Quantification of 5-Methylcytosine in DNA by the Chloroacetaldehyde Reaction

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ABSTRACT

The study of changes in genome-wide levels of DNA methylation has become a key focus for understanding the epigenetic regulation of gene expression. Many procedures exist to study DNA methylation, falling into two categories: gene-specific and genome-wide. Genome-wide methylation analysis is best performed by DNA hydrolysis followed by HPLC; however, it requires access to an HPLC machine, which is not always available. Alternative procedures, such as the radioactive labeling of CpG sites using Sss*I DNA methyltransferase, have been developed to address this problem, but it can only monitor CpG methylation changes, and CpNpG methylation is not detected. Here, we present a method for the analysis of DNA methylation in any sequence context by fluorescent labeling. We present control analyses using synthetic oligonucleotides of known methylation levels and a comparison of genomic DNA from two transgenic tobacco lines known to differ in their methylation levels. The results indicate that hygromycin-induced hypermethylation acts equally on all classes of methylatable cytosine, perhaps indicating a common mechanism.*

INTRODUCTION

Current procedures for genome-wide methylation analysis are often time-consuming or expensive to perform. The classical procedure uses DNA hydrolysis coupled to reverse-phase (RP)-HPLC (4,7,17). However, not every laboratory has access to an HPLC machine, so alternative procedures are required. The most common of these uses the bacterial enzyme *Sss*I DNA methyltransferase to transfer tritium-labeled methyl groups from *S*-adenosylmethionine (SAM) to unmethylated cytosines in CpG sites (18). The amount of tritium incorporated is inversely proportional to the level of CpG methylation (14,18).

We report a procedure for quantifying DNA methylation by combining the sodium bisulphite reaction (5,11) with the ability of chloroacetaldehyde to fluorescently label DNA. Sodium bisulphite catalyzes the deamination of cytosine into uracil (50°C, pH 5.0 for 4 h) (11) with negligible m5C deamination (Figure 1A). Chloroacetaldehyde reacts quantitatively with the cytosine and adenine bases to give the fluorescent adducts ethenocytosine and ethenoadenine, respectively (Figure 1B) (1). Uracil, thymine and guanine do not undergo this reaction (1). DNA is rapidly depurinated under acidic conditions (15), so we can remove any adenine with acid and any cytosine with bisulphite. The extent of fluorescence after reaction with chloroacetaldehyde will be proportional to the amount of m5C in the genome.

MATERIALS AND METHODS

Oligonucleotides Used

Oligonucleotides with the basic sequence, 5′-GXTTXGTGXTGGTXXT-TGTGXTGTXT-3′ were synthesized, in which X = C (C-oligo); X = 5-methylcytosine (m5C-oligo); X = U (GTU-oligo) and X = A (A-oligo). The oligonucleotides C-oligo and A-oligo were produced in-house (Friedrich Miescher Institute, Basel, Switzerland), and the m5C-oligo and GTU-oligo were produced by Microsynth AG (Balgach, Switzerland) to test the specificity of the reaction.

Origin of the Genomic DNA

Transgenic *Nicotiana tabacum* var. SR1 seeds containing the *GUS-HPTII* construct pAT2 (6) were germinated and cultured in liquid Linsmaier and Skoog (LS) medium (Sigma, Buchs, Switzerland) (9,16). The plants were subdivided into two groups: those cultured in LS medium (Hyg-0) and those cultured in LS medium supplemented with 20 mg/L hygromycin B (Hyg-20) (Calbiochem-Novabiochem, San Diego, CA, USA). Full details of growth conditions and DNA isolation may be found in Reference 14. This earlier paper had established that tobacco plants grown in the presence of 20 mg/L hygromycin were subject to genome-wide DNA hypermethylation (14), making it a good control for our assay.

Preparation of the Bisulphite Solution

We mixed 1.9 g of sodium metabisulphite ($Na_2S_2O_4$; Fluka AG, Buchs, Switzerland) with 2 mL sterile water and 0.7 mL 2 M NaOH by inversion for 20 min (not all of the bisulphite will dissolve at this point). At the same time, 350 mg of hydroquinone (Fluka AG) was mixed with 1 mL sterile water to give a saturated solution (not all of the hydroquinone will dissolve). Then, 500 µL of the hydroquinone was added to the bisulphite solution and mixed further for 5 min. The pH was checked and adjusted to 5.0 with 2 M NaOH. The final volume was then adjusted to 4 mL with sterile water. This solution contains a free bisulphite concentration of 5 M.

Bisulphite Reaction on Genomic DNA

We then mixed 10 µg of NaOH-denatured, *Eco*RI-digested genomic DNA with 200 µL of fresh bisulphite solution and incubated it for 4 h, essentially following the protocol of Raizis et al. (11), except that a DNA Thermal Cycler (PE Biosystems, Foster City, CA, USA), rather than in a water bath, was used to regulate sample temperature.

*Sss*I Methyl-Accepting Assay

Performed as described by Schmitt et al. (14), this is a modification of the methyl-accepting assay of Wu et al. (18) and uses 300 ng of phenol:chloroform-extracted, *Eco*RI-digested tobacco genomic DNA.

Citrate Buffer

A 2× citrate-buffer solution (pH 3.5) was prepared by dissolving 43 g/L $Na_2HPO_4 \cdot 12\ H_2O$ and 28.4 g/L citric acid in double-distilled (dd)H_2O (3).

The solution was warmed to 37°C, and the pH adjusted to 3.5, using either Na_2HPO_4 or citric acid as appropriate.

Chloroacetaldehyde Reaction

Because this reaction forms the basis of the results for this paper, the experimental procedure is described in detail in that section.

RESULTS

Protocol for the Fluorescent Determination of m5C in DNA

Step 1. Depurination of bisulphite-treated DNA. We mixed 25 µL of bisulphite-treated genomic DNA (10 µg dissolved in water) with 5 µL of 0.3 M H_2SO_4, covered with 30 µL of liquid paraffin (Merck, Darmstadt, Germany), heated it to 94°C for 1 min and then incubated the mixture at 37°C for 4½ h. This eliminated more than 99% of the adenine from the DNA, based on rate constants in Reference 15.

Step 2. Alkaline hydrolysis of apurinic sites (12). The sample (oil included) was transferred onto a strip of Parafilm "M" (American National Can, Norwalk, CT, USA). The aqueous phase floated above the paraffin and was transferred to a fresh tube. The apurinic sites were hydrolyzed with 1 µL 10 M KOH, followed by 15 min at 25°C, and the pH was adjusted to approximately 1.0 by the addition of 3 µL 2 M H_2SO_4.

Step 3. Silver nitrate precipitation of purines (8,10,13). The sample was heated to 50°C and mixed with 3.4 µL 20% $AgNO_3$. The sample was cooled to 25°C in 75 mL of water (starting temperature 50°C) and then incubated overnight on ice. The sample was centrifuged at 14 000× *g* for 10 min, and the supernatant collected. In agreement with published data (8,10), >99% of the free purines were found in the pellet, but <1% of the polypyrimidine DNA (data not shown) were found.

Step 4. Recovery and quantification of purines after precipitation. The pellet was dissolved in 500 µL of water, mixed and acidified with 4 µL of 12 M HCl. The sample was vortex mixed and heated to 95°C for 5 min.

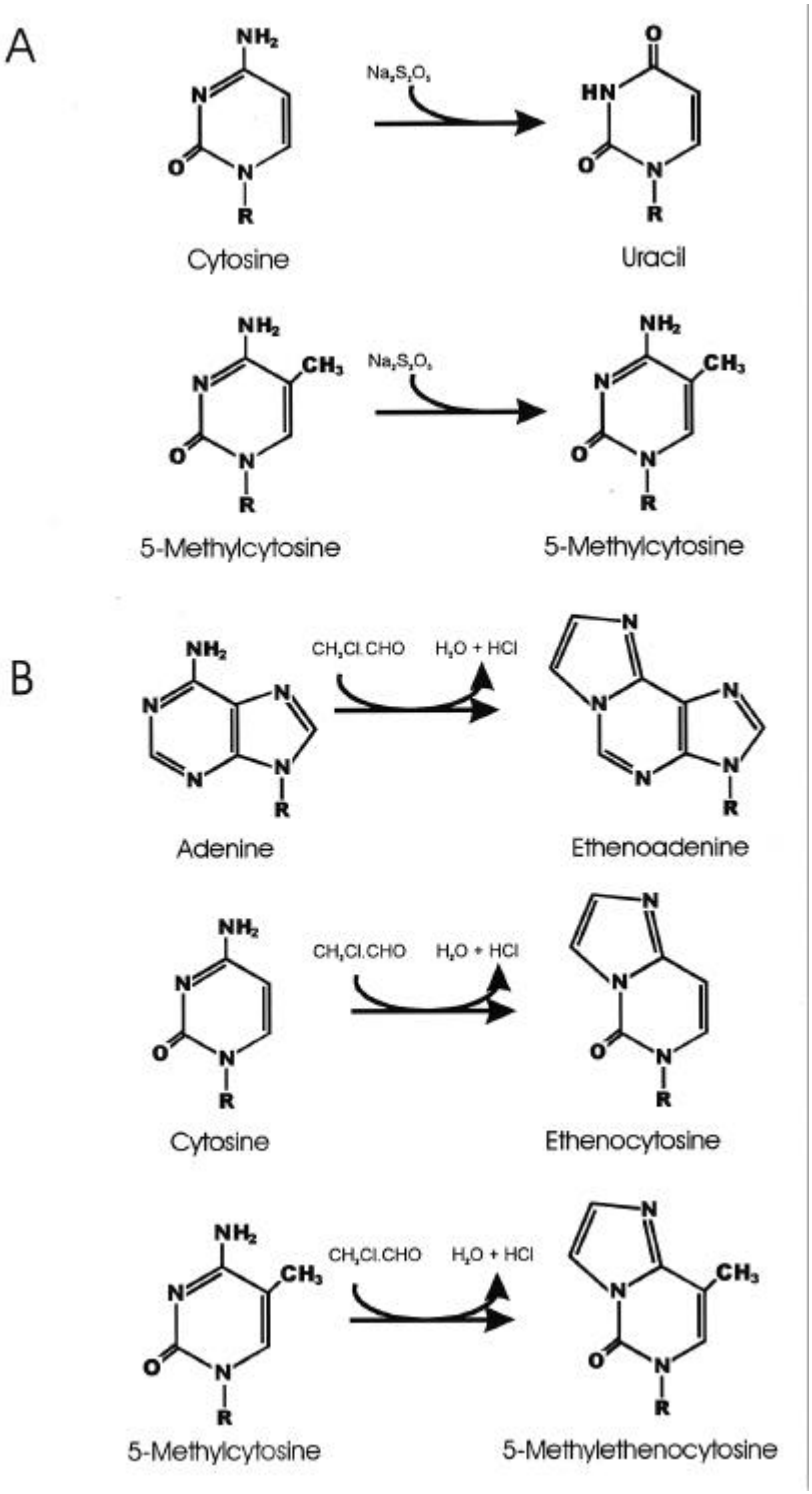

Figure 1. Products of the bisulphite and chloroacetaldehyde reactions. (A) Shown are the key events of the bisulphite reaction on DNA. Cytosine is efficiently deaminated to uracil, whereas 5-methylcytosine is not. (B) Shown are the fluorescent reaction products of the chloroacetaldehyde reaction. Once the cytosine has been converted into uracil, only the 5-methylcytosine and adenine can be labeled.

The silver chloride precipitate was removed by centrifugation, and the A_{276} absorbance was measured. The expected purine concentration was estimated to be: [Purine] = 3.106 × DNA mass.

Step 5. Removal of residual silver from solution and subsequent DNA labeling. The pH of the DNA solution (step 4) was adjusted to 3.5 with 200 µL citrate buffer (pH 3.5) (3). Residual silver nitrate was precipitated with 250 µL chloroacetaldehyde (6.8 M; Fluka AG) and spun at 14000× *g* for 10 min. The supernatant was transferred to a light-safe microcentrifuge tube (Eppendorf, Hamburg, Germany) and incubated at 37°C for 20 h.

Care should be taken with chloroacetaldehyde, as it is both toxic and corrosive. Gloves, eye protection and a good fume hood should always be used. In addition, the use of safe-lock, light-safe microcentrifuge tubes is recommended to reduce the risk of inhalation and to protect the samples from bleaching. Higher temperatures accelerate the labeling, but can cause thermal fluorophore decomposition (1).

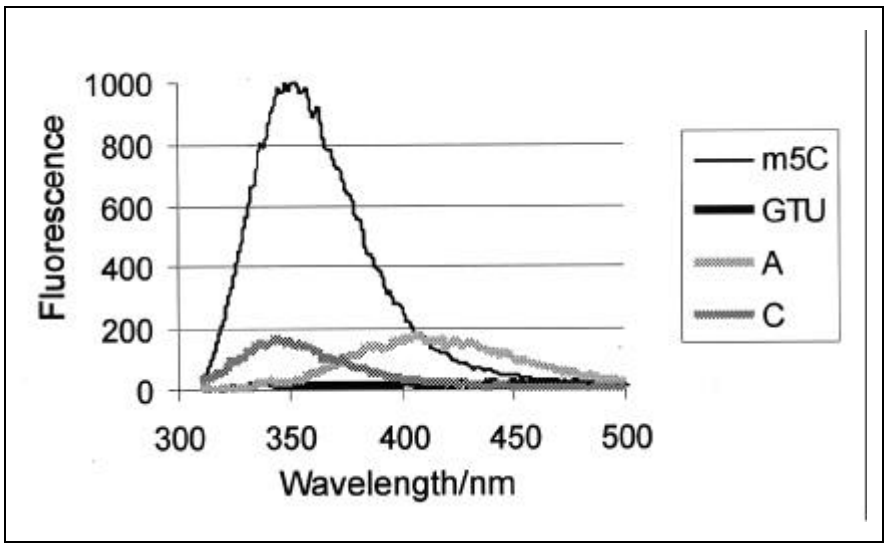

Figure 2. Fluorescence emission spectra of control oligonucleotides. The basic oligonucleotide sequence is in the form of 5′-GXTTXGTGXTGGTXXTTGTGXTGTXT-3′, in which X is 5-methylcytosine (m5C), uracil (GTU), adenine (A) or cytosine (C).

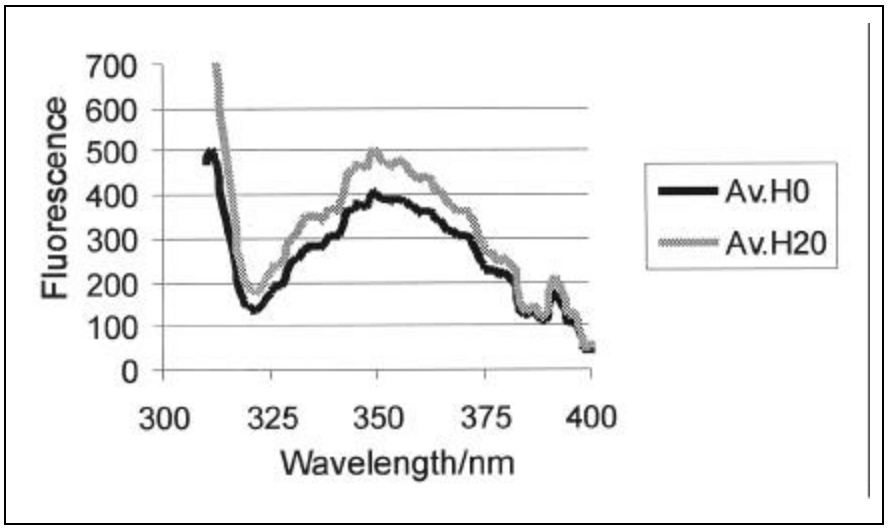

Figure 3. Fluorescence emission spectra of tobacco genomic DNA. The traces plotted here have been normalized using the observed DNA concentrations, and the residual ethenoadenine signal has been subtracted in comparison with the control signal for the A-oligo. Here, Av.H0 is the average trace of three repetitions on Hyg-0 plants, and Av.H20 is the same for Hyg-20 plants.

Step 6. Detoxification of the chloroacetaldehyde solution. The solution from step 6 was transferred to a 2-mL microcentrifuge tube and placed on ice. The aldehyde was reduced to chloroethanol by the dropwise addition of 200 µL of sodium borohydride (25 mg/mL). After the effervescence had stopped, the sample was mixed and incubated in the dark at 25°C, with the top open for 15 min. This step converts any residual chloroacetaldehyde into chloroethanol, which is both less toxic and less volatile. The reaction should be performed on ice in a fume hood and away from open flames, as the mixture can generate much hydrogen.

Step 7. Inactivation of the remaining $NaBH_4$. The residual $NaBH_4$ was inactivated by the dropwise addition of 300 µL 0.6 M HCl, followed by a 10-min incubation in the dark with the top open. HCl was added until no further effervescence was observed. Once the decomposition was complete, the sample was diluted to a final volume of 1.5 mL with ddH_2O. HCl catalyzed the exothermic decomposition of $NaBH_4$ to hydrogen gas.

Step 8. Fluorescence scanning of the sample. The sample and blank were excited at 302 nm, and the resultant fluorescence was measured between 310–450 nm in a Model LS50B Luminescence Spectrometer (PE Biosystems). A peak at 413 nm indicated residual ethenoadenine contamination (1). Genomic methylation was proportional to F_{354}, once the ethenoadenine signal had been subtracted (see below).

Application of the Chloroacetaldehyde Reaction to Synthetic Oligonucleotides

The specificity of this procedure was determined using 10 µg each of the oligonucleotides C-oligo, A-oligo, m5C-oligo and GTU-oligo (Figure 2). Only cytosine, adenine and m5C gave rise to fluorescent products. The m5C-oligo was approximately five times as fluorescent as the equivalent C-oligo, which increased the reaction sensitivity. The normalized fluorescence spectrum from the A-oligo can be subtracted from sample fluorescence to

improve the signal noise for m5C.

The reaction sensitivity was assessed with mixtures of C-oligo, A-oligo and m5C-oligo to simulate different levels of methylation. We used a total of 15 µg of oligonucleotide in each assay, which consisted of triplicate reactions containing 10 µg A-oligo, plus 5 µg of a mixture of C-oligo and m5C-oligo, to assess different levels of percentage methylation (Table 1). These reactions were performed identically to those on genomic DNA. Table 1 shows that the measured methylation levels agreed with those expected, with an average deviation of 1.88 ± 1.06%. Thus, our technique was sensitive enough to detect changes in methylation levels of >3%. This result was comparable to the sensitivity of HPLC reported for m5C detection in pBR322 (1), although our base line "noise" level of 2.8% (Table 1) is higher than that of HPLC.

Application of the Chloroacetaldehyde Reaction to Genomic DNA

To test our procedure on genomic DNA, we isolated DNA from transgenic tobacco grown either in the presence or absence of the antibiotic hygromycin (see Materials and Methods). Then, 15 µg of DNA were reacted with bisulphite and chloroacetaldehyde as described above. After bisulphite treatment, the DNA concentration was measured and found to be 34.2 µM for the Hyg-0 plants and 37.3 µM for the Hyg-20 plants, a result that was in agreement with the purine concentrations found after silver nitrate precipitation (data not shown).

The sample fluorescence was scanned between 310 and 450 nm using a luminescence spectrometer. The excitation wavelength was 302 nm, the excitation slit set to 5.0 and the emission slit to 15.0. A blank, using water instead of DNA, was processed in parallel as a control. The residual ethenoadenine noise was subtracted by comparing it with the A-oligo standard, as described above, and the data were normalized by dividing the measured DNA concentration. Figure 3 shows the resultant emission profiles (average of three experimental repetitions). The area under the curves was integrated between 325 and 375 nm and was found to be:

$$\int_{325}^{375} F_{\text{Hyg-0}} = 32\,135 \pm 2238 \text{ and}$$

$$\int_{325}^{375} F_{\text{Hyg-20}} = 39\,164 \pm 3043$$

This is a methylation change of 1.22 (39164/32135) or a 22% hypermethylation after hygromycin treatment.

***Sss*I Methyl-Accepting Assay**

Triplicate analyses were performed on 300 ng of genomic DNA as described in the Materials and Methods section. Hyg-0 plants incorporated 12.786 ± 1.483 pmol CH_3·(µg DNA)$^{-1}$ and Hyg-20 plants incorporated 2.182 ± 0.217 pmol CH_3·(µg DNA)$^{-1}$. Tobacco (genome mass = 3.9

Table 1. Control Analysis of DNA Methylation

Sample	A-Oligo	C-Oligo	m5C-Oligo	%m5C[a]	Fluorescence[b] (325–375 nm)	%Max[c]
1	10 µg	5.0 µg	0.0 µg	0%	3125 ± 1285	2.8%
2	10 µg	4.5 µg	0.5 µg	10%	13 540 ± 2522	12.3%
3	10 µg	4.0 µg	1.0 µg	20%	20 266 ± 1658	18.3%
4	10 µg	3.5 µg	1.5 µg	30%	33 817 ± 1795	30.6%
5	10 µg	3.0 µg	2.0 µg	40%	48 346 ± 2088	43.7%
6	10 µg	2.5 µg	2.5 µg	50%	57 805 ± 2629	52.3%
7	10 µg	2.0 µg	3.0 µg	60%	69 390 ± 2047	62.8%
8	10 µg	1.5 µg	3.5 µg	70%	78 213 ± 1514	70.8%
9	10 µg	1.0 µg	4.0 µg	80%	89 664 ± 1965	81.1%
10	10 µg	0.5 µg	4.5 µg	90%	98 509 ± 2113	89.1%
11	10 µg	0.0 µg	5.0 µg	100%	110 527 ± 2268	100%

This table shows a control experiment to assess the sensitivity of the chloracetaldehyde reaction using mixtures of the oligonucleotides, A-oligo, C-oligo and m5C-oligo.

[a]%m5C shows the predicted methylation level based on the ratio of C-oligo to m5C-oligo in the reaction.

[b]Fluorescence (325–375 nm), shows the average integrated fluorescence value for 5-methyl ethenocytosine, after subtraction of the ethenoadenine signal, between 325 and 375 nm. The errors given are standard deviations for three repetitions.

[c]%Max, shows the ratio of the fluorescence values, measured to the fluorescence observed from sample 11 (multipled by 100 to get a percentage).

pg) (2) contains 63.9 pmol CpG·(µg DNA)$^{-1}$. Thus, the incorporation translated to a CpG methylation change of 1.20 [(63.9-2.182)/(63.9-12.786)] or a 20% CpG hypermethylation. This result confirmed that both our procedure and the *Sss*I procedure showed similar increases. The chloroacetaldehyde reaction measured changes in total cytosine methylation, whereas *Sss*I measured only CpG methylation changes. Thus, we concluded that the hypermethylating effect of hygromycin caused a similar percentage effect on all methylatable classes of cytosine (CpG, CpNpG and CpX).

DISCUSSION

Our procedure had two significant advantages over the *Sss*I methyl-accepting assay: *(i)* It produced a chemical, rather than an enzymatic, reaction, which used quite stable and inexpensive reagents (the *Sss*I assay is sensitive to both enzyme and *S*-adenosyl methionine instability). *(ii)* It was not restricted to m5Cs located in the CpG context (*Sss*I will only label CpG sites), so it could be used to monitor non-CpG methylation changes. Chloroethanaldehyde is not fluorescent, so it will not interfere with the data collection. The *Sss*I assay also required the use of tritiated SAM that is difficult to dispose of and expensive. After the borohydride reduction of the aldehyde, the solutions are nontoxic and can be safely discarded. HPLC offers many advantages in terms of experimental simplicity over our procedure, but again, such machines are not always available.

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Edward J. Oakeley, Frédéric Schmitt[1] and Jean-Pierre Jost[2]
University of Exeter
Exeter, England, UK
[1]Rhône-Poulenc Agro
Lyon, France
[2]Friedrich Miescher Institute
Basel, Switzerland

Changing Functionality of Surfaces by Directed Self-Assembly Using Oligonucleotides—The Oligo-Tag

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ABSTRACT

A method is presented to modify surfaces for biotechnological applications. Oligonucleotides have been coupled covalently to a pre-activated surface. Complementary oligonucleotides hybridize to the surface, which are conjugated with functionalities. The oligonucleotides serve as "Oligo-Tags" for these functionalities that now are linked specifically and reversibly. The approach might be used to change DNA-arrays into arrays of arbitrary ligands. We demonstrate the method with an optical wave guide grating coupler as a sensing surface using two different haptens as examples for a variety of functionalities. The haptens were 2,4-dichlorophenoxyacetic acid and atrazin and are recognized by specific antibodies. The surface created was completely regenerable by alkaline washing or temperature increase without any loss of binding capacity. Specificity was demonstrated by competitive binding of antibody in presence and absence of analyte; unspecific binding has not been observed.

INTRODUCTION

The highly ordered microstructure of the living world serves as a guide for either the construction of biomimetic devices or by giving access to the direct use of biomolecules. In biotechnology, it is often necessary to couple the biomolecule to a solid surface. Such a surface might be a silicium wafer, a metal or organic polymer or any kind of glass, as is used in this paper. Several methods have been developed to couple biomolecules to solid supports for a variety of applications (1,15,19). Most of these methods link the molecules irreversibly to the surface, e.g., by physisorption or by covalent coupling of receptors to an activated surface. As a consequence of such methods, the molecules are statistically distributed and often only poorly oriented. Active biomolecules, binders as well as catalysts, are highly flexible molecules with a limited lifetime in their natural environment. The living cell overcomes this problem by continuous exchange of functional molecules, i.e., destruction and synthesis of active macromolecules. The lifetime of devices relying on biomolecules is restricted by the lifetime of the isolated biomolecule. Attempts to stabilize the molecules and restore their functionality have been only partially successful (21). What we can learn from the living cell for biotechnological in vitro applications is to exchange the biomolecule without changing the device.

In this report, we introduce a method for directed self-assembly of defined surface coverage by the use of oligonucleotides, making use of the high specificity of the double-stranded formation of natural nucleic acids. Two oligonucleotides form a strong dimer by hydrogen bonds called hybrids when both strands are complementary. The strength of the dimerization depends on (*i*) the number of matching bases in a sequence, (*ii*) the number and loci of mismatches and (*iii*) the base composition. Double-strand stability is well investigated and applied in all types of gene probe analysis, which is hybridization-based (20). To separate the two complementary strands, destabilizing conditions have been proven in numerous applications such as high temperature, high ionic strength or alkaline environment (6). Separability is given for all kinds of homo- and het-