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Bioengineering and Characterization of DNA–Protein Assemblies Floating on Supported Membranes

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Summary

This chapter describes the design, practical construction, and characterization of P-DNA and their applications in building a new generation of DNA chips. P-DNAs are artificial covalent assemblies involving a histidine tag head able to bind to modified phospholipids, a core protein domain derived from cytochrome b5 by genetic engineering that features specific spectroscopic and electrochemical properties useful for detection, a synthetic linker acting as a spacer, and an oligonucleotide acting as a probe. P-DNA has the property of being able to efficiently self-associate to a supported bilayer including nickel-iminodiacetate-modified phospholipids. The construction of P-DNA and its interaction with a complementary oligonucleotide sequence can be monitored in real time by surface plasmon resonance using a Biacore system or equivalent. P-DNA chips feature unique properties including tunable surface density of probes; very low nonspecific interaction with external DNA; lateral mobility, minimizing–steric interaction; optimization of hybridization efficiency; and, potentially, recognition by multiple probes of a single target and perfectly defined and homogeneous structure, permitting high density up to a compact monolayer. Potential applications of this new device are multiple, including high-sensitivity and high-selectivity chips for DNA–DNA, DNA–RNA, or DNA–protein interactions.

Key Words: DNA chip; supramolecular assemblies; surface plasmon resonance; artificial protein-DNA structure; supported membrane; lateral mobility; cytochrome; dynamic.

1. Introduction

Designing artificial supramolecular assemblies containing both protein and nucleic acid is still a challenge. Few models in the literature deal with this kind of molecular construction in order to develop DNA or bioelectronic devices. Most DNA chip sensors are designed using poorly controlled adsorption or

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covalent attachment of the probes, which leads to uncontrolled pattern and geometry, severely limiting applications. A critical aspect for bioinorganic devices is the contact of the inorganic template (or substrate) with the biological components. Strong interaction of biomolecules with the support yields a random immobilization that impairs self-organization. Biomolecular structures by themselves are frequently denatured on interaction with the support, leading to loss of function.

To isolate inorganic support from biomolecules, several types of support functionalization have been evaluated, including supported lipid bilayers that mimic natural membranes. Most of the common substrates can be covered with a supported membrane resulting from vesicular spreading or Langmuir-Blodgett layer deposition. Such a bilayer has several advantages: (1) it acts as a cushion isolating and protecting the metallic or inorganic support, (2) it constitutes a biomimetic environment limiting nonspecific adsorption and denaturation of biomolecules, and (3) it can be easily functionalized to allow specific attachment of biomolecules through a floating artificial anchor attached on one side to the bilayer by hydrophobic interactions and on the opposite side to the biomolecule by a specifically engineered link. This interfacing confers unique properties such as lateral mobility of biomolecules that are maintained close to the surface by the anchor while not interacting directly with the support.

This chapter describes an original molecular device associating protein and nucleic acid components with a supported membrane interface to set up highly ordered nanostructures of interest for biosensors. This molecular Lego is composed of two parts: a supramolecular assembly composed of a modified oligonucleotide covalently linked to a genetically modified protein via a homobifunctional linker, and a supported bilayer including functionalized phospholipids that can specifically capture the previous assembly. Unique properties of this device result both from the lateral mobility permitted by the floating structure that allows in turn some easy bidimensional structural reorganization and from the specific properties of the embedded recombinant protein that was designed to give the device additional optical and electronic properties of critical interest for biosensors. This chapter also describes how such assembly can be implemented to set up conformation-sensitive devices with potential application in the detection of the interaction of a single molecule or in the detection of geometric transition in nucleic acids in response to interacting proteins or ligands.

2. Materials

Dimyristoyl phosphatidyl choline (DMPC), dioleoyl phosphatidyl choline (DOPC), and octyl glucoside were purchased from Sigma (St. Louis, MO).

1,2-Dioleoyl-*sn*-glycero-3[(*N*(5-amino-1-carboxypentyl)] iminodiacetic acid succinyl (DOGS) was purchased from Aventi Polar Lipids. Cis-[$PtCl_2(NH_3)_2$] was kindly provided by Johnson Matthey (London, UK). 1,4-Di-[3'-(2'-pyridyldithio)propionamido]-butane (DPDPB) was provided by Pierce (Rockford, IL). DPDPB is insoluble in aqueous buffers and was first dissolved in dimethyl sulfoxide (DMSO) (20 m*M*) before dilution. Oligonucleotides were provided by Eurogentec.

Surface plasmon resonance (SPR) experiments were run on BIAcore X and 3000 (BIAcore, Uppsala, Sweden) at 25°C, with a flow rate of 2–50 µL/min, in 50 m*M* sodium phosphate solution (pH 7.5) (buffer P1). Sensor chip HPA precoated with octadecylmercaptan (OM) was purchased from BIAcore or prepared from a standard J1 chip or SIA kit as follows: a 1 m*M* solution of OM in an ethanol/water solution (4:1 [v/v]) was sonicated for 20 min using a Transsonic 310 at maximum power. J1 sensor chips were treated overnight and rinsed with ultrapure ethanol and water prior to drying under nitrogen.

Single-stranded DNA (ssDNA) (5'-CTATCATTTGCTTACTATTC-3') and the complementary oligonucleotide were provided by Eurogentec. DPDPB is insoluble in aqueous buffers and was first dissolved in a stock solution of DMSO (20 m*M*). The concentration of DPDPB can be calculated by spectroscopy using $A_{237\ nm} = 1.2 \times 10^4\ M^{-1}\ cm^{-1}$ and $A_{287\ nm} = 8.8 \times 10^3\ M^{-1}\ cm^{-1}$. Its functionality was monitored directly or by the absorbance ($A_{344\ nm} = 8.08 \times 10^3\ M^{-1}\ cm^{-1}$) of pyridine-2-thione released on reduction. A mixture of modified ssDNA and cytochrome b5 with DPDPB must contain <10% (v/v) DMSO in buffer solution.

3. Methods

3.1. Protocols and Instrumentation

3.1.1. Molecular Engineering of Cytochrome b5

Cytochrome b5 is an electron carrier protein encompassing an iron protoporphirin IX as a redox cofactor. This protein is naturally anchored onto the endoplasmic reticulum membrane of cells through a C-terminal hydrophobic amino acid sequence (**Fig. 1**). The globular redox domain is hydrophilic and does not interact with membranes. This domain features a high absorbancy at 413 nm for the oxidized form that shifts to 423 nm on reduction. An engineered protein (Hb5-$[His]_4$) was derived from human microsomal cytochrome b5 by genetic engineering resulting in the substitution of the 26 C-terminal amino acid residues by the –NGHHHH–COOH sequence. This engineered protein was soluble and presented a C-terminal histidine tag that can coordinate a nickel-iminodiacetate-ended synthetic linker.

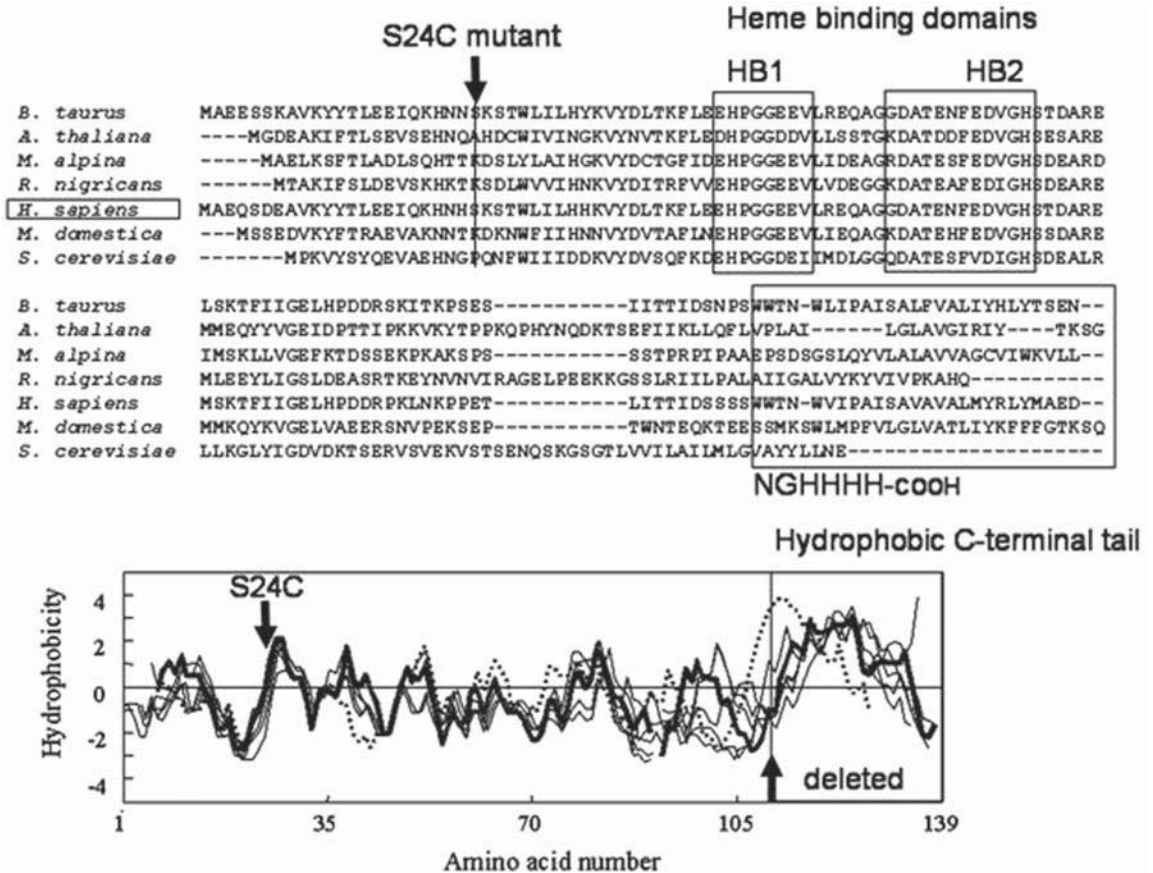
S24C mutant
Heme binding domains
HB1
HB2
B. taurus MAEESSKAVKYYTLEEIQKHNNSKSTWLILHYKVYDLTKFLEEHPGGEEVLREQAGGDATENFEDVGHSTDARE
A. thaliana ----MGDEAKIFTLSEVSEHNQAHDCWIVINGKVYNVTKFLEDHPGGDDVLLSSTGKDATDDFEDVGHSESARE
M. alpina -----MAELKSFTLADLSQHTTKDSLYLAIHGKVYDCTGFIDEHPGGEEVLIDEAGRDATESFEDVGHSDEARD
R. nigricans ------MTAKIFSLDEVSKHKTKSDLWVVIHNKVYDITRFVVEHPGGEEVLVDEGGKDATEAFEDIGHSDEARE
H. sapiens MAEQSDEAVKYYTLEEIQKHNHSKSTWLILHHKVYDLTKFLEEHPGGEEVLREQAGGDATENFEDVGHSTDARE
M. domestica ---MSSEDVKYFTRAEVAKNNTKDKNWFIIHNNVYDVTAFLNEHPGGEEVLIEQAGKDATEHFEDVGHSSDARE
S. cerevisiae -------MPKVYSYQEVAEHNGPQNFWIIIDDKVYDVSQFKDEHPGGDEILIMDLGGQDATESFVDIGHSDEALR
B. taurus LSKTFIIGELHPDDRSKITKPSES----------IITTIDSNPSWWTN-WLIPAISALFVALIYHLYTSEN--
A. thaliana MMEQYYVGEIDPTTIPKKVKYTPPKQPHYNQDKTSEFIIKLLQFLVPLAI------LGLAVGIRIY----TKSG
M. alpina IMSKLLVGEFKTDSSEKPKAKSPS----------SSTPRPIPAAEPSDSGSLQYVLALAVVAGCVIWKVLL--
R. nigricans MLEEYLIGSLDEASRTKEYNVNVIRAGELPEEKKGSSLRIILPALAIIGALVYKYVIVPKAHQ----------
H. sapiens MSKTFIIGELHPDDRPKLNKPPET----------LITTIDSSSSWWTN-WVIPAISAVAVALMYRLYMAED--
M. domestica MMKQYKVGELVAEERSNVPEKSEP----------TWNTEQKTEESSMKSWLMPFVLGLVATLIYKFFFGTKSQ
S. cerevisiae LLKGLYIGDVDKTSERVSVEKVSTSENQSKGSGTLVVILAILMLGVAYYLLNE----------
NGHHHH-COOH
Hydrophobic C-terminal tail
S24C
Hydrophobicity
4
2
0
-2
-4
deleted
1
35
70
105
139
Amino acid number

Fig. 1.

Engineering was performed on human b5 cDNA by polymerase chain reaction amplification using the 5'-GGCTGGATCCTTAATGGTGGTGATGTC CGTTACTGGAACTAGAATCAATAGTAGTG-3' oligonucleotide as a reverse primer and an oligonucleotide covering the 24 first bases of the 5' end of the cDNA open reading frame as a direct primer. The amplified cDNA was subcloned into pCR®2.1-TOPO® using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and was checked by sequencing. The Hb5-$(His)_4$ coding sequence was extracted from pCR2.1 by *Sph*I-*Bam*HI double digestion and was ligated into the *Sph*I-*Bam*HI-digested pUHE25-2 expression vector ***(1)*** giving pUHE-[Hb5-$(His)_4$]. Owing to the construction, this vector expresses a cytochrome b5 protein containing an additional leucine residue immediately following the initiation codon in addition to the C-terminal substitution.

Serine 24 in Hb5-$(His)_4$ (serine 23 of original human b5) was changed to cysteine by site-directed mutagenesis on pUHE-[Hb5-$(His)_4$] using a QuickChange site-directed mutagenesis kit (Stratagene) following the manufacturer's recommendations. Mutation was checked by sequencing.

3.1.2. Expression of Engineered Cytochrome b5

The same procedure was used for expression of [Hb5-$(His)_4$] and its S24C derivative. XL1-Blue™ cells transformed by pUHE-[Hb5-$(His)_4$] were grown in 1 L of Terrific Broth supplemented with ampicilin (100 mg/L) at room temperature for 48 h. Isopropyl thiogalactoside and δ-amino levulinic acid hydrochloride (Sigma) were added at a final concentration of 0.5 m*M* when the OD_{600} reached 10. Induction took place for 30 h at 24°C. Cells were harvested and resuspended in 50 mL of 20 m*M* Tris-HCl buffer, pH 8.0. Cell walls were broken by the conjugated effects of lysozyme (1.25 mg/mL) and by sonication (4×180s with 50% active cycles, using a Vibracell from Bioblock-Scientific) at 4°C in the presence of Cocktail Protease Inhibitors (Sigma), DNase (Sigma), and RNase (Boehringer). The 10,000*g* supernatant was applied to a 1.5×7 cm column of Ni^{2+}/iminodiacetate immobilized on agarose (Sigma) equilibrated with 25 mL of buffer P1. After thoroughly washing the column with buffer P1,

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Fig. 1. *(previous page)* Sequence alignments of microsomal cytochromes b5. Aligned sequences are from mammalian, insect, plant, and fungal origins. (**Top**) Boxes HB1 and HB2 indicate conserved sequences surrounding the histidinyl residues that are ligands of the heme iron. S24C labels the position mutated in this work in the human sequence to introduce a unique cysteinyl residue. The large box indicates the hydrophobic C-terminal sequence tail anchoring the natural enzyme to the membrane and the engineered C-terminal sequence (bold large letters). (**Bottom**) Corresponding hydrophobic index profiles. The arrow under S24C indicates the mutated position. The box labeled "deleted" indicates the deleted hydrophobic C-terminal sequence.

[Hb5-$(His)_4$] was eluted with a 1 mg/mL solution of L-histidine. After purification, the protein was characterized by mass spectroscopy analysis (observed = 13,109; expected = 13,129).

3.1.3. Coupling of ssDNA to Engineered Cytochrome b5 Via a cis-Platin Bridge

To constitute a well-defined covalent link between the ssDNA of interest and cytochrome b5, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ was used as a crosslinking reagent. This approach has the advantage of forming a short linkage between the protein and the nucleic acid while preserving most of the hybridization properties of the ssDNA moiety. A drawback is the requirement for specific nucleic acid sequences that include a unique guanine that acts as a target at its N7 position for the cisplatin link ***(2)***. *Cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (cisplatin) was prepared by dissolving a suspension of *cis*-$[Pt(NO_3)_2(NH_3)_2]$ in water, formed by reaction of *cis*-$[PtCl_2(NH_3)_2]$ with silver nitrate. The ssDNA oligonucleotide (5'-CTA TCATTTGCTTACTATTC-3') was chosen as a model DNA fitting the previous requirements. Methylation analysis and Maxam-Gilbert sequencing ***(3,4)*** indicated that a single platination occurred at the N7 position of the unique guanine present on this oligonucleotide.

For formation of the cisplatin link, [Hb5-$(His)_4$] and ssDNA (100–200 μ*M*) were incubated in the presence of 5 molar eq of platinum complex in buffer P1 for 5 h at 25°C. The resulting complex (90 μ*M*) was incubated with 1.5 eq of ssDNA for 50 h at 25°C. The [Hb5-$(His)_4$]-Pt-ssDNA (P-DNA) was purified from the excess reagent using Ni^{2+} iminodiacetic acid immobilized on agarose (Sigma). The protein was eluted with 1 *M* imidazole acetate in P1 buffer. The purification steps were monitored using the radiodetection of the 5'-end ^{32}P-labeled DNA and optical absorption monitoring at 413 nm for the cytochrome b5 and at 260 nm for the DNA. Results were consistent with formation of an equimolecular complex between [Hb5-$(His)_4$] and the ssDNA based on $\varepsilon_{413\ nm} = 117\ mM^{-1}\cdot cm^{-1}$ for [Hb5-$(His)_4$] and $\varepsilon_{260\ nm} = 180\ mM^{-1}\cdot cm^{-1}$ for the ssDNA. Control of P-DNA structure was routinely assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis ***(5)***.

3.1.4. Coupling of ssDNA to Engineered Cytochrome b5 Through Homobifunctional Crosslinker

An alternate strategy was conceived to make it possible to combine any type of nucleic acid with cytochrome b5. This strategy consisted of creating, by site-directed mutagenesis, a unique attachment site on cytochrome b5 for a bifunctional crosslinker also able to couple with a modified nucleic acid. The homobifunctional linker DPDPB is a 16Å linker that can form disulfide bonds with both thiolate-containing ssDNA and protein (**Fig. 2**). ssDNA modified by

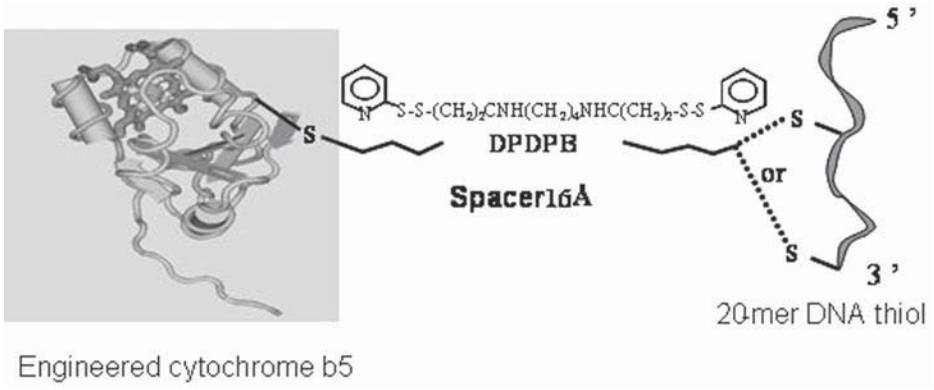

Fig. 2. Schematic structure of DPDPB-coupled P-DNA. Cytochrome b5 was coupled to ssDNA through a DPDPB bifunctional linker. DPDPB reaction led to insertion of a 16Å linker between the cytochrome b5 thiolate and the thiol-modified oligonucleotide.

a thiol group linked to the 3' or 5' end via a $(–CH_2–)_3$ or $(–CH_2–)_6$ spacer was provided by Eurogentec. The unmodified cytochrome b5 contains no endogenous cysteinyl residue. The S24C mutation was introduced to add a solvent-accessible cysteine at the periphery of the protein without disturbing the cytochrome folding (preservation of its spectroscopic and electrochemical properties). Based on the three-dimensional structure, this substitution localizes far away from the C-terminal histidine tag, thus allowing two independent and noninteracting links of cytochrome b5 with external elements.

3.1.4.1. Oligonucleotide Coupling With DPDPB

A specific protocol has been designed to optimize the yield of adduct synthesis. A solution of the thiolated ssDNA was incubated at 30°C for 10 min with dithiothreitol (DTT) at a 1:100 molecular ratio to cleave potential preexisting disulfide bridges. Excess unreacted DTT had to be eliminated before carrying out ssDNA coupling with the DPDPB reagent. For this purpose, a gel-exclusion chromatography on Sephadex G25 (Pharmacia) was performed. The coupling step was for 4 h at 30°C at a molar ratio of 1:20 between the desalted ssDNA and DPDPB. Following this step, coupled ssDNA was purified from excess reagent by separation onto a weak anion-exchange column (DEAE cellulose; Pharmacia). After a low-salt (0.1 *M* NaCl) wash, the ssDNA-PB adduct was eluted with 1.5 *M* NaCl. This led to highly concentrated fractions of the ssDNA-PB adduct from which a small amount (1 nmol) was withdrawn to evaluate the coupling efficiency. This was determined by monitoring the change in absorption at 344 nm following the addition of excess DTT. This chemical

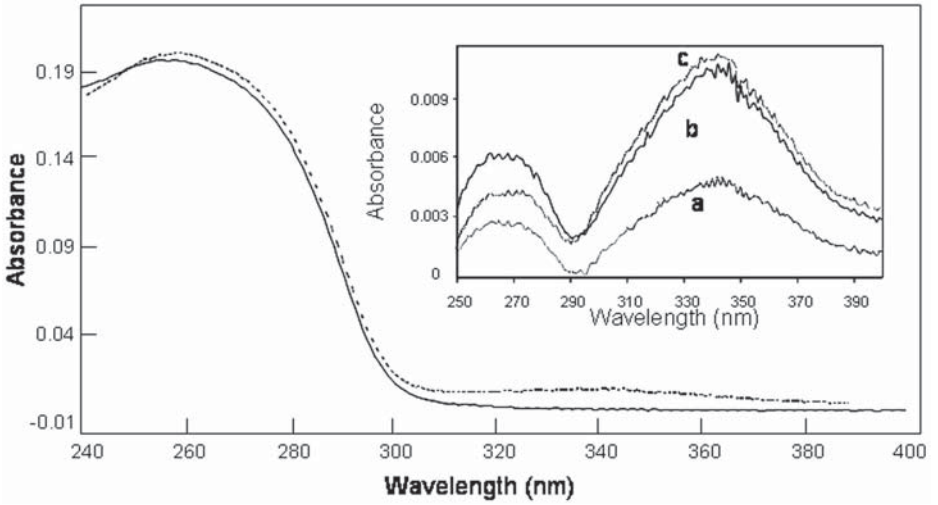

Fig. 3. Spectral characterization of adducts between DPDPB and ssDNA. Coupling and purification were performed as described in the text. Spectra were recorded for ssDNA prior to mixing with DPDPB (solid line) and after coupling and purification (dashed line). Titration of the remaining thiopyridine functionalization on the adduct can be performed by cleavage with DTT, which allows the release of the strongly absorbing pyridine 2-thione chromophore ($\varepsilon_{343\ nm}$ = 8080 M^{-1}·cm^{-1}). Increasing the molar ratio of the DTT/adduct gave rise to corresponding increases in the $A_{343\ nm}$ signal until saturation: (a) 10/1, (b) 50/1, (c) 100/1.

cleaves the uncoupled end of the linker releasing the pyridine-2-thione that was monitored at 344 nm (8.1×10^3 M^{-1} cm^{-1}), as presented in **Fig. 3**. Up to 80% of the initial ssDNA was found to be coupled to DPDPB, and the ssDNA-PB adduct was kept at 4°C for a few days without any detectable loss of reactivity (*see* **Note 1**).

3.1.4.2. Coupling of S24C Mutant of Hb5(His)$_4$ Protein to ssDNA-PB Adduct

The second step of the assembly formation involves reaction of the protein with the ssDNA-PB adduct. During the purification process of the cysteine-containing protein, we noticed a high level of spontaneous dimer formation through a disulfide bond. This dimer was reduced by incubation of the purified protein with DDT as already described for the modified oligonucleotide (same time, temperature, and molar ratios). Similarly, the excess DTT was removed immediately before the coupling step by a rapid gel chromatography on Sephadex G50. The protein eluted into the dead volume of the column and was directly collected into the solution of purified ssDNA-PB adduct to reach a final molar ratio of 1 protein for 10 modified oligonucleotides. After at least

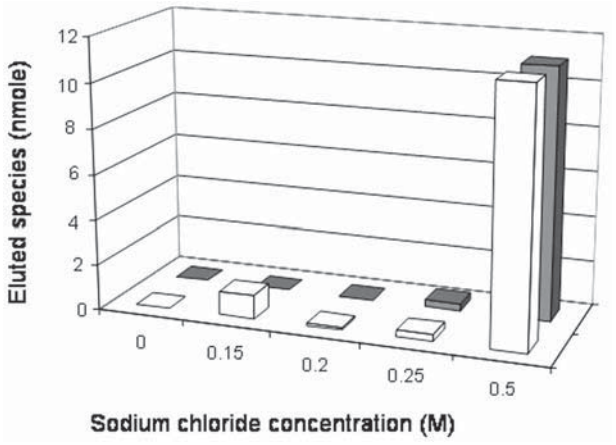

Fig. 4. Elution profile of DPDPB-coupled P-DNA purification on DEAE-cellulose column. As described in the text, a combination of chromatographic steps was required to obtain pure P-DNA. The last step involved an anion-exchange column (DEAE-cellulose) and was used in order to separate the different negatively charged species. A salt gradient allowed resolution of free cytochrome b5 from P-DNA. Solid boxes represent the ssDNA elution profile as deduced from ultraviolet absorbance; open boxes represent the cytochrome b5 elution profile as deduced from visible light absorbance.

2 h of coupling at room temperature in buffer P1, the final adduct was purified by combined chromatographies. Metal chelate chromatography was used to eliminate all the histidine tag–free species (i.e., nonprotein species). Following a wash step with a saline solution, the nickel-iminodiacetate column was eluted by a solution of histidine (1 mg/mL) or of EDTA (1 m*M*). Three species may still coexist in the eluate: monomeric and dimeric uncoupled proteins and the complex. Final purification was achieved by loading the previous eluate onto a DEAE Sepharose gel column that binds the three species but with differential affinities. Owing to the negative charges on DNA phosphate groups, the P–DNA complex is much more tightly bound than uncoupled proteins. A salt gradient allowed gradual elution of the species, as illustrated in **Fig. 4**. P-DNA eluted at the higher salt concentration. Routinely, this procedure resulted in a high yield of P-DNA synthesis (up to 75%). Spectrophotometric analysis of P-DNA was performed, and spectroscopic properties were compared with those of the uncoupled cytochrome b5 and ssDNA macromolecules (**Fig. 5**). P-DNA exhibits two major absorbance peaks corresponding to the nucleic acid and protein moieties, at 260 and 412 nm, respectively. For calculations, the absorbance at 260 nm has to be corrected to take into account the contribution of the Hb5-$(His)_4$ protein at this wavelength (about 20% of the 413-nm absorbance value).

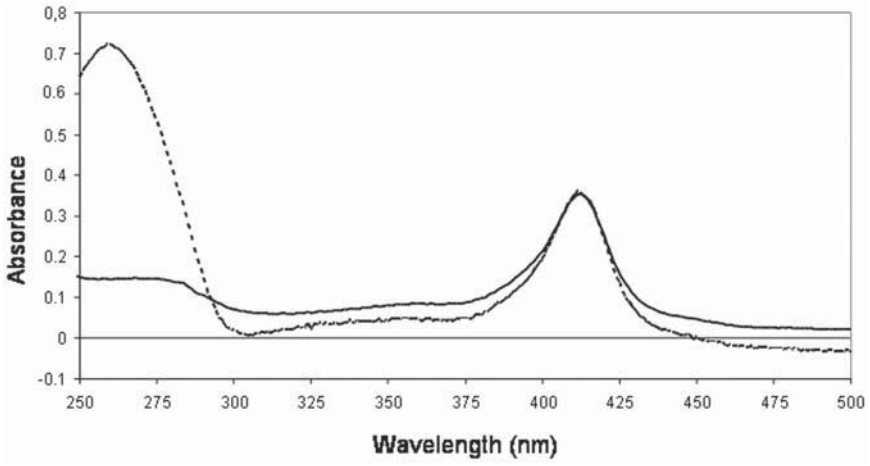

Fig. 5. Spectral characterization of [Hb5]His_4 and P-DNA. Absorption spectra of Hb5(His_4) (solid line) and of purified P-DNA resulting from DPDPB-mediated coupling of Hb5(His_4) with ssDNA (dashed line).

3.1.5. Conclusions

The formation of highly ordered and homogeneous supramolecular assemblies requires fully defined molecular components able to associate together in a fully defined and unique manner. We thus engineered a small globular protein, cytochrome b5, that exhibits a stable folding as well as useful specific spectroscopic and redox properties facilitating detection and characterization. Molecular engineering was used to introduce two types of functionalization: one allowing the controlled association of the final complex with a supported bilayer, and the other allowing a unique linkage to a nucleic acid moiety. This link was designed to avoid any disturbances of the protein structure, which, in the final complex, conserves unchanged redox and spectral properties. Moreover, this link allows a sufficient intramolecular flexibility to limit and even avoid any interference from the protein moiety that could affect the hybridization properties of the nucleic acid domain. The use of a distal homobifunctional synthetic crosslinker enabled further reduction of steric hindrance and, thus, higher yield and increased specific DNA base-pairing recognition.

A specific optimized protocol was designed to implement the reaction of the single-strand oligonucleotide with the DPDPB coupling agent under mild reaction conditions. Prior to reacting the oligonucleotide-spacer adduct with the protein, a spectroscopic assay requiring only a very limited amount of adduct (<1 nmol) was designed in order to quantify coupling efficiency. The yield of the coupling between the oligonucleotide-spacer adduct and the pro-

tein could also be assessed by spectroscopic methods taking advantage of the well-defined absorption of DNA and cytochrome b5 at 260 and 412 nm, respectively.

3.2. P-DNA Applications

A biosensor must combine biological recognition processes with signal transduction, allowing detection. Sensitivity of this detection increases when the size of the detection system decreases. SPR technology is a suitable detection system that requires no preliminary labeling of samples to monitor, and that provides real-time kinetics of biomolecule assembly ***(6)***. The generation of an evanescent wave requires a metal-coated template that also acts as an assembly support. These devices detect time-dependent changes in the refractive index at the surface of the sensor and give a signal (the sensorgram) proportional to the mass of the molecules bound to the surface. The sensitivity of SPR exponentially decreases with the distance between the interface and the recognition assemblies and becomes negligible for distances exceeding 300 nm. The classic BIAcore technology, which is essentially based on chips exhibiting a 100-nm polymer cushion (a dextran matrix on a gold surface), is not optimal for sensitivity. Supported lipidic membranes allow a closer distance, and thus a potentially optimized limit of detection, and also feature biomimetic properties limiting nonspecific interactions.

3.2.1. Setup of Hybrid Bilayer Onto Gold Surface

A hybrid bilayer composed of a dense octadecyl mercaptan monolayer self-assembled onto a gold substrate overlaid by a free-floating phospholipid upper layer could be obtained by interaction of a liposome suspension with the OM-functionalized gold substrate ***(7)***. Kinetics of the hybrid bilayer formation was monitored by using SPR technology. Based on experimental and theoretical calibrations, a signal of 1 RU was found to correspond to a mass of 1 pg/mm^2 ***(8)*** and 700 resonance unit (RU) to a monolayer 1-nm thick ***(9)***. Fusion of small unilamellar vesicles of phospholipids (1 m*M* in buffer P1 at 25°C) with the self-assembled OM monolayer was performed. To reach a highly packed monolayer, a 20-min incubation was required. To remove excess material resulting from vesicular interdigitations, a 20 m*M* sodium hydroxide solution is injected until a stable signal is reached (*see* **Note 2**). Routinely, we measured values of 1700 and 1900 ± 300 RU for fusion of, respectively, DMPC and DOPC vesicles. This is in good agreement with previously published values ***(10)***. Nevertheless, it appeared important to control the integrity of the planar lipidic upper layer by injecting some polar proteins. Injection of a 1 μ*M* solution of Hb5[His]$_4$ did not produce any signal, indicating the absence of nonspecific interactions with uncovered gold or hydrophobic layers ***(5)***. To confer

metal chelate affinity properties to the supported membrane, 10% (by mole) of a synthetic modified phospholipid (DOGS) bearing a nickel-iminodiacetate head was incorporated into the DMPC- or DOPC-containing vesicles used for the fusion step. Functionalized and nonfunctionalized vesicles behaved similarly toward the OM monolayer (*see* **Note 3**) (formation of uniform hybrid bilayer).

3.2.2. Interaction of Various Types of Cytochrome b5 With Supported Membranes

3.2.2.1. Native Human Cytochrome b5

Native human cytochrome b5 is an extrinsic membrane protein with a natural transmembrane anchor domain composed of 26 amino acids at its carboxy terminus. We studied the incorporation of this protein into a hybrid bilayer by SPR in a 0.1 to 3 μ*M* range of concentration. Cytochrome b5 exhibited a high affinity for the supported bilayer but, surprisingly, at the highest concentrations tested acted as a macromolecular detergent by destroying the lipidic layer. Based on the Langmuir model proposed in the BIAevaluation software (BIAcore), a dissociation constant of 20 n*M* was evaluated, which is the same order of magnitude as the value calculated for rabbit cytochrome b5 ***(11)***. With an appropriate concentration (i.e., <1 μ*M*), a monolayer of cytochrome b5 can be reconstituted highly packed onto the biosensor surface, giving an SPR signal of 1000 RU, which represents a coverage of 8 pmol/cm^2 ***(12)***. Although this membranous protein has a strong affinity for the hybrid bilayer, several factors make its use difficult, such as aggregation in solution, solubilization with detergent, and destruction of the bilayer.

3.2.2.2. Hb5(His)$_4$ Protein

It was previously reported that a membrane anchor–free cytochrome b5 containing a hexa-histidine tag could be incorporated with a relatively high affinity (>1 μ*M*) into a metal chelate monolayer ***(13)***. In our case, the –NGHHHH C-terminal extension was added to a modified human microsomal cytochrome b5 deleted of its natural C-terminal polypeptidic membrane anchor. Binding of the Hb5(His)$_4$ protein to the DOGS-doped supported bilayer was analyzed by SPR (**Fig. 6**, curve B). Typically, a unique injection of Hb5(His$_4$) was performed at 1 μ*M* concentration (**Fig. 6**, curve B, **step 1**). A small decrease in the signal was observed on washing with buffer (**Fig. 6**, curve B, **step 2**), which probably reflected conditions of thermodynamic equilibration. Classically, the signal reaches values of 200 RU in the condition of excess lipid anchor inside the lipidic monolayer (10% DOGS). Injections of free L-histidine strongly decreased the signal (not shown), as expected for a nickel-chelating agent having the ability to disrupt the metal-mediated linkage. A dissociation constant of

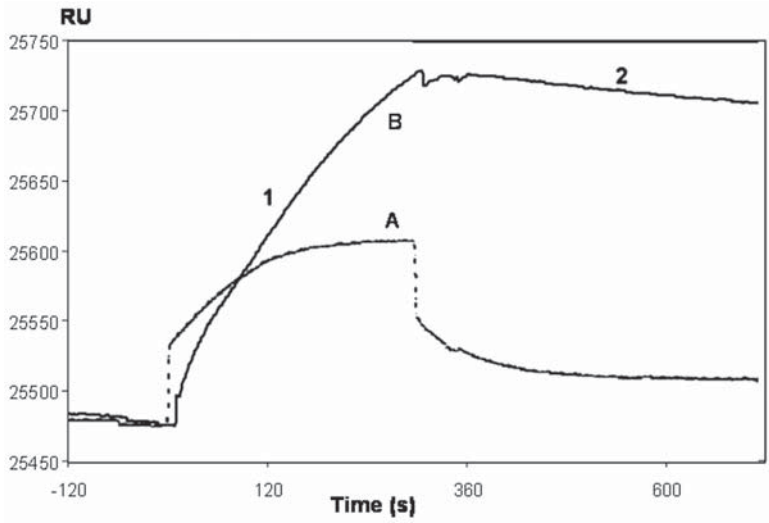

Fig. 6. Sensorgram of DNA-free [Hb5]His_4 and P-DNA interaction with membrane. The left baseline corresponds to the hybrid bilayer (DPMC/DOGS [10/1 by mole]) structure. Two injections were performed in order to analyze the kinetics of Hb5(His_4) (curve B) and P-DNA (curve A) association/dissociation: 1. 1 μ*M* Hb5(His_4) and P-DNA were injected at 25°C with a flow rate of 10 μL/min; 2. washing step.

50 n*M* was deduced for Hb5$[His]_4$. This value is very close to the value for the native protein and is much lower than the one (>1 m*M*) reported for the acylated soluble proteins ***(14)***. This is an important result that validates the strategy of cytochrome b5 molecular engineering because (1) the modification of the type of membrane attachment (chelate vs hydrophobic binding) did not alter the affinity for the lipid matrix; (2) in contrast to the natural b5, the engineered protein used did not tend to destroy the hybrid bilayer (even for large concentrations above 25 μ*M*) and did not interact with a DOGS-free hybrid bilayer; and (3) the protein layer can be removed in gentle aqueous conditions by chelate competitors, which makes biosensor regeneration easy to carry out.

3.2.3. Building of P-DNA Chips

3.2.3.1. Interaction of Various P-DNAs With Hybrid Bilayer

SPR experiments have been performed to test the ability of *cis*platin- and DPDPB-coupled P-DNA to form supramolecular assemblies with the nickel-iminodiacetate-functionalized bilayer. As illustrated in **Fig. 6**, curve A, when a 1 μ*M* solution of DPDPB-coupled P-DNA was injected in the same conditions as for Hb5$[His]_4$, a 30-RU response signal was observed. To reach a level of coverage similar to the one observed with Hb5-$(His)_4$, a longer injection was

necessary. Under experimental conditions that give rise to a saturation with uncoupled Hb5-(His_4), the response for P-DNA was far from saturation with a coverage density about twofold lower. A value of 300 RU corresponding to coverage of 1.25 pmol/cm^2 of P-DNA was observed after a binding time of 20 min ***(5)***.

Kinetic constants were evaluated in a range of concentration between 0.1 and 2.5 μ*M* at 25°C and illustrated a lower affinity (K_D = 0.5–1 μ*M*) of P-DNA for the supported bilayers compared with what was observed with the DNA-free protein. The decrease in the affinity could be explained by the larger size and the strong negative charge of the P-DNA compared with the uncoupled protein. Therefore, the ssDNA moiety of the complex probably introduces some steric hindrance as well as some electrostatic repulsive interactions with the negatively charged membrane surface that together reduce the affinity of the complex for the bilayer. However, this reduced affinity is not a major drawback because the supramolecular complex once formed was found to be stable enough to allow most interactions to take place. This indicates that the large size of the complex does not cause a rapid extraction of the lipid anchors from the phospholipid matrix, or a rapid break of the chelate-mediated link. Preliminary experiments indicated that the complex can be further stabilized by lowering the temperature, probably because the induced phase transition of the lipid layer likely slows down the residual extraction of the anchor lipid from the bilayers.

3.2.3.2. Spectroscopic and SPR Analysis of Hybridization

To evaluate the influence of protein coupling on the hybridization properties of the ssDNA, the melting temperature of the double-stranded DNA formed by hybridization of a free ssDNA sequence complementary to the nucleic acid part of the P-DNA was determined. Melting temperatures of DNA duplexes were determined on a Uvikon 941 spectrophotometer using Bio-Tek software (Bio-Tek Kontron, St Quentin en Yvelines, France). Free ssDNA or P-DNA (cisplatinum coupled) was incubated with the complementary ssDNA at a final concentration of 0.5 μ*M* in 0.1 *M* $NaClO_4$, pH 4.0. The duplex sample was heated at 80°C (for the free ssDNA) or 65°C (for P-DNA), and the temperature was allowed to decrease to 2.5°C at a rate of 1°C/min. Absorbance changes were monitored at 260 nm. Samples were then heated at a rate of 1°C/min from 2.5 to 60°C in order to check that hybridization and fusion curves were consistent. The melting temperatures in 0.1 *M* $NaClO_4$, pH 4.0, were found to be 45 and 23°C for the free duplex and the [Hb5-$(His)_4$] complex, respectively (**Fig. 7**). This result suggested that the stability of the duplex DNA was significantly reduced on coupling to [Hb5-$(His)_4$]. Steric interaction with the protein

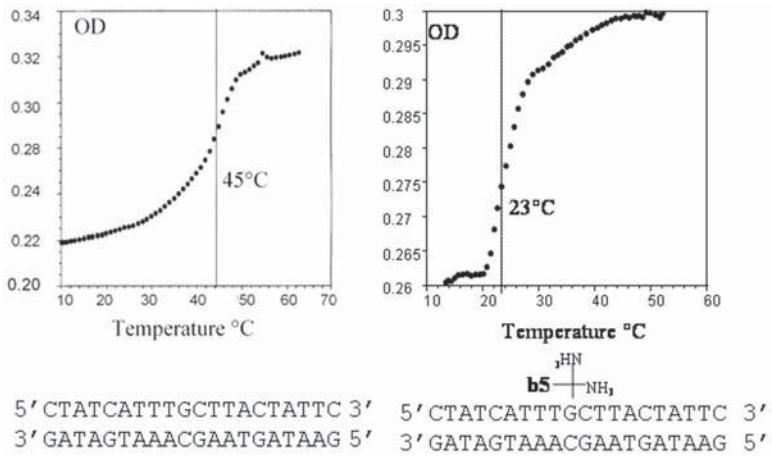

Fig. 7. Melting analysis of double-stranded free DNA and cisplatinum-coupled P-DNA. The melting of each 20-base-long double-stranded oligonucleotide was analyzed by duplex optical adsorption at 260 nm in 0.1 $NaClO_4$, pH 4.0, with the temperature scanning rate set at 1°C/min.

moiety and/or geometric distortion of duplex DNA owing to guanine platination likely contributed to the phenomenon ***(15)***. Nevertheless, the fusion curve for P-DNA showed a clear single-step transition, suggesting that a real hybridization and not some nonspecific interactions had occurred. In addition, control experiments with unrelated DNA sequences of similar length did not exhibit any signal ***(5)***.

Hybridization of the bilayer-coated P-DNA (1 pmol/cm^2 of membrane) with the complementary nucleotide sequence was realized at different temperatures below 30°C. In such conditions, the duplex DNA within the complex in solution was found to be stable based on the previous fusion curve analysis. Complementary ssDNA (50 µg/mL in buffer P1 or phosphate-buffered saline) was injected onto the membrane-bound device. Based on RU signal, hybridization was only observed for sodium chloride concentrations >150 m*M* (**Fig. 8**). This result is consistent with the expected stabilization of DNA duplex by reduction of the DNA negative-charge electrostatic repulsion following increases in ionic strength. Under such conditions, the observed response of 55 RU was half that expected for a full hybridization. Furthermore, when a noncomplementary ssDNA was injected, no signal was observed, highly suggesting that observed signals resulted from a specific interaction.

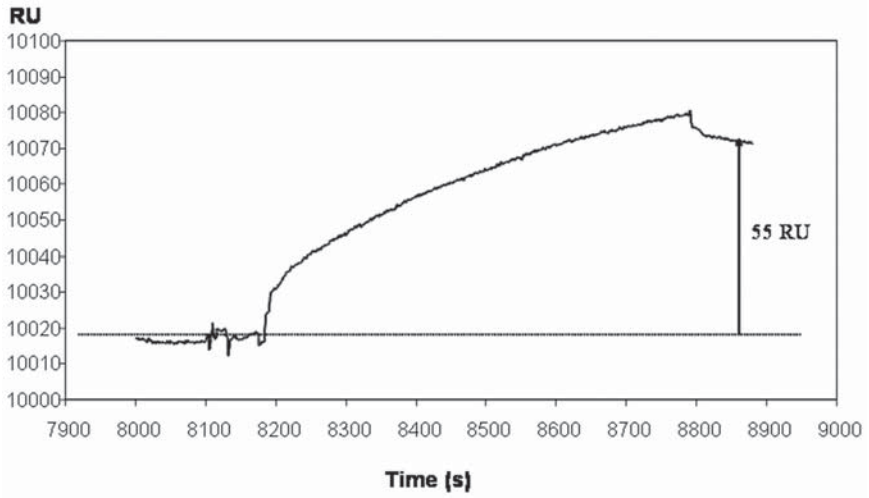

Fig. 8. SPR hybridization of DPDPB-coupled P-DNA with complementary oligonucleotide. The initial baseline corresponds to the deposition of 1.25 pmol/cm^2 of P-DNA onto the lipidic upper layer. A single injection of complementary ssDNA yielded a specific stable association response (55 RU) after washing. The complementary ssDNA concentration was 25 µg/µL, and the flow rate during the injection was 10 µL/min at 25°C.

3.2.4. Unique Properties of P-DNA Chips

The designed device is a much more well-defined molecular organization than the stochastic one observed in classic DNA chips resulting from the random deposit of oligoprobes on a poorly defined support layer generally constituted of a positively charged polymer that acts as a nonspecific binder for the nucleic acids. Because of the limited affinity of such interaction, additional crosslinking of nucleic acids is generally required to stabilize the device. Such crosslinking could partially depreciate the nucleic acids' properties. Data indicate that in such a classic chip only a few percent of probes are really accessible for hybridization, thus dramatically limiting sensibility. The P-DNA concept minimizes nonspecific interactions since the negatively charged supported membrane does not interact with DNA and, on the contrary, it maximizes the base-pairing specific recognition by presenting the probe oligonucleotide in solution away from the interface. Additionally, the unique lateral mobility properties of the device favor probe accessibility even in conditions leading to a high level of probe saturation.

P-DNA chip design includes the possibility of accurately controlling the density of immobilized probe onto the biomimetic layer, allowing optimization of the ratio between sensitivity and specificity for particular applications.

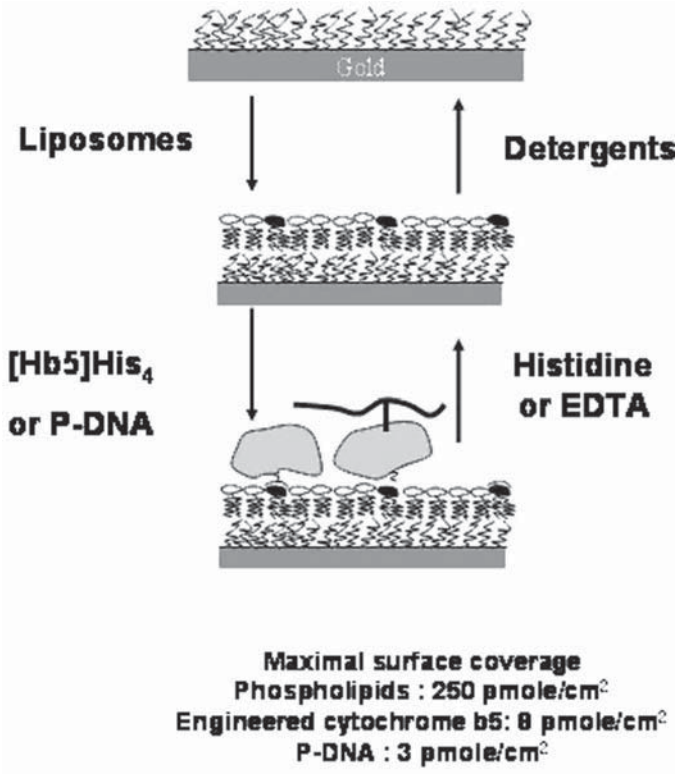

Fig. 9. Schematic for reversible self-assembly of P-DNA.

The coverage density can be accessed by SPR. Calculated saturating densities for phospholipids, cytochrome b5, and P-DNA on the sensor are summarized in **Fig. 9**. A remarkable advantage of SPR is that it allows real-time monitoring of the variation in density when varying the DOGS dosage in the lipid layer. Another advantage is the controlled reversibility of the self-assembling reactions that make chip regeneration possible (**Fig. 10**).

4. Notes

1. The presented strategy for the coupling of engineered cytochrome b5 with the oligoprobes involved a homobifunctional linker and thiolated oligonucleotides. This approach works well but is rather tricky to implement because of the difficulty in reproducibly synthesizing thiolated oligonucleotides of good quality, the rather high cost of such synthesis, the need to deal with oxygen-dependent oxidation of thiolated DNA, the need to tightly control the coupling step with the homofunctionalized linker to avoid formation of cytochrome b5 or oligoprobe homodimers, and the resulting need for a good purification that leads to some

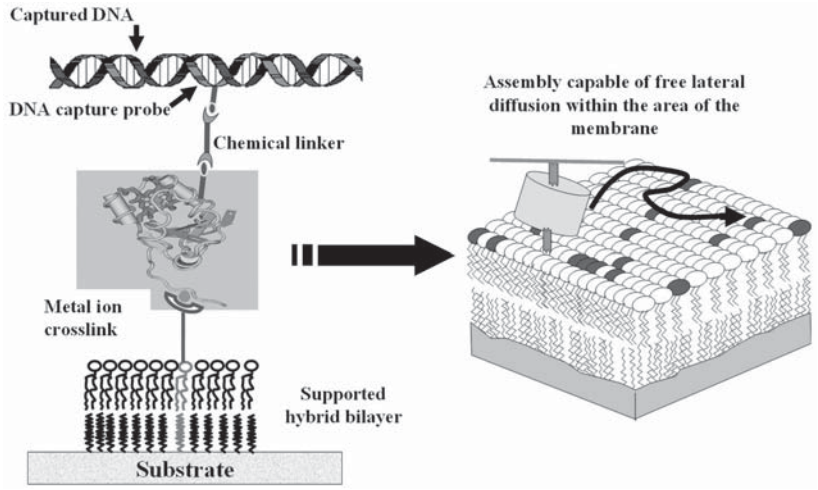

Fig. 10. P-DNA as floating DNA sensor capable of lateral diffusion.

decrease in final complex yields. To solve these drawbacks, we recently modified the procedure by using amino-modified instead of thiolated oligonucleotides. Such amino-modified oligoprobes (commercially available as 3'-, 5'-, or internal base modifications) are widely use in classic chip technology; they also are simpler to synthesize at much lower costs. The homobifunctional linker has to be changed for a heterobifunctional linker including both a thiol-reactive and an amine-reactive extremity. A large number of such linkers are available from commercial sources. The coupling of the linker with the oligoprobe was first performed. This led to a single possible product with no possibility of dimer formation. The purified adduct was then coupled to the modified cytochrome b5 as previously described for the homobifunctional linker. Globally, the yield is improved and the costs and number of building steps are reduced.

2. Self-assembly of supported lipid bilayers by liposome (or small unilamellar vesicle) fusion is classic and well described in the literature. However, this process can lead to a coverage being incomplete owing to the presence of impurities in the substrate or in the vesicle solution. An alternate cause can also be a too large dispersion of the vesicle sizes. The process can also lead to multilayer formation when incubation time for the fusion step is too long or when an unadapted phospholipid composition is used. These multilayers introduce heterogeneities and instabilities in chips. To limit and control such phenomena during SPR monitoring of vesicle fusion, each injection of phospholipid (typically 1 m*M* at 25°C for 20 min) was followed by repeated injections of 20 m*M* sodium hydroxide in order to remove phospholipid digitations resulting from incomplete vesicle fusion. This can account for up to 20% of the SPR signal and causes major signal instabilities. The preparation of vesicle solutions implies repeated sonications, which can cause a progressive chemical degradation of phospholipids, particu-

larly metal dissociation from those modified with nickel iminodiacetate. It is thus useful following the construction of bilayer to reload the modified lipid (DOGS) with nickel by injecting a 50 m*M* nickel chloride solution in sodium acetate buffer, pH 6.0. This step is also required following biochip regeneration by histidine or EDTA injections.

3. Each step of the whole chip setup has to be carefully controlled by systematic SPR measurements. For example, the binding of Hb5$(His)_4$ is evaluated on a supported phospholipid bilayer including 10% mol/mol DOGS. It is highly recommended that one test that no interaction can be detected when only DOPC or DMPC but no modified lipid is included, and also that one check the integrity of the supported layer by verifying the absence of interaction of a protein such as cytochrome-*c*, which normally does not interact with bilayers of good quality.

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