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## Invasive Cleavage Reactions on DNA-Modified Diamond Surfaces

**Abstract:** Recently developed DNA-modified diamond surfaces exhibit excellent chemical stability to high-temperature incubations in biological buffers. The stability of these surfaces is substantially greater than that of gold or silicon surfaces, using similar surface attachment chemistry. The DNA molecules attached to the diamond surfaces are accessible to enzymes and can be modified in surface enzymatic reactions. An important application of these surfaces is for surface invasive cleavage reactions, in which target DNA strands added to the solution may result in specific cleavage of surface-bound probe oligonucleotides, permitting analysis of single nucleotide polymorphisms (SNPs). Our previous work demonstrated the feasibility of performing such cleavage reactions on planar gold surfaces using PCR-amplified human genomic DNA as target. The sensitivity of detection in this earlier work was substantially limited by a lack of stability of the gold surface employed. In the present work, detection sensitivity is improved by a factor of ~100 (100 amole of DNA target compared with 10 fmole in the earlier work) by replacing the DNA-modified gold surface with a more stable DNA-modified diamond surface. © 2004 Wiley Periodicals, Inc. *Biopolymers* 73: 606–613, 2004

**Keywords:** stability; stable surfaces; DNA chips; diamond; SNPs; invasive cleavage reaction

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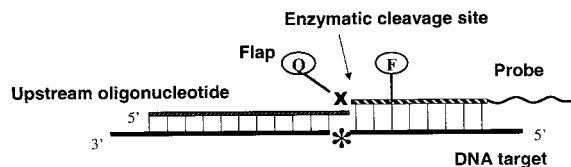
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## INTRODUCTION

In recent years, DNA microarray technology has become one of the most powerful methodologies available for genetic analysis, with applications in the areas of gene expression, genotyping of polymorphisms and mutations, DNA–protein interactions, and determination of protein structural alterations.<sup>1–4</sup> The main advantage of DNA arrays is the capability of high-throughput analysis in that samples can be queried by thousands of different surface elements in a single experiment. One dynamic area in the development of this technology is the fabrication of biologically modified substrates. These substrates should be well characterized, thermally and chemically stable, and amenable to DNA hybridization and to enzymatic manipulations of immobilized DNA molecules in reactions such as ligation, polymerase extension, invasive cleavage and restriction enzyme digestion.<sup>5,6</sup> A variety of planar substrates have been employed for this purpose, such as glass, gold, and silicon.<sup>5–9</sup> However, the degradation of these surfaces in long-term storage and under certain harsh conditions, such as high-temperature incubation, has been a persistent problem, hampering their applications in many biological analyses. A new attachment chemistry was recently developed to immobilize DNA molecules to thin film diamond surfaces.<sup>10–12</sup> Direct comparisons of these DNA-modified diamond surfaces with other commonly used surfaces, such as gold, silicon, and glassy carbon, showed that the modified diamond surfaces possessed excellent specificity and chemical stability under the conditions of repeated DNA hybridization/denaturation cycles.<sup>11,12</sup>

DNA molecules attached to diamond surfaces are accessible to enzymes, and can be modified in surface enzymatic reactions. One example is the surface invasive cleavage reaction for single nucleotide polymorphism (SNP) analysis. SNPs are the most abundant and stable type of genetic variations found in the human genome. They can be used as markers in linkage and association studies aimed at identifying and characterizing genes involved in human diseases.<sup>13–16</sup> The invasive cleavage assay is a useful tool for sensitive and specific genotyping of SNPs. A two-stage version of the solution-phase invasive cleavage reaction has sufficient sensitivity for direct detection of as few as 600 target molecules with no prior DNA amplification.<sup>17–19</sup> The reaction requires hybridization of two short DNA oligonucleotides, referred to as the upstream oligonucleotide and the probe oligonucleotide, to a target sequence (Figure 1). The probe contains two regions: a hybridization region to the target sequence and an unpaired region, referred to as a



**FIGURE 1** The invasive cleavage reaction acts on a three-piece DNA complex, consisting of an upstream oligonucleotide, a probe oligonucleotide containing a donor fluorophore (fluorescein, F) and an acceptor dye (Dabcyl, Q), and a single-stranded DNA target. The 3' terminal nucleotide of the upstream oligonucleotide overlaps (or invades) the first base pair of the downstream probe–target duplex. The probe oligonucleotide is then cleaved by a structure-specific 5' nuclease at the position indicated by the arrow. The cleavage rate is 300 times higher when the bases at positions × and \* of the probe and target sequences are complementary than when they are not. Separation of the fluorophore/quencher pair by invasive cleavage causes the fluorescence of the system to increase substantially. In the solid-phase version of this reaction, the 3' end of the probe oligonucleotide is attached to a planar surface through a series of chemical linkers (see Materials and Methods section).

“flap.” Cleavage of the probe by a class of structure-specific 5' nucleases will occur only when the 3' end of the upstream oligonucleotide invades or overlaps at least one base into the downstream duplex formed between the probe and target.<sup>20</sup> Watson–Crick base-pairing between the probe and target at the overlapping position (denoted as × and \* in Figure 1) is required for efficient enzymatic cleavage. Probes with a nucleotide complementary to the target at the position of overlap are cleaved at a rate up to 300 times greater than those with mismatched bases.<sup>21</sup> The extraordinary specificity of this assay, a product of the combination of structure-specific enzymatic recognition and sequence-specific hybridization, enables the discrimination of single base differences in the target DNA strands. The use of a thermostable 5'-nuclease allows the reaction to be performed near the melting temperature ( $T_m$ ) of the probe-target duplex, usually designed to be  $>60^\circ\text{C}$ , so that with excess probe present, one target molecule can produce multiple cleavage events, resulting in a linear accumulation of cleavage product with respect to both time and target amount. Under optimal reaction conditions in solution,  $\sim 3000$  cleaved probes can be generated per target molecule in 90 min.<sup>17</sup> This format of signal amplification is well controlled and eliminates carryover contamination, which is one of the major drawbacks of target amplification in the polymerase chain reaction (PCR) process.<sup>22–24</sup> One convenient way to monitor the reaction in a homogeneous format

is using a fluorescence resonance energy transfer (FRET) mechanism, where the fluorescence emitted by a donor fluorophore is quenched by a nearby acceptor dye on the probe sequence.<sup>18</sup> The cleavage reaction physically separates the quencher-fluorophore dye pair and generates subsequent fluorescence signal. The invasive cleavage assay has been adapted to a variety of different formats, including the use of mass spectrometry<sup>25</sup> and microparticles.<sup>26</sup> We previously proposed a very powerful approach for large-scale SNP genotyping on surface DNA microarrays using this invasive cleavage assay; we demonstrated the feasibility of performing such reaction on DNA-modified gold surfaces using PCR-amplified genomic DNA as target.<sup>27,28</sup> However, the detection sensitivity of this new approach suffered from instability of the attachment chemistry of DNA oligonucleotides to the solid substrate, particularly at elevated temperatures.

In this report, we investigate the chemical and thermal stability of the DNA-modified diamond, gold, and silicon surfaces subjected to high-temperature incubation in biological buffers. A similar attachment chemistry was used in each case to facilitate comparison of the substrates. The diamond thin film substrate was the only surface that exhibited excellent stability under the conditions employed. Invasive cleavage reactions were performed on the three surfaces. The modified diamond surfaces demonstrated not only the feasibility for performing such enzymatic reactions, but greatly improved detection sensitivity (2 orders of magnitude) due to their superior stability at the reaction temperature.

## MATERIALS AND METHODS

### Oligonucleotide Synthesis

A SNP site in codon 158 of the human ApoE gene was used as the model system. The wild-type allele has a cytosine nucleotide C at the polymorphic site (C-allele target), indicated with a capital letter in the target sequence (see below), and the mutant allele has a thymine nucleotide T (T-allele target). The corresponding probes to these two alleles are referred to as the C-allele probe and the T-allele probe, respectively. The upstream oligonucleotide (5'-ccccggcctgtactactccaggct-3') and target strands (5'-cgcgatccgatgacctgcagaag (T/C)gcctggcagtgaccaggccgggccccgca-3') were obtained polyacrylamide gel electrophoresis (PAGE) purified from Integrated DNA Technologies (Coralville, IA). The surface-bound FRET probe (5'-dabcyl-(A/G)cctt-(fluorescein-dT)tgacgtcatcgg(spacer phosphoramidite 18)<sub>10</sub>-SH-3') was synthesized at the University of Wisconsin Biotechnology Center (Madison, WI). The 5'-dabcyl phosphoramidite, fluorescein-dT, spacer phosphoramidite 18, and 3'-thiol modifier C3 S-S CPG500 used in the

synthesis were all purchased from Glen Research (Sterling, VA). A series of ten 18-atom spacer moieties, providing a total spacer length of 240 Å, were incorporated at the 3' end of the probe sequence followed by a thiol group for covalent coupling to a maleimide group present on the surface. The use of such a spacer region between an oligonucleotide and a surface is often critical to obtaining good performance in surface hybridization.<sup>29</sup> The 3' thiol-modified probe oligonucleotide was deprotected with 100 μL 100 mM dithreitol (DTT) in Tris-HCl solution (pH 8.4) for 30 min to generate free thiol groups,<sup>30</sup> followed by purification on reverse-phase binary gradient elution high-pressure liquid chromatography (HPLC) (Shimadzu SCL-6A). Oligonucleotide concentrations were determined by measuring absorbance at 260 nm with an HP8453 ultraviolet (UV)-vis spectrophotometer.

### DNA Surface Attachment Chemistry

#### Generation of Free Amine-Terminated Surfaces.

**Diamond.** Nanocrystalline diamond thin films (boron doped, 10<sup>18</sup> atoms/cm<sup>3</sup>) were deposited on silicon substrates in a microwave plasma reactor using purified H<sub>2</sub> and CH<sub>4</sub> at the Naval Research Laboratories. The surfaces were then cleaned in a series of acid baths (3:1 HCl and HNO<sub>3</sub> diluted 1:1 with distilled water, and 3:2 H<sub>2</sub>SO<sub>4</sub> and HNO<sub>3</sub>) to remove metals and amorphous carbon. Immediately before each use, the surfaces were heated to ~850°C in a 13.56-MHz inductively coupled hydrogen plasma (15 Torr H<sub>2</sub>) for 20 min to generate a hydrogen-terminated surface. In a nitrogen-purged reaction chamber, these H-terminated diamond surfaces were photochemically reacted with trifluoroacetamide-protected 10-aminodec-1-ene (TFAAD, ~5 μL per slide, covered with quartz window) for 15 hours under the illumination of a 254-nm low-pressure mercury lamp (UVP, Upland, CA). The removal of the protecting group was accomplished by using a solution of 0.36 M HCl in methanol at 65°C for 24 h, leaving behind a primary amine.<sup>11,12</sup>

**Gold.** The gold-coated glass substrates (GenTel, Madison, WI) were immersed in 1 mM alkanethiol 11-mercaptoundecylamine (MUAM; Dojindo Laboratories, Japan) in ethanol for at least 18 h to form a self-assembled monolayer (SAM) via gold-sulfur chemisorption.<sup>31</sup>

**Silicon.** Si(111) (Virginia Semiconductor, Martinsville, VA) surfaces were ultrasonically cleaned in acetone followed by methanol (5 min each), and then oxidized with a 1:1:5 hydrogen peroxide-ammonium hydroxide-water solution and a 1:1:5 hydrogen peroxide-hydrochloric acid-water solution (10 min each at 80°C). The oxidized samples were then immersed in NH<sub>4</sub>F solution (40% by weight) for 5 min to produce hydrogen-terminated Si(111) surfaces. The photochemical functionalization of these H-terminated surfaces was accomplished by covering the surfaces with *t*-butyloxycarbonyl (*t*-BOC)-protected aminoalkene (10-amino-1-ene) using quartz windows, and illuminating with 254 nm UV light for ~2 h in a nitrogen-purged reaction chamber. 50% trifluoroacetic acid was used to remove the

protecting group and generate free amine terminated surfaces.<sup>7,32</sup>

**Attachment of DNA Oligonucleotides to the Free Amine-Terminated Surfaces.** The resultant primary amine groups on surfaces were covalently reacted with 1 mM (pH 7.0) heterobifunctional cross-linker sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC) (Pierce), creating a thiol-reactive, maleimide-terminated surface that can covalently interact with thiol-modified DNA oligonucleotide. 0.5  $\mu$ L of 1 mM thiol-modified probe oligonucleotides was deposited at discrete locations on this maleimide-terminated surface. The surface attachment reaction was permitted to occur for  $\sim$ 20 h in a humid chamber at room temperature. Afterward, the surfaces were soaked in  $2\times$  SSPE (0.02 M phosphate buffer containing 0.3 M NaCl and 0.002 M EDTA)/0.2%SDS (pH 7.4) at 37°C for 1 h to remove nonspecifically bound DNA molecules.<sup>31</sup>

### Stability Test

To study the stability of DNA-modified diamond, gold, and silicon surfaces for a variety of enzymatic reactions, the three surfaces modified with the probe oligonucleotide (fluorescein-dabcyl labeled) were subjected to incubation in  $2\times$  SSPE/0.2%SDS buffer at three different temperatures; 37°C, at which exonuclease digestion and some polymerase reactions are performed; 45°C, at which ampligase ligation is performed; and 60°C, at which the invasive cleavage reaction is performed. The fluorescence intensity on the surfaces was recorded at 1-h intervals over a 9-h period with a FluorImager 575 scanner (Molecular Dynamics, Sunnyvale, CA).

### Surface Invasive Cleavage Reaction

The 20- $\mu$ L reaction solution contained 10 mM MOPS (pH 7.5), 7.5 mM MgCl<sub>2</sub>, 0.25  $\mu$ M upstream oligonucleotide, 1000 ng *Afu* FEN 1 (commercially available in the Factor V Leiden RUO Kit from Third Wave Technologies, Madison, WI), and 100 fmole or 100 amole synthetic target DNA. The surfaces were fully covered by the 20- $\mu$ L reaction mix, and incubated at 58.5°C for up to 10 h in a humid chamber. The surface fluorescence was measured both before and after the reaction.

## RESULTS AND DISCUSSION

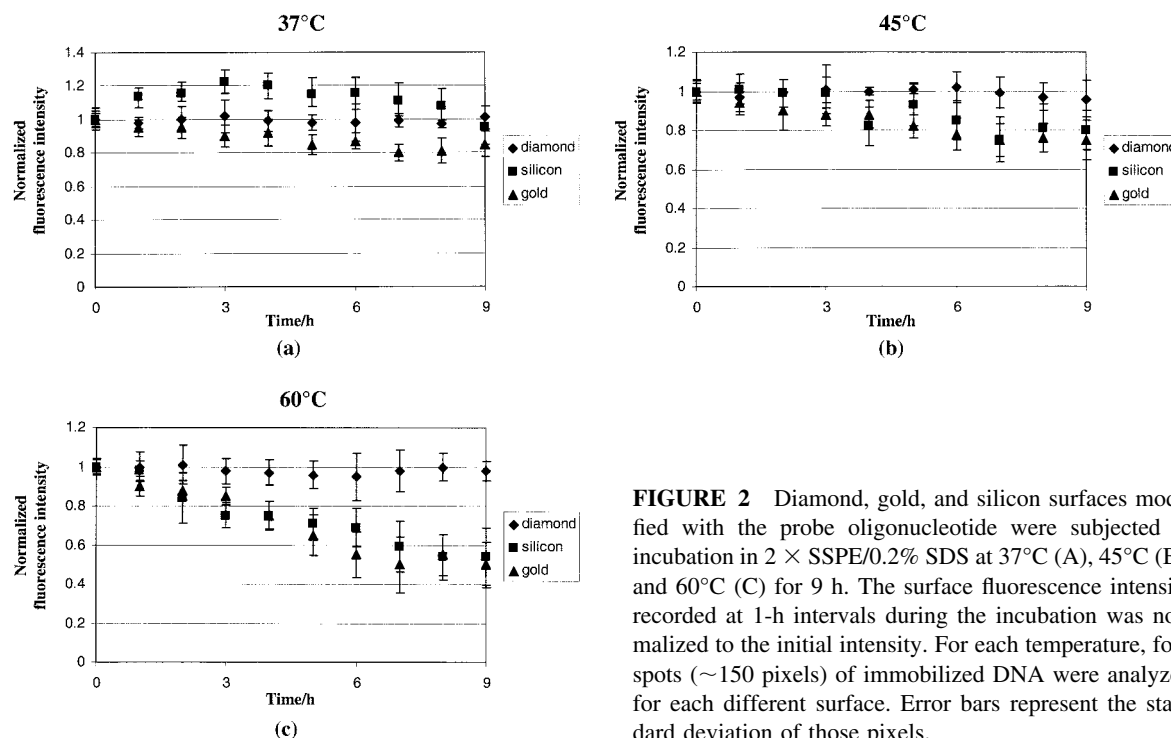
### Chemical Stability to High-temperature Incubation

Many enzymatic manipulations of surface-immobilized DNA oligonucleotides require a higher operating temperature than room temperature, such as deep vent polymerase extension at 37°C, ligation using

ampligase at 45°C and the invasive cleavage reactions at 60–63°C. Therefore, the performance of the surface arrays at elevated temperatures is critical for these types of biological assays. We investigated the chemical stability of DNA-modified diamond, gold, and silicon surfaces under the conditions of incubation in biological buffers at a broad range of elevated temperatures for a period of 9 h. The biological buffer used in these studies was  $2\times$  SSPE/0.2%SDS, which is one of the most commonly used buffers in the process of hybridization. The immobilized DNA probe oligonucleotides were fluorescently labeled, and the surface stability was interpreted in terms of the stability of the surface fluorescence.

The fluorescence intensities of the surface-bound probes recorded during the incubation were normalized to their initial fluorescence intensities,  $585 \pm 65$  relative fluorescence units (RFU) on diamond,  $622 \pm 58$  RFU on gold, and  $598 \pm 51$  RFU on silicon. The fluorescence intensity on the gold and silicon surfaces was much less stable than that on the diamond surfaces (Figure 2). At the end of the 9-h experiments, the signal on the gold surface dropped approximately 15% at 37°C, 25% at 45°C, and 50% at 60°C, whereas the signal on the silicon surface dropped 5% at 37°C, 20% at 45°C, and 46% at 60°C. The loss of surface fluorescence is largely due to the loss of surface-bound DNA probes (as determined by a previously described method,<sup>12</sup> Dr. Dora Bodlaki, unpublished data). Both gold and silicon surfaces showed greater loss of fluorescence signal at higher temperature. In contrast, the modified diamond surfaces exhibited excellent stability at all three temperatures. For the diamond substrates, the surface fluorescence signal only fluctuated around the same intensity level during the 9-h incubation, and nearly 100% of signal remained at the end of the incubation at 37°C, 96% at 45°C, and 98% at 60°C.

Since identical chemistry was used to attach the thiol-modified DNA oligonucleotides to all three amine-terminated surfaces, and as the amine-terminated chemical linkers employed are all similar, the difference in surface stability is very likely to be related to the bonds coupling the chemical linkers to the starting solid substrates (a C—C bond for diamond, a Si—C bond for silicon and a Au—S bond for gold). It is widely accepted that gold–sulfur bonds are highly susceptible to oxidation.<sup>33–34</sup> The oxidation of the thiolate species into an alkylsulfonate is severe particularly at high temperature, and eventually leads to partial desorption of the organic phase from the gold surface. In addition, the increased mobility of the alkane-thiols that form the SAM on gold surfaces at higher temperature might also disrupt the previously



**FIGURE 2** Diamond, gold, and silicon surfaces modified with the probe oligonucleotide were subjected to incubation in  $2 \times$  SSPE/0.2% SDS at 37°C (A), 45°C (B), and 60°C (C) for 9 h. The surface fluorescence intensity recorded at 1-h intervals during the incubation was normalized to the initial intensity. For each temperature, four spots ( $\sim 150$  pixels) of immobilized DNA were analyzed for each different surface. Error bars represent the standard deviation of those pixels.

highly ordered packing.<sup>34</sup> Similarly, the degradation of the modified silicon surface may start from the oxidation of silicon atoms on the surface, particularly under basic conditions,<sup>35–38</sup> resulting in subsequent hydrolysis and the loss of surface-bound DNA molecules. Methods to achieve greater stability for the gold and the silicon surfaces are currently under investigation. The DNA-modified diamond surfaces employed for the present study have extremely stable C—C bonds linking the solid substrate and the alkyl monolayer, and the diamond itself is highly stable at elevated temperatures. These DNA-modified diamond surfaces show excellent specificity and selectivity in hybridization<sup>11,12</sup> and enzymatic manipulation of the immobilized DNA oligonucleotides, as discussed below.

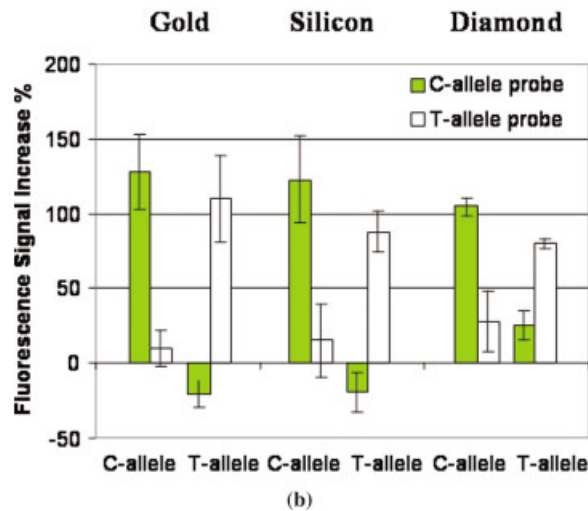
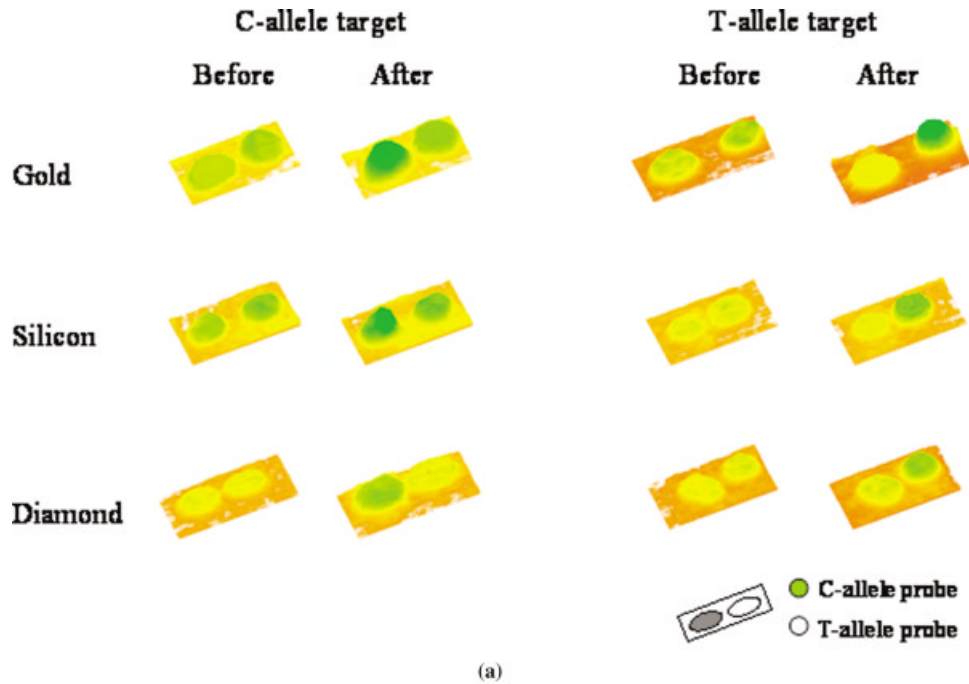
### Surface Invasive Cleavage Reactions

The excellent stability of the DNA-modified diamond surfaces at elevated temperatures makes them very attractive candidates for enzymatic assays which require such high operating temperatures. To be employed in such enzymatic reactions, the surfaces must be compatible with the biological environment, and the surface-bound DNA probe oligonucleotides must be accessible to the enzyme. To demonstrate the feasibility of performing enzy-

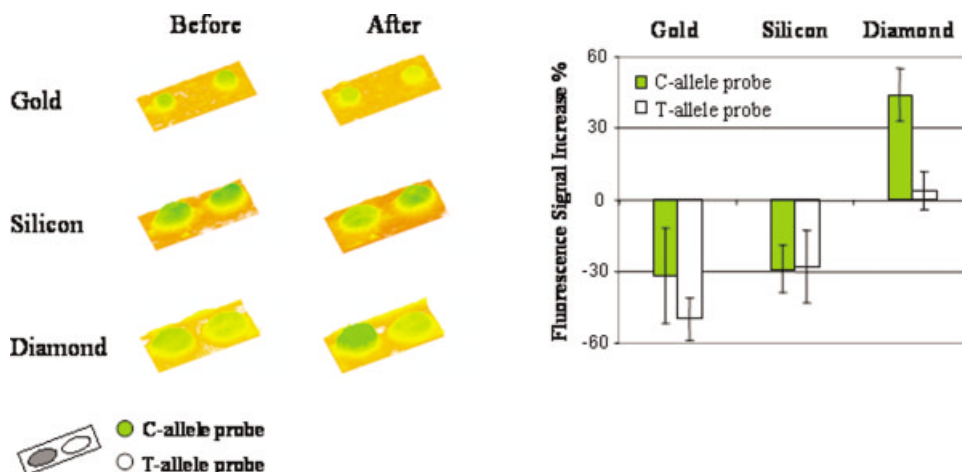
matic assays on this newly developed diamond surface, we conducted the invasive cleavage reaction on the diamond surface in a format for SNP analysis, and directly compared the results with those obtained on gold and silicon surfaces. Figure 3 shows the results obtained using three surfaces to which both the C-allele probe and the T-allele probe were attached. In these 3-h reactions, 100 fmole of either the T-allele or the C-allele target was used. The appropriate results were observed across all three surfaces, with the T-allele or C-allele target yielding significant fluorescence signal increase only for the corresponding probe oligonucleotides (T-allele probe or C-allele probe, respectively). These results demonstrate that the DNA-modified diamond surface is suitable for performing the invasive cleavage reaction while retaining the reaction specificity.

### Improved Detection Sensitivity With a More Stable Surface

One important aspect of the solution phase invasive cleavage assay is genotyping SNPs directly from genomic DNA without the requirement for prior PCR amplification.<sup>17,18</sup> A first step in pursuing this goal in the surface assay is to detect a reasonable amount of



**FIGURE 3** (A) Fluorescence images of DNA-modified diamond, gold, and silicon surfaces both before and after surface invasive cleavage reactions. All three surfaces exhibited excellent specificity for potential single nucleotide polymorphism (SNP) analysis. With 100 fmole T-allele target and incubation at 58.5°C for 3 h, the fluorescence intensity of the T-allele probe increased, on average, 80% on diamond, 110% on gold, and 88% on silicon surfaces. No significant signal increase was observed from the noncomplementary probe oligonucleotides. Similarly, with 100-fmole C-allele target, the fluorescence intensity of the C-allele probe increased 105% on diamond, 128% on gold, and 123% on silicon surfaces. (B) Histogram shows the fluorescence intensity changes of the surfaces (gold, silicon, and diamond, as indicated at the top) shown in (A), where gray columns represent the changes in the C-allele probe and white columns for the T-allele probe. Targets used are indicated on the x-axis. Approximately 40 pixels from each DNA spot were analyzed for each reaction on the three different surfaces. Error bars represent the standard deviation of those pixels.



**FIGURE 4** Increased detection sensitivity for the surface invasive cleavage reaction (100 amole target) was achieved on DNA-modified diamond surfaces. With 100 amole C-allele target and incubation at 58.5°C for 10 h, the fluorescence intensity for both the C-allele probe (the correct probe) and the T-allele probe (the wrong probe) decreased, on gold (32% and 50%, respectively) and silicon surfaces (29% and 28%, respectively). These results are therefore inconclusive. In contrast, a clear differentiation of the same two probes on diamond surfaces was observed with a 45% increase for the correct probe and a 4% increase for the wrong one. The percentage signal change for each surface is shown in the corresponding histogram. Approximately 40 pixels from each DNA spot were analyzed for the three different surfaces. Error bars represent the standard deviation of those pixels.

target, for example, 100 amole, which is equivalent to the amount of genomic DNA extracted from ~10 mL whole blood.<sup>39</sup> However, the detection sensitivity in our previous study<sup>27,28</sup> was limited by the long-term degradation of the DNA-modified surfaces at elevated temperature. At relatively high target concentration, a very short reaction time can generate enough signal increase for detection (Figure 3), whereas at low target concentration, as is the case for the analysis of genomic DNA samples, much longer reaction times are needed as the accumulation of the cleavage product, or the signal generation increases linearly as a function of time.<sup>17</sup> The ability of the surface to withstand incubation in the reaction buffer for a lengthy period is thus essential. Figure 4 shows the results of surface invasive cleavage reactions in which 100 amole C-allele target was employed, and the reaction was allowed to proceed for 10 h. The results obtained on gold and silicon surfaces were inconclusive for such low concentrations and long incubations as the signal obtained for both the T-allele probe and the C-allele probe decreased to a similar degree. In contrast, with the DNA-modified diamond surfaces, clear and correct results were obtained from 100 amole of synthetic target, which is a hundred-fold less target than that required for the previous work on gold surfaces.<sup>27</sup>

## CONCLUSION

In the present work, a newly developed DNA-modified diamond surface showed excellent chemical stability compared with modified gold and silicon surfaces using similar attachment chemistry under the high-temperature (60°C) incubation conditions employed. It was demonstrated that the DNA-modified surface is compatible with the conditions of surface invasive cleavage reactions yielding single base cleavage discrimination. The superior stability of the diamond surface and the resultant ability to employ a long reaction time resulted in a hundred-fold increase in detection sensitivity.

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