display in a single colour format. The background-subtracted signal intensities were analysed using two-way analysis of variance. The effects of glass surfaces on background-subtracted signal intensities were significant ($p = 0.003$). Multiple comparisons showed that differences existed mainly between the AACA-Copolymer surface and the other glass surfaces, and that the AACA-Copolymer surface had the highest background-subtracted signal intensity. The probe GC content had no significant effect on signal intensities in the narrow range of GC content represented ($p = 0.07$). The results suggested that the AACA-Copolymer surface may be a novel choice of microorganism survey based on long oligonucleotide microarray.

Key words: glass surfaces, oligonucleotide probe, ANOVA, diagnostic microarray.

INTRODUCTION

DNA microarray has been developed as a high throughput screen to detect the microbial diversities of various environments. Its use has a broad applicability including medical clinics, microbial ecology, agriculture, and biodefense surveys (Zhou and Thompson, 2002; Zhou, 2003; Bodrossy *et al.*, 2004; Lemarchand *et al.*, 2004; Sergeev *et al.*, 2004). Previous studies have shown that long oligonucleotide diagnostic microarrays (50-70 mer) are more efficient than short oligonucleotide (20-30 mer) or cDNA microarrays in microbial diversity detection (Zhou, 2003). Compared to the short oligonucleotide diagnostic microarrays, the long oligonucleotide diagnostic microarrays do not require high-redundancy probe design. Compared to the cDNA diagnostic microarrays, the long oligonucleotide diagnostic microarrays have many advantages including a broader range of uniform

hybridisation conditions, lower overall costs, they are less labour intensive, and the potential for quantisation (Bodrossy *et al.*, 2004). The long oligonucleotide diagnostic microarrays have been used widely to identify microorganisms from soil (Zhou and Thompson, 2002), water (Lemarchand *et al.*, 2004), the intestinal tract (Vora *et al.*, 2004), and the respiratory tract (Wang *et al.*, 2002).

Long oligonucleotide diagnostic microarray experiments are a multi-step process and each step is a potential source of variation for signal intensities (Mo *et al.*, 2006). Variations affecting the signal intensities can be generally classified into three categories: biological variation, technical variation, and residual error. Biological variation is intrinsic to all microorganisms; it may be introduced by the genetic characteristics of microorganisms. For example, pathogens sequence divergence affected signal intensities of long oligonucleotide diagnostic microarray (Zhou, 2003; Reed *et al.*, 2007). Technical variation is introduced during microarray fabrication, sample preparation, hybridisation and washing procedure, array scanning, data analysis,

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and so on. Although many efforts have been made to develop uniform microbial separation, extraction, and purification of sample nucleic acids, sample labelling methods, and mathematical methods (Lemarchand *et al.*, 2004; Vora *et al.*, 2004; Mo *et al.*, 2006; Reed *et al.*, 2007; Tembe *et al.*, 2007), a standard protocol for long oligonucleotide diagnostic microarrays has not been well-established. In order to achieve a standardised methodology it is crucial to first evaluate the effects of the factors at each experimental step that contribute to the variability in signal intensities of long oligonucleotide diagnostic microarrays.

The glass surface and its immobilised probe are very important technical factors during microarray fabrication (Halliwell and Cass, 2001; Hessner *et al.*, 2004). Recently, two different chemically-modified glass surfaces, a poly-amine coating activated by 1,4-phenylene diisothiocyanate (Poly-Amine surface) and an acrylic acid-co-acrylamide copolymer coating activated by 1-(3-dimethylamino propyl)-3-ethylcarbodiimide hydrochloride /N-hydroxysuccinimide (AACA-Copolymer surface), have been developed for microarray fabrication (Wu *et al.*, 2005). The \bar{I} phase cDNA diagnostic microarrays experiments on the Poly-Amine surface and the AACA-Copolymer surface showed spots that were uniform and regular (Wu *et al.*, 2005). However, the immo-

bilisation of long oligonucleotide probes on these two glass surfaces has not been investigated. In this report we compared the Poly-Amine and AACA-Copolymer surfaces with the commercial Corning CMT-GAPS and Telechem SuperAmine amino surfaces, and we examined the effects of glass surfaces and probe GC content on signal intensities of a 60-mer diagnostic microarray were studied.

MATERIALS AND METHODS

Experimental design. To investigate the factors that affect signal intensities of long oligonucleotide diagnostic microarray, two different variables were chosen: probe GC content ($< 50\%$ and $\geq 50\%$) (Table 1), and glass surface (the Poly-Amine surface, the AACA-Copolymer surface, the commercial Corning CMT-GAPS amino surface and the commercial Telechem SuperAmine amino surface). Full factorial design with these two variables with different levels gave a total of eight different experimental procedures. Each set of experiments were performed in four replicates.

Preparation of glass surfaces. The Poly-Amine surface slides (Fig. 1) were prepared as follows (also described by Wu *et al.*, 2005): (1) slides were

TABLE 1 - Sequences and characteristics of SARS-CoV 60-mer oligonucleotides probes

Number	Sequences	5'-oligonucleotide terminus	GC contents
1	TCGTGAGCTCAATGGAGGTGCAGTCACTCG CTATGTCGACAACAATTTCTGTGGCCAG TGTGTGTTTGCCTATGTTGGCTGCTATAAT	771	$\geq 50\%$
2	AAGCGTGCCTACTGGGTTCCCTCGTGCTAGT ACTTCAGTCTTTACAAGTGTGCGTGCAGAC GGTTCGTACACAGGTTTATATTGCAGTCAA	1474	$< 50\%$
3	CCAGCTTCACGAGAGCTATCTGTACATT TTCCAGACTTGAATGGCGATGTAGTGGCT ATTAGTGATGAAGTTGCTCGTGATTTGTCA	3657	$< 50\%$
4	CTCCAGTTAAAAGACCAATCAACCCTACT GTGAGCTTCGTCCAGACTCGTTATGTGC TTATGGATGGTTCCATCATAAGTTTCCTA	6028	$\geq 50\%$
5	TGCTGTAGACATTAATAGTTGTGCGAGGA AATGCTCGATAACCGTGCTACTCTTCAGGC GTTTCTACAGGTTAGCTAACGAGTGTGCGC	7573	$< 50\%$
6	AAGTATTAAGTGAGATGGTCATGTGTGGCG TTTATCACCCGGAAGAAGCTATTCGTAC GTTTCGTGCGTGGATTGGCTTTGATGTAGAG	9017	$< 50\%$
7	GGTGGTTATTCTGAGGATAGGCACTCAGGT GTAAAGACTATGTCGTTGTACATGGCTAT TAGCGGAGGTGGTGAAGTGCCTCGCGC	11949	$< 50\%$
8	TATTGCTGCTAGACAGATTGAACCAGCTTGA TGCTCCAAGTGCCTCTGCATTCTTTGGAAT GTCACGCATTGGCATGGAAGTCACACCTTC	15307	$< 50\%$
9		18171	$< 50\%$
10		25811	$< 50\%$
11		28739	$\geq 50\%$
12		29028	$\geq 50\%$

silanised using $(\text{C}_2\text{H}_5\text{O})_3\text{-Si-(CH}_2)_3\text{-NH}_2$, (2) silanised slides were amidated with $\text{CH}_2=\text{CHCO-Cl}$, (3) amidated slides were coated with a solution containing several different chemical compounds with -NH_2 , (4) finally coated slides were activated by 1,4-phenylenediisothiocyanate (PDITC).

The AACA-Copolymer surface slides (Fig. 2) were prepared as described previously (Wu *et al.*, 2005). Three steps were involved in this process: (1) slides were silanised using $(\text{C}_2\text{H}_5\text{O})_3\text{-Si-(CH}_2)_3\text{-NH}_2$, (2) silanised slides were coated with acrylic acid-co-acrylamide copolymer, (3) finally coated slides were activated by 1-(3-dimethylamino propyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS).

Oligonucleotide probes design and microarray fabrication. Twelve virus-specific 60-mer oligonucleotide probes were designed using SARS-CoV genome sequence from NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>) (Accession number: AY274119) and illustrated in Table 1. These 60-mer oligonucleotide probes were designed according to published criteria (Mo *et al.*, 2006), and synthesized using 3900 DNA Synthesizer (Applied Biosystems, Foster City, USA). The oligonucleotide

probes were resuspended in 50% DMSO to a final concentration of $0.5 \mu\text{g}/\mu\text{l}$. The probes were divided into a higher GC content group ($\geq 50\%$) and a lower GC content group ($< 50\%$), and then each group was spotted from 384-well microtiter plates onto each of the different glass surfaces: the Poly-Amine surface, the AACA-Copolymer surface, the CMT-GAPS amino surface (Corning, New York, USA) and the SuperAmine amino surface (Telechem, Atlanta, USA) in a four replicate spots format using Cartesian Pixsys 5500 (Cartesian, Newton, USA). Finally, the slides were processed using BIO-RAD crosslinker (BIO-RAD, Hercules, USA).

Viral RNA isolation. SARS-CoV was cultured in safety level three laboratories in the Centre for Disease Control and Prevention (CDC) in Guangdong Province, China. Viral infections were undertaken using Vero E6 cells, which were cultured in MEM supplemented with 10% FCS and antibiotics. Viral RNA extraction was performed using Qiagen viral RNA kit (Qiagen, Valencia, USA) and then dissolved in $50 \mu\text{l}$ DEPC water. RNA quality and concentration were determined by ultraviolet spectrophotometer DU530 (Beckman, Fullerton, USA). Viral RNA ($2 \mu\text{g}$) was used in procedures described below.

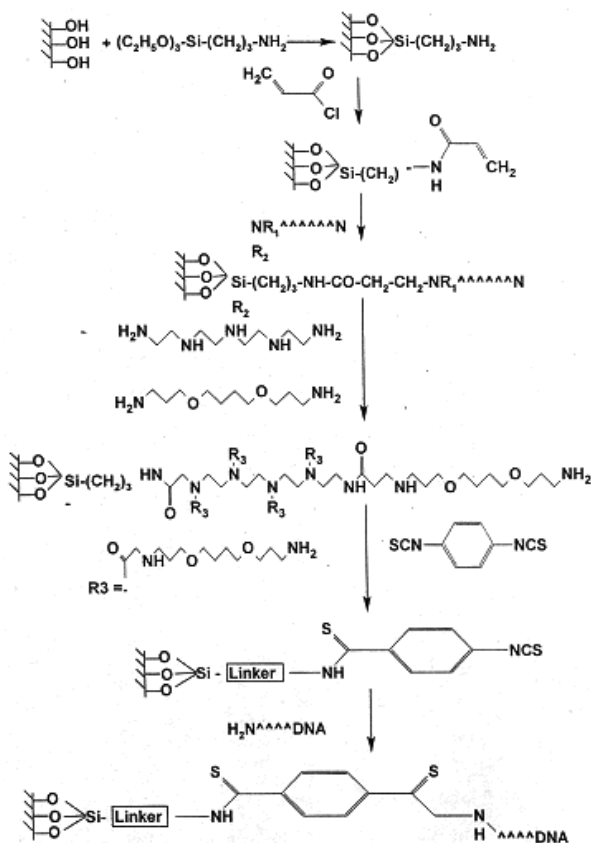


FIG. 1 - The chemical processes of the poly-amine surface. The silanised slides were coated with poly-amine, and then activated by 1,4-phenylene diisothiocyanate.

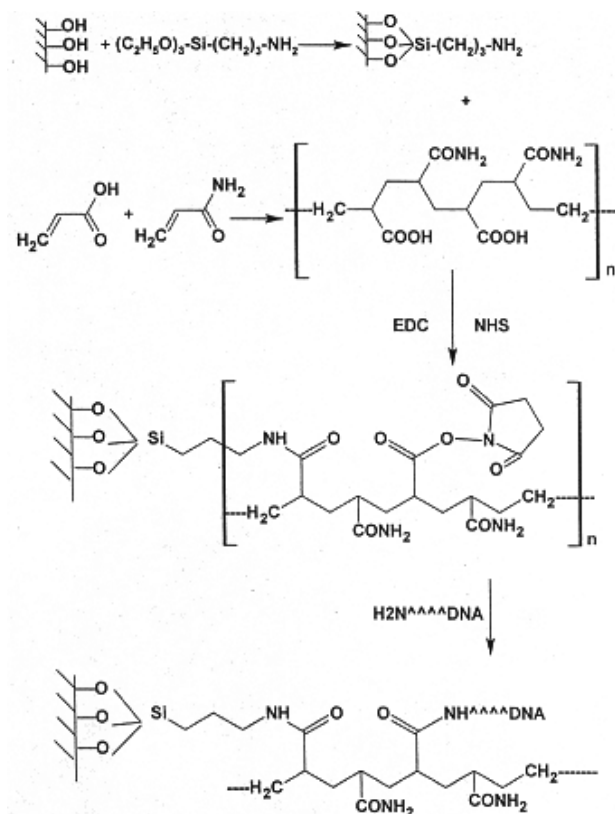


FIG. 2 - Chemical processing of the AACA-copolymer surface. The silanised slides were coated with acrylic acid-co-acrylamide copolymer, and then activated using 1-(3-dimethylamino propyl)-3-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide.

Sample labelling. Direct cDNA labelling method with RD (DL-RD) was carried out as described previously (Li *et al.*, 2003; Mo *et al.*, 2006) using TaKaRa products (TaKaRa, Shiga, Japan). The synthesis of the first cDNA chains was conducted in 20 μ l of reaction mixture containing viral RNA 2 μ g, oligo-dT 2 μ l (40-100 pmol), reverse transcriptase XL 10 U, 5x reverse transcriptase XL buffer 4 μ l, dNTP 8 μ l (2.5 M) and RNase inhibitor 5 U. The reaction mixture of the second cDNA chains contained the first reaction products 10 μ l, *E. coli* polymerase 4 μ l (3-6 U/ μ l), *E. coli* ligase 4 μ l, RNase H 1 μ l (20-60 U/ μ l), dNTP 8 μ l (2.5 M) and 10 \times second reaction buffer 5 μ l. Double-strand cDNAs (5 μ g) were digested with restriction endonucleases *Sau3A* I at 37 °C for 2 h, and linked to universal adapters using T4 DNA ligase. The Cy3-labeled universal primers (Cy3-UP) were used to amplify the linked cDNA fragments; the 20 μ l of labelling reaction mixture contained 2 μ l linked double-strand cDNA, 2 μ l of 5'-Cy3-UP, and 10 μ l 2 \times pre-mixture. PCR amplifications were performed at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min with a final extension at 72 °C for 7 min.

Hybridisation and washing. Four replicate hybridisation experiments were carried out for each set of procedure. The slides were washed at 42 °C for 25 min in a pre-hybridisation solution containing 25% formamide, 5 \times SSC, 0.1% SDS and 0.5% BSA. The labelled targets (3 μ l) were mixed with the same volume of 2 \times hybridisation solutions at 42 °C, and neutralised at 95 °C for 5 min, and then centrifuged at 14000 rpm for 2 min followed by snap-cool on ice. Three microlitres of the hybridisation mixture were applied to each pre-hybridised microarray slide. The slides were inserted in a sealed hybridisation chamber (Corning), and incubated in a water bath at 42 °C for 4 h. After hybridisation, the slides were transferred to a washing buffer I (2 \times SSC, 0.2% SDS), and washed at 42 °C for 5 min; the slides were subsequently washed in washing buffer II (0.1 \times SSC, 0.1% SDS) at room temperature for 10 min, followed by incubation in washing buffer III (0.1 \times SSC) for 5 min. Finally, the slides were dehydrated in 100% ethanol, and dried at room temperature.

Scanning and data analysis. The ScanArray Lite (Gsi Lumonics, Moorpark, USA) was used for slides scanning, and the data were collected from the scanned images using the Array-Pro Analyzer (Media Cybernetics, Silver Spring, USA).

The average background-subtracted signal intensity of each probe was calculated based on the signal intensities of the replicate spots and slides. The average background-subtracted signal intensity of each probe was considered as a random and independent variable. The SPSS program (version

11.0) was used to perform the regular tests. The coefficients of variation (CV) of the signal intensities of the probes and glass surfaces were calculated based on the following formula: $CV = \text{standard deviation} / \text{mean of background-subtracted signal intensities}$. The mean background-subtracted signal intensities were log₂-transformed. Normality of signal intensities was examined by the Kolmogorov-Smirnov test, and homogeneity of variances was examined using the Levene test. The Kruskal-Wallis test was used to examine the differences of background signal intensities among glass surfaces. Two-way analysis of variance (two-way ANOVA) was performed to test the main effect of glass surfaces, the main effect of GC contents, and interaction effect between glass surfaces and GC contents. Least significant difference (LSD) was used for multiple comparisons. Cubic curve estimation was used to examine signal intensity variations resulted from the glass surfaces. The significance level was set at $p < 0.05$.

RESULTS

The basic hybridisation characteristics of twelve 60-mer oligonucleotide probes on four glass surfaces are shown in Table 2. The average background-subtracted signal intensities on different glass surfaces indicate that the AACA-Copolymer surface gave the highest signal intensities. Except for the commercial Telechem SuperAmine amino surface, the average background-subtracted signal intensities on other three glass surfaces of the higher GC content group ($\geq 50\%$) were higher than those of the lower GC content group ($< 50\%$). Although the difference was significant ($p < 0.001$), the mean background signal intensities on the different glass surfaces were very low. With the low background signal intensities, the average background-subtracted signal intensities of the higher GC content group on the self-fabricated slides surfaces, especially the signal intensities on the AACA-Copolymer surface, were higher than those on the commercial slides surfaces. The coefficients of variation of the high GC content groups ($\geq 50\%$) were lower than those of the low GC content groups on all glass surfaces, with the Poly-Amine surface having the lowest coefficient of variation and the commercial Telechem SuperAmine amino surface having the highest one (Table 2).

The background-subtracted signal intensities of the probes showed a normal distribution and homogeneity ($p = 0.150$). Multiple comparisons indicated that the differences existed mainly between background-subtracted signal intensities on the AACA-Copolymer surface and background-subtracted signal intensities on the other three glass surfaces. The effects of glass surfaces on background-subtracted

TABLE 2 - Signal intensity comparisons with different glass surfaces and probe GC content using a 60-mer diagnostic microarray

Glass surfaces	Background signal intensities	Background-subtracted signal intensities of different GC contents groups		Total background-subtracted signal intensities; n = 12 (CV %)	p
		< 50%; n = 8 (CV %)	≥ 50%; n = 4 (CV %)		
Poly-Amine	44.39 ± 1.75	2044.54 ± 1093.52 (53.5)	3439.96 ± 1317.85 (38.3)	2509.68 ± 1306.39 (52.1)	
AACA-Copolymer	45.76 ± 1.13	10268.03 ± 8856.06 (86.3)	11410.90 ± 7660.45 (67.1)	10648.99 ± 8138.23 (76.8)	
Corning CMT-GAPS	49.50 ± 1.28	2122.89 ± 1598.51 (75.3)	3412.81 ± 1476.97 (43.3)	2552.87 ± 1619.99 (63.5)	
Telechem SuperAmine	47.65 ± 1.09	3838.87 ± 5745.04 (149.7)	3132.98 ± 1940.08 (62.0)	3603.57 ± 4706.46 (130.1)	
Total		4568.58 ± 6141.48 (134.4)	5349.16 ± 5133.52 (96.0)		0.070*
p	< 0.001 [#]			0.003*	0.981**

Twelve virus-specific oligonucleotide probes were divided into a high GC contents group (n = 4) and a low GC content group (n = 8), and spotted onto four different chemically-modified glass surfaces. All data are presented as *mean* ± *SD*. The coefficients of variation (CV) of signal intensities for the probes and glass surfaces were calculated based on the following formula: CV = standard deviation/ mean of background-subtracted signal intensities. The *p*-value ([#]) indicated local background signal intensity difference between glass surfaces and was generated by Kruskal-Wallis test. Two-way ANOVA was used to evaluate the main effects (*) and the interaction effect (**).

signal intensities were significant ($p = 0.003$), but the GC content ($p = 0.07$) and its interaction with the glass surfaces ($p = 0.981$) had marginal or no significant effect on signal intensity. Within narrow ranges of GC content represented from 38% to 53%, no substantial differences in hybridisation signal were observed due to GC bias.

Cubic curve estimation indicated glass surface variations explained 30.5% of the variation in background-subtracted signal intensities (using cubic equation: $y = 0.47 + 16.80 X - 7.07 X^2 + 0.88 X^3$, $p = 0.001$). The 95% confidence intervals for \log_2 -transformed background-subtracted signal intensities on the Poly-Amine surface, the AACA-Copolymer surface, the Corning CMT-GAPS amino surface and the Telechem SuperAmine amino surface were 8.55 to 13.60, 10.32 to 15.37, 8.56 to 13.61 and 8.55 to 13.63, respectively.

DISCUSSION

The challenges for using diagnostic microarrays are due to the multiple sources of variation as a result of differences in array fabrication, sample preparation, microarray hybridisation, image scanning, and data analysis. It has been shown that glass surfaces are a critical factor in microarray signal intensities. A factorial analysis of silanisation condition showed the number of surface-bound thiol groups are dependant on both postsilanisation thermal curing and silanisation time, and are relatively independent of silane concentration, reaction temperature, and sample pretreatment (Halliwell and Cass, 2001). Another study indicated there were consid-

erable differences in DNA retention characteristics among commercially available poly-L-lysine and amino-coated slides (Hessner *et al.*, 2004). In this paper, the effects of four different glass surfaces, the Poly-Amine surface, the AACA-Copolymer surface, the Corning CMT-GAPS amino surface, and the Telechem SuperAmine amino surface, as well as the effects of oligonucleotide probe GC content on signal intensities of a 60-mer diagnostic microarray were studied. The signal intensities clearly varied between glass surfaces, with the AACA-Copolymer surface showing the highest background-subtracted signal intensities. Within the narrow range of GC contents represented, the average signal intensities for the high GC contents group was not statistically different from that of the low GC content group. The signal intensity variations we observed were due mainly to differences among glass surfaces used in this study.

The virus-specific probes were randomly determined using the known SARS-CoV genome sequence in public database, and the virus nucleic acid targets were from cultured SARS-CoV strain. Spot replicates of each probe and slide replicates of each glass surface were applied in this study. Two-way ANOVA was used to identify the main effect of glass surfaces, the main effect of probe GC content, and their interaction effect, while other possible sources of variation due sample genetic characteristics, virus RNA extraction, sample fluorescent labelling, microarray hybridisation, image scanning, and data analysis were controlled. Unfortunately, because only twelve SARS-CoV-specific probes were fit for the criteria (Mo *et al.*, 2006), the potential limitation in this study is that investigating the influ-

ence of GC content using only twelve probes split unevenly. The fact that we see a marginally significant effect of probe GC content on signal intensities ($p = 0.07$) suggests further study with a more balanced design using more probes and in other viral species.

Many aspects of glass surfaces may produce the differences in microarray signal intensities observed in this study. The highest background-subtracted signal intensities were seen on the AACA-Copolymer surface suggesting that signal intensities differences between glass surfaces arose from the intrinsic properties of chemical modification methods themselves. First, the acrylic acid-co-acrylamide copolymers with dendrimeric structure and swellable characteristics were used as the coating agents on the AACA-Copolymer surface, which greatly increased the immobilising area for oligonucleotide probes. Second, the 1-(3-dimethylamino propyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were used as activators on the AACA-Copolymer surface. The EDC speeds the covalent linkage reactions between amino groups on the silanised glass surface and the acrylic acid-co-acrylamide copolymers. In addition to the similar function as the EDC, the NHS provided the chemical groups to link covalently with the amino groups of oligonucleotide probes.

Combined with the results from previous reports on \bar{I} phase cDNA diagnostic microarrays (Wu *et al.*, 2005), these findings indicate that self-fabricated glass surfaces (the AACA-Copolymer surface and the Poly-Amine surface) have several advantages. First, the self-fabricated glass surfaces produced low background signal intensities after hybridisation (Wu *et al.*, 2005); this is the result of repulsion between the increasing hydrophilic amines and hydrophobic dye molecules (e.g., cyanine). Second, introducing covalent bonds into poly ammoniates and/or dendrimeric copolymer system on the self-fabricated glass surfaces clearly increased the covalent linking ability and loading capacity (Wu *et al.*, 2005). Finally, the self-fabricated glass surfaces, which contain with more positive charges, were more suitable for immobilising long oligonucleotide probes.

In conclusion, the effects of glass surfaces and probe GC contents on signal intensities of a diagnostic microarray were examined in this study. The results show that glass surfaces can have a significant influence on signal intensities in diagnostic microarrays, but the probe GC content may not significantly affect intensities. The AACA-Copolymer surface, with low background signal intensity and the highest background-subtracted signal intensity, is likely to be the optimal choice for the environmental and clinical surveillance of pathogens using long oligonucleotide microarray.

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