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Use of Modified Glasses for a Microarray-based Diagnosis of *BRCA* Mutations

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Abstract

In this paper, we describe a microchip-based *BRCA* diagnosis using slide glasses modified with covalent functional groups. First, we activated pre-synthesized aminosilylated glasses with isothiocyanate (NCS) and epoxide functional groups, respectively. The two kinds of modified glasses were then evaluated for their immobilization efficiency using capture probe oligonucleotides labeled with biotin at the 3′ end, which can directly generate a fluorescence signal using the staining of the immobilized probes with streptavidin-Cy3 instead of their hybridization. The immobilization efficiencies of both the epoxide- and NCS-activated glasses were good enough to be useful as a substrate for an oligonucleotide microchip. Next, we examined their clinical utility for a reliable diagnosis of selected *BRCA* mutations through the hybridization of Cy3-labeled target DNA. All of the genotypes on the *BRCA* mutation sites were successfully identified on the two types of modified glass chips, providing reliable discrimination ratios (Qpm) between perfectly matched and mismatched probes of higher than 3.0 for all the mutation sites. This work demonstrates that both epoxide- and NCS-activated glasses can be good candidates for the preparation of a DNA chip for the diagnosis of human genetic mutations such as those found in *BRCA* genes.

Keywords: Modified glass, Oligonucleotide chip, Immobilization efficiency, Mutation detection

Introduction

Single nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide is changed in the genome sequence. The technology for an efficient SNP detection has been attracting remarkable attention, as SNPs are known to be closely related with hereditary diseases, response to drug regimens, and so on. Recently, DNA microarray technology has emerged as a general method for detecting such a single-base variation[1,2]. Up until now, various strategies for increasing the diagnostic ability of the DNA microarray have been developed, focusing on various factors that can affect its performance. One of the most important considerations influencing this performance is immobilization chemistry on the microchip substrate. Therefore, finding suitable immobilization chemistry may be critical to achieve a DNA microarray with high performance.

Generally, immobilization chemistry can be divided into non-covalent methods[3-7] and covalent methods[3-5,8-14]. A non-covalent immobilization strategy utilizing an electrostatic interaction between negatively charged DNA and a positively charged surface, such as a polylysine- or aminopropylamine-coated surface, has been commonly used for gene expression microarrays. However, non-covalently immobilized DNA probes can be detached from the surface under harsh hybridization and washing conditions, and show lower immobilization and hybridization efficiency compared to covalently immobilized probes[15]. Among the covalent coupling methods, the most commonly achieved strategy is using aldehyde-, epoxide-, or isothiocyanate (NCS)-activated glass[16,17]. These covalent couplings are considered to be stronger and more stable than the non-covalent attachment methods.

Here, we describe the use of modified glass for a microarray-based diagnosis of Korean-specific *BRCA* mutations. Mutations in *BRCA* are characterized by a predisposition to breast and ovarian cancers, as well as to prostate and other cancers. Breast cancer is the main cause of death by cancer in women, and *BRCA1* and *2* genes are reported to be related to over 50% of all breast and ovarian cancers[18]. In this study, we prepared two modified glasses, epoxide- and NCS-activated glasses, to utilize the covalent coupling strategy, and then investigated their performances for

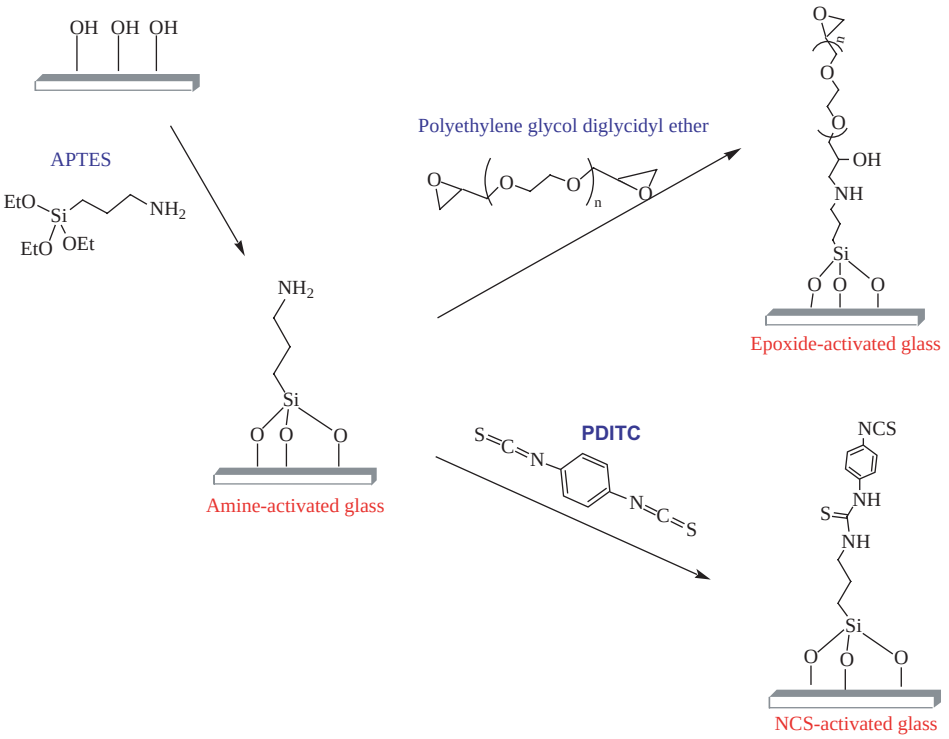

Figure 1. Preparation of epoxide- and isothiocyanate (NCS)-activated glasses.

Table 1. Capture probe sequences (a) and target probe sequences (b) employed in this study.

(a)

Probe No.	Region	Capture sequence (5′→3′)		Position	Change	Mutation effect
		Wild-type	Mutant			
1	*BRCA1* Exon11	GAGGACTCTAATTTC	AGGACTCTTAATTTC	3746	insA	1818X
2	*BRCA1* Exon11	TCCCTTGGGGTTTTC	TCCCTTGGGTTTTCA	2552	delC	814X
3	*BRCA2* Exon22	CTTACCTGAATCTTT	CTTACCTCAATCTTT	9179	C>G	S2984X
4	*BRCA2* Exon11	GTTGCTTGTTTATCA	GGTTGCTTATCACCT	3036	del ACAA	958X

(b)

Probe No.	Target sequence (5′→3′)
1	Cy3-GCCAAGAAATTAGAGTCCTCAGAAG
2	Cy3-CATTTGAAAACCCCAAGGGACTAAT
3	Cy3-AAGAAAAAGATTCAGGTAAGTATGT
4	Cy3-ACAGGTGATAAACAAGCAACCCAAG

*The bases that are complementary to the wild-type capture probe are underlined.

the application of a DNA microarray, including the immobilization capacity and discriminating ability between the perfectly matched and mismatched probes.

Results and Discussion

Coupling Chemistry

Among several immobilizing chemistries to covalently bind DNA onto a glass surface, we selected two coupling methods, epoxide- and NCS-activated glasses, as they can be easily prepared from the same amine-modified glasses, and amine-modified capture DNAs can be efficiently immobilized onto their surfaces. First, silane glasses were treated with an APTES solution for the preparation of amine-activated glass followed by a reaction between the amine groups and the corresponding linker molecules, yielding the epoxide- and NCS-activated glasses (Figure 1). Although additional procedures are needed to produce epoxide-

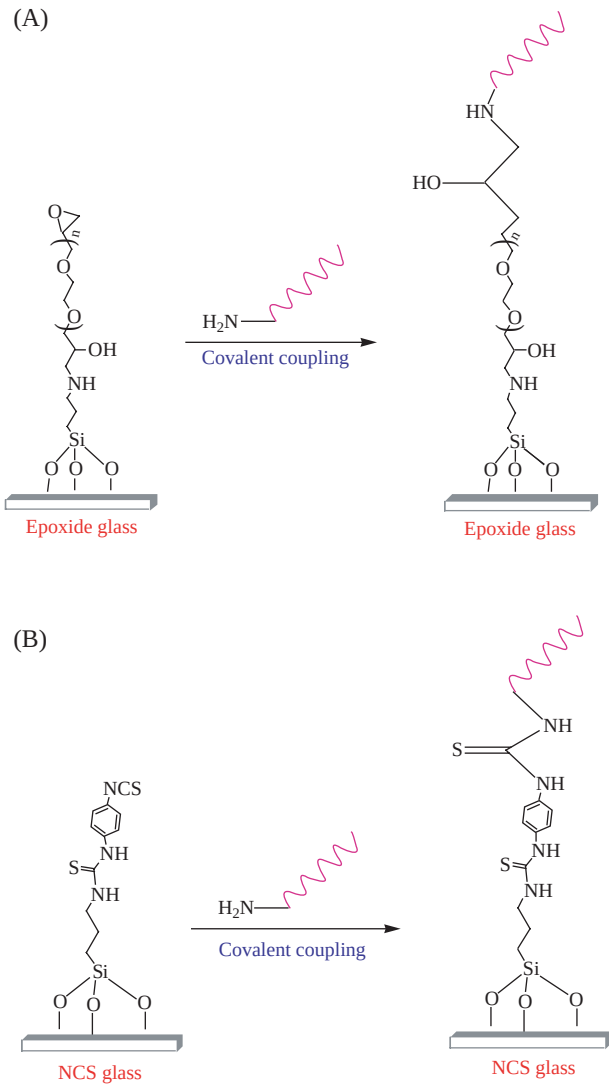

Figure 2. Immobilization strategy of capture DNA on (A) epoxide- and (B) NCS-activated glasses.

and NCS-activated glass, covalent binding methods are considered to be more advantageous due to the high chemical stability compared to non-covalent coupling methods. The amine-modified capture DNAs were covalently immobilized on the activated glasses, as represented in Figure 2.

Immobilization Efficiencies of Capture Probe DNAs

To investigate the effects of concentration of probe DNAs on efficient immobilization, 15 mer wild-type capture probes, shown in Table 1(a), were immobilized on the two activated glasses at concentrations ranging from 0.01 to 50 µM. A streptavidin-Cy3 conjugate was then applied on each glass containing immobilized

Table 2. DNA chip analysis of mutations in a *BRCA* gene.

Probe No.	Discrimination ratio (Qpm)	
	Epoxide glass	NCS glass
1	4.69	4.13
2	5.69	6.15
3	3.68	3.43
4	12.08	9.77

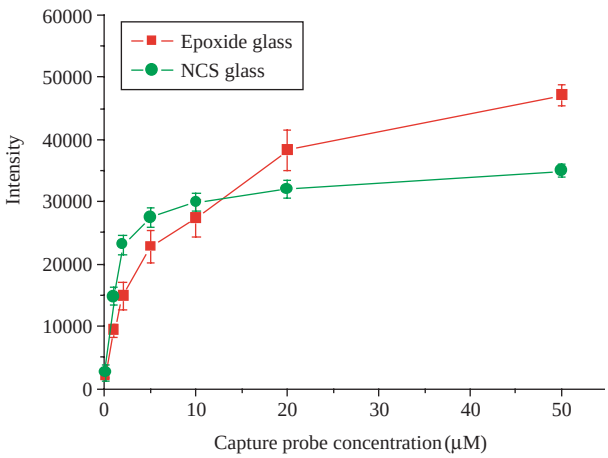

Figure 3. Immobilization capacities of epoxide- and NCS-activated glasses.

capture DNAs. As shown in Figure 3, the immobilization capacity on both of the two glasses increased as the concentration of applied oligonucleotide increased, and reached a plateau from about 10 µM. The further increase in concentration over 20 µM did not cause any significant enhancement in the immobilization capacity, indicating that 20 µM is near the saturation concentration. Therefore, in subsequent studies, the capture oligonucloetides were spotted at a concentration of 20 µM. With a 50 µM capture probe, the fluorescence intensity from the epoxide glass was about 1.5 fold higher than that from the NCS glass, but the overall immobilization capacities of the two glasses seem to be comparable to each other. Based on the results in Figure 3, both of the two modified glasses are considered to be good substrate candidates for a DNA microarray in the aspect of immobilization efficiency.

BRCA Mutation Detection on Activated Glass Microchips

To examine the clinical utility of the activated glasses for DNA chip application, four typical types of *BRCA* mutations were selected, as shown in Table 1(a). They include a single-base insertion, single-base deletion, single-base substitution, and multiple-base deletion.

To prepare the DNA chip for determining the genotypes at the four mutation sites, the capture oligonucleotides corresponding to both wild-type and mutant were spotted on the two activated glasses. On the glass DNA chips, we applied Cy3-labeled synthetic target probes (Table 1(b)), which are complementary to the wild-type capture probes, allowing them to hybridize with the capture probes on the chip. The fluorescence signals resulting from the hybridization of the target DNAs were measured, and the signal intensities obtained from the wild-type capture probes (perfectly matched) were compared to the respective intensities obtained with the corresponding mutant capture probes (mismatched). To evaluate the genotyping reliability, the discrimination ratio (Qpm) for a mutation site was defined as the ratio of the hybridization signal intensity from the perfectly matched capture probe with that of the mismatched capture probe. For a reliable determination of the mutations of the *BRCA* gene, a high Qpm value is required.

The results from both epoxide- and NCS-activated glasses are summarized in Table 2. All of the Qpm values were greater than 3, indicating a reliable identification of the genotypes for all four of the mutation sites. Very high Qpm values were obtained with the multiple-base deletion mutation compared with the other three single-base change mutations, which is very consistent with our intuition. Among the three single-base change mutations, the Qpm from single-base substitution was the lowest, but their differences were not great. Overall, the epoxide- and NCS-activated glasses showed comparable discrimination capacities for *BRCA* mutations.

Conclusion

We investigated the utility of both epoxide- and NCS-activated glasses as a substrate for a DNA microarray. The immobilization capacities of the two modified glasses were good enough to be used as a DNA microarray support. Selecting four typical *BRCA* mutations, we were able to reliably determine the genotypes at the mutation sites through a chip test on the modified glasses. Therefore, the epoxide- and NCS -activated glasses could be used as good substrates for efficient clinical chip testing for human genetic mutations including those of *BRCA* genes.

Materials and Methods

Chemicals, Glass Supports and Oligonucleotides

Saline sodium citrate (SSC) and Saline sodium phosphate EDTA (SSPE) buffers were from Sigma-Aldrich Co. (St. Louis, MO). Other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). All reaction solvents were of reagents grade from Merck (Darmstadt, Germany). Untreated glasses (26 × 76 mm) were purchased from Knittel Glaser (Braunschweig, Germany). All oligodeoxynucleotides were synthesized and purified from Genotech (Daejeon, Korea).

Modification of Glass Surfaces

Step 1: Aminosilylation of the Glasses

Glasses pre-cleaned using a piranha solution (sulfuric acid/30% hydrogen peroxide) (7 : 3) (v/v)) were immersed in a solution consisting of 1% 3-aminopropyl trimethoxysilane (APTES) in anhydrous ethanol at room temperature under constant agitation for 4 h. After silanization, the glasses were washed twice in 95% ethanol followed by three washings in distilled water. They were stirred for 5 min per wash to remove any excess silane agent.

Step 2: Preparation of Modified Glasses

Preparation of the NCS-activated glasses: Aminosilanated glasses were placed in a dichloromethane solution containing DIEA (N,N′-diisopropylethylene amine) and stirred for 30 min at room temperature. The resulting substrates were thoroughly washed with dichloromethane three times and dried in a vacuum oven for 20 min. Next, the glasses were immersed in an acetonitrile solution containing 1% 1,4-phenylene diisothiocyanate (PDITC) overnight at room temperature under gentle agitation. Then, the glasses were washed three times with dichloromethane.

Preparation of the epoxide-activated glasses: Aminosilanated glasses were placed in a 1% polyethylene glycol diglycidyl ether solution in a 10 mM sodium bicarbonate buffer (pH 8.5) for 2 h at room temperature under agitation[19]. The glasses were then washed three times with distilled water.

Immobilization Capacity Test

The wild-type capture probes shown in Table 1(a) were modified using an aminohexyl group at the 5′ end and labeled with biotin at the 3′ end. The probes were diluted with a sodium bicarbonate buffer solution (pH 9.5) and printed at concentrations from 0.01 to 50 μM on activated glasses using a Vers Array (Bio-Rad laboratory, CA), and then allowed to bind covalently to the glass surfaces in a 70% humid chamber for 6 h. To verify the reproducibility, four spots were printed for each capture probe on the same glass surface. After incubating in a humid camber, the printed glasses were washed twice with 0.2% SDS and once

with distilled water, submerged in water at 95-100˚C for 2 min, and rinsed with 0.2% SDS and distilled water for 5 min each. The efficiency of the immobilization was evaluated by staining the biotin-labeled capture probes with streptavidin-Cy3 (0.833 μg/mL)[20] followed by measuring the fluorescence signal at 532 nm.

Mutation Detection Experiment

The wild-type and mutant capture probes shown in Table 1(a), which were modified with an aminohexyl group at the 5′ end, were immobilized on activated glasses according to the same procedure described in previous section, *Immobilization Capacity Test*. 50 nM of Cy3 labeled target probes (Table 1(b)) were prepared using a 6 × SSPE (0.9 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA [pH 8.0]) buffer containing 0.1% (v/v) Triton X-100, and directly hybridized to the capture probes for 3 h at 25˚C. After hybridization, the glasses were washed once in a rotating mixer with a 3 × SSPE buffer (pH 7.4) containing 0.005% (v/v) Triton X-100 for 10 min, and then washed once with distilled water for 5 min.

Scanning and Data Analysis

The fluorescence signals of the glasses were detected using a laser scanner (GenePix 4000 A from Axon Instrument, CA). The spot intensity was quantified using GenePix Pro 3.0 software (Axon).

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