Dendrislides, dendrichips: a simple chemical functionalization of glass slides with phosphorus dendrimers as an effective means for the preparation of biochips

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Materials with tailored surface properties are useful for diverse applications, in particular for adsorption and/or covalent immobilization of various biomolecules such as nucleic acids and proteins. One of the main challenges is to design a reactive surface chemistry for stable binding of biomolecules on the support, which in addition keeps away the biomolecules from the support to reduce interference. We report here a novel support functionalization that is based on the formation of a reactive layer, covalently grafted on glass slides onto which amino-modified DNA probes were covalently fixed. The layer is composed of spherical home-made neutral phosphorus dendrimers containing a large number of aldehyde functions at their periphery. The method of preparation of these dendrimer-activated glass slides (so-called dendrislides) is performed in a few steps and results in stable activated supports, since aldehyde dendrimers are stable compounds. After immobilization of nucleic acids the so-called dendrichip products were investigated by means of hybridization experiments using complementary fluorescent labelled-oligonucleotide targets. Our results indicate that this novel grafting technology leads to surfaces with a high binding capacity for amino-modified oligonucleotides compared to commercially available aldehyde slides and with a detection limit of 1 pM labelled targets. Since links between the surface, the dendrimers and the nucleic acids are covalent, the dendrichips could be re-used up to 10 times without significant change in the fluorescence signal intensity. Finally, the performance of dendrichips in detection of a single base mutation was demonstrated.

Introduction

Microarraying[1] is rapidly becoming the technique of choice for gene expression analysis[2] and has also applications in diagnostics,[3,4] genotyping of Single Nucleotide Polymorphisms (SNPs) and pharmacogenomics.[5,6] The technique is conceptually simple – involving the arraying of nucleic acids (Polymerase Chain Reaction (PCR) products, oligonucleotides, cDNAs) onto solid support, each in a well-determined position. The array is then hybridized using a labelled target and the matched sequences are identified by position and fluorescent signal intensity.[7,8] However, the production of microarrays involves a number of steps, all of which can affect the quality of the array and the data produced. Some of the factors known to affect the quality of microarrays are the purity and the concentration of DNA to be arrayed, the slide surface chemistry, the target purification and labelling, the hybridization method, and the software used to interpret the large amount of data which can be produced.

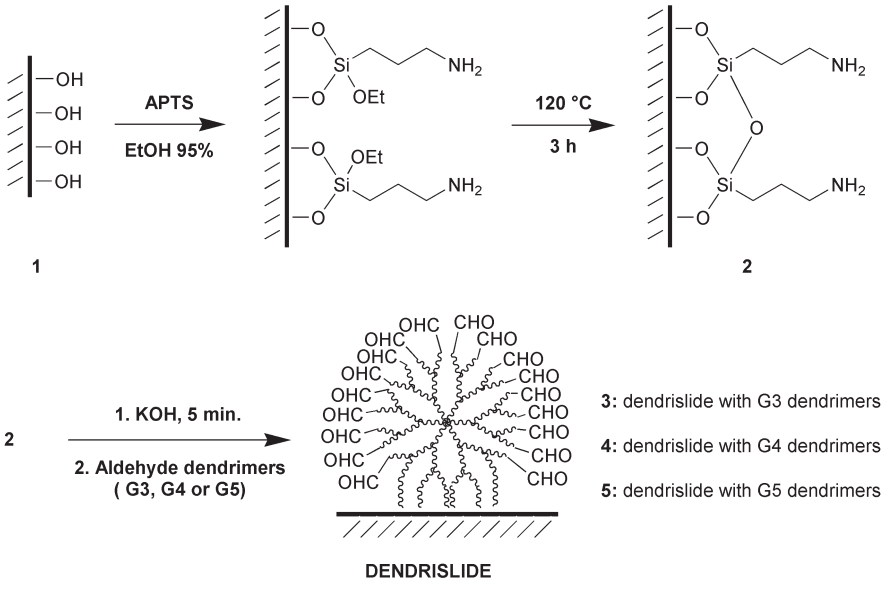
Due to its low fluorescence and cost, transparency, and resistance to high temperature and organic solvents, glass has been often used for DNA array technology.[9] Microscope slides have been commonly used because they are easy to handle and adaptable to automatic readers. Two main strategies have been developed. The first one involves the *in situ* spatially addressable parallel synthesis of oligonucleotide probes by photochemistry.[10] The second method involves the immobilization of pre-synthesized oligonucleotides or PCR products by means of spotting devices[11] or ink-jet technology.[12] This method has also the potential to be applied to arrays containing proteins[13] and low-molecular-weight molecules.[14] In the case of DNA microarrays, the pre-synthesized probes could interact with the support *via* noncovalent or covalent links. The most popular commercially available substrates for spotting DNA are aminosilane or polylysine coated glass slides[15] which allow electrostatic interactions between nucleic acids and the surface. However, in these cases, DNA molecules are susceptible to being removed from the surface under high salt or high temperature conditions. In this type of immobilization the probes are generally spread on the support, which can reduce the accessibility to the targets during hybridization. To solve this problem, a variety of covalent coupling reactions have been used to bind pre-synthesized probes onto a reactive surface. Glass surfaces can be modified by silane-chemistry to introduce specific functions such as amino, epoxide, carboxylic acid or aldehyde groups. The nucleic acid probes are immobilized through introduction of functional groups on either the 5′- or 3′- terminus. Amino-terminal oligonucleotides can be bound to isothiocyanate-activated glass,[16] aldehyde-activated slides,[17] or epoxide-modified glass surfaces.[18]

An ideal chemically-derivatized support should allow the stability of DNA attachment throughout hybridization and washing steps together with a high binding capacity by increasing the number of reactive sites to which the probes can be covalently bound. Furthermore, probes should be spaced out from the surface to reduce steric interferences between the support and the immobilized oligonucleotides and solid–liquid phase perturbations. Shchepinov *et al.*[19] described that an optimal spacer should possess 40 atoms in length and if possible should not contain positive or negative charges in its structure. Two major achievements have been reported in the past few years that have progressively replaced the low efficient non-covalent binding of DNA on poly-L-lysine coated glass slides.[15] The first progress was the covalent binding of nucleic acids probes onto silanized glass slides *via* 4 to 12-carbon units spacers, ending up with an epoxy, aldehyde or isothiocyanate reactive function.[16,17,18] The grafting of the DNA probes occurs by their 5′-termini, which must be amino or thiol-modified, depending on the chemical reactive group on linkers. While good quality, accuracy and reliability of these microarrays were demonstrated,[20] the linear structure of the linker still leads to a planar surface structure on the glass-slide, which neither increases very much the accessibility of the targets to the probes nor the loading capacity of the solid support. Moreover, these microarrays cannot withstand frequent regeneration steps. To overcome these failures at least in part, a second major advancement has been to build up a pseudo-three-dimensional structure using the so-called 'dendrimeric' linker system. A significant contribution to this achievement was provided by the work of Beier and Hoheisel who generated a dendritic linker by direct chemical reaction on the glass slide or on polypropylene surfaces.[21] With this structure, they could show an increased binding capacity of the support for the probe beyond that of the glass slide, a high stability of the bonding of the dendrimer to the support, and a capacity of the microarray produced with these activated supports to withstand thirty consecutive cycles of hybridization and stripping without loss of signal intensity. However, the dendritic system on the support must be build '*in situ*' by several-time-consuming chemical steps, precluding any quality control of the linkers during the process. Another work describes the use of prefabricated polyamino-functionalized 'Polyamidoamine (PAMAM) starbust' macromolecules for the manufacture of biochips, which includes an activation of dendrimer surface layer prior to coupling with the DNA probes.[22] Though this procedure likely provides the best quality for DNA chips in terms of homogeneity of the spots, high sensitivity, low background and reusability, this technique involves several reaction steps to produce the dendrimer-activated slides with intermediate activated slides that have to be stored at −20 °C. Furthermore, after grafting of PAMAM dendrimers and their activation, as succinimidyl- or isothiocyanate- intermediates, for the reaction with the nucleic acids, the probes must be grafted immediately, which might preclude a large scale development and any storage.

We report here a method based on the formation of a reactive layer, covalently grafted on glass slides onto which amino-modified DNA probes were covalently fixed.[23] The layer was composed of spherical home-made neutral phosphorus dendrimers that contain a large number of aldehyde functions at their periphery.[24] The method of preparation of these dendrimer-activated glass slides (so-called dendrislides) was performed in few steps (Scheme 1) and leads to stable activated supports, as we have already shown.[25] The dendrislides can be stored at room temperature for at least three months and can then be used for the covalent attachment of 5′ or 3′-amino-modified nucleic acids. The resulting products, so-called dendrichips, were investigated by means of hybridization experiments.

Experimental section

Deionized water was used in all experiments. Oligonucleotide probes were synthesized by standard phosphoramidite methods by Eurogentec (Seraing, Belgium). An amine was attached to the 5′-terminus *via* a 6-carbon alkyl spacer. APTS (Aldrich) was used without further purification. Dendrimers with CHO end groups, generation 3 to 5 (G3, G4 or G5), were synthesized according to a method developed in the laboratory and published earlier.[24] Sequences used: doubly functionalized oligonucleotide: 5′-Cy5-TTT AGC GCA TTT TGG CAT ATT TGG GCG GAC AAC TT-NH_2-3′ 35-mer probe sequence: 5′-NH_2-GTG ATC GTT GTA TCG AGG AAT ACT CCG ATA CCA TT-3′. Target: 5′-Cy5-AAT GGT ATC GGA GTA-3′ or 5′-Cy3- AAT GGT ATC GGA GTA-3′. Sequences used for mutation studies: 35-mer probe sequence above;



Scheme 1 Schematic representation of dendrislides preparation. The commercially available glass surface **1** was functionalized by reaction with 3-aminopropyltriethoxysilane (APTS) to give amino-slides **2**. After an alkaline regeneration step, the dendrislides were obtained by reaction with aldehydic-phosphorus dendrimers. Dendrimers of generations 3, 4 or 5 (G3, G4 or G5) were used to produce the corresponding dendrislides **3**, **4** and **5**, respectively.

target T (matched): 5′-Cy5- AAT GGT ATC GGA GTA-3′ target A: 5′-Cy5- AAT GGT A*A*C GGA GTA-3′ target G: 5′-Cy5- AAT GGT A*G*C GGA GTA-3′ target C: 5′-Cy5- AAT GGT A*C*C GGA GTA-3′. Mismatched nucleotides are italicized.

Aminosilanization of glass slides

Glass cleaning. The glass substrates (Gold Seal Microslides) were pre-cleaned by treatment in an alkaline solution consisting of NaOH (50 g) in water (200 mL) and 95% ethanol (300 mL) for at least 2 h at room temperature.[31] They were thoroughly washed with water until the pH of the washing medium was neutral and were stored in 95% ethanol until required.

Silanization. Pre-cleaned microscope slides were treated with a 10% (v/v) solution of APTS in 95% ethanol for 12 h at room temperature with gentle agitation. Slides were removed and rinsed two times with 95% ethanol, then two times with water. They were dried under a stream of nitrogen or by centrifugation (500 rpm, 5 min) and finally baked at 120 °C for 3 h.

Functionalization with dendrimers: preparation of dendrislides. The slides were dipped in an aqueous 1.3 M solution of KOH for 5 min. They were extensively washed with water and dried under a nitrogen stream or by centrifugation. The dendrislides were prepared by incubation in a solution containing phosphorus dendrimers (0.1%, w/v, generation required) in dichloromethane for 7 h at room temperature under gentle agitation. The slides were washed two times with dichloromethane, then with 95% ethanol and finally with absolute ethanol. They were dried by centrifugation or under a nitrogen stream. The dendrislides can be stored for at least three months without any particular precautions. The solution of dendrimers was evaporated under reduced pressure and the dendrimers were precipitated by addition of pentane. They can be used again for another functionalization of glass slides.

Spotting onto "dendrislides". Aminated oligonucleotide (probe 1) was diluted to the appropriate concentration in 300 mM sodium phosphate buffer (Na_2HPO_4, pH 9.0) and was spotted onto the "dendrislides" with a robotic spotter (Eurogridder, ESI/Eurogentec). The spots were approximately 100 μm in diameter and the volume dispensed was about 2 nL. The distance between two adjacent spots was 300 μm. The quality of the printing was optimal at 45% relative humidity and constant temperature of 22 °C.

Reduction of the imine functions. After incubation overnight, the slides were incubated for 3 h with sodium borohydride solution (3.5 mg mL^{-1} of water). They were rinsed once with a 0.2% SDS aqueous solution and three times with water. They were dried by centrifugation or under a stream of nitrogen. The dendrichips can be stored in a slide container at 4 °C for later use.

Hybridization. Hybridizations were carried out by adding a solution containing the complementary target Cy5-labeled oligonucleotide in saline-sodium phosphate-EDTA (SSPE: 20 mM NaH_2PO_4, 0.3 M NaCl, 2 mM EDTA) buffer, containing sodium dodecyl sulfate (SDS, 0.1%, w/v), pH 7.4, in an hybridization chamber (Corning Inc), for 30 min at room temperature. The dendrichips were then washed twice for 10 min with the washing buffer (20 mM NaH_2PO_4, 0.3 M NaCl, 2 mM EDTA, 0.1% SDS), then with diluted buffer (2 mM NaH_2PO_4, 0.03 M NaCl, 0.2 mM EDTA). After the hybridization and washing process, the dendrichips were analysed using the GenePix 4000 (Axon Instruments) which simultaneously scans microarray slides at two wavelengths (532 nm and 635 nm, photomultiplier PMT = 600) using a dual laser scanning system.

Stripping procedure. For re-use, "dendrichips" were incubated in stripping buffer (2.5 mM Na_2HPO_4, 0.1% SDS) at 95 °C for 5 min. In case of partial removal of the targets the procedure had to be repeated two or three times. The dendrichips were then washed with water and dried by centrifugation. Each DNA chip was checked by fluorescence-scanning for complete target removal prior to the next hybridization.

Autoradiography experiments. A 3′-[^{32}P]-labelled and 5′-amino-modified oligonucleotide was used to carry out the autoradiography experiments. 0.2 μL of various solutions of 10 μM, in phosphate buffer 0.3 M, unlabelled oligonucleotide containing 10^6 to 10^4 cpm of labelled oligonucleotide were spotted. The dendrislides were incubated overnight. The imine functions were reduced by immersion in an aqueous solution of $NaBH_4$ (3.5 mg mL^{-1}) for 3 h and the slides were washed first with an aqueous solution of 0.2% SDS, then with water. The radioactive slides were imaged and analyzed by phosphoimaging using Image Quant Version (Molecular Dynamics).

Results and discussion

Characteristics of the dendrimers

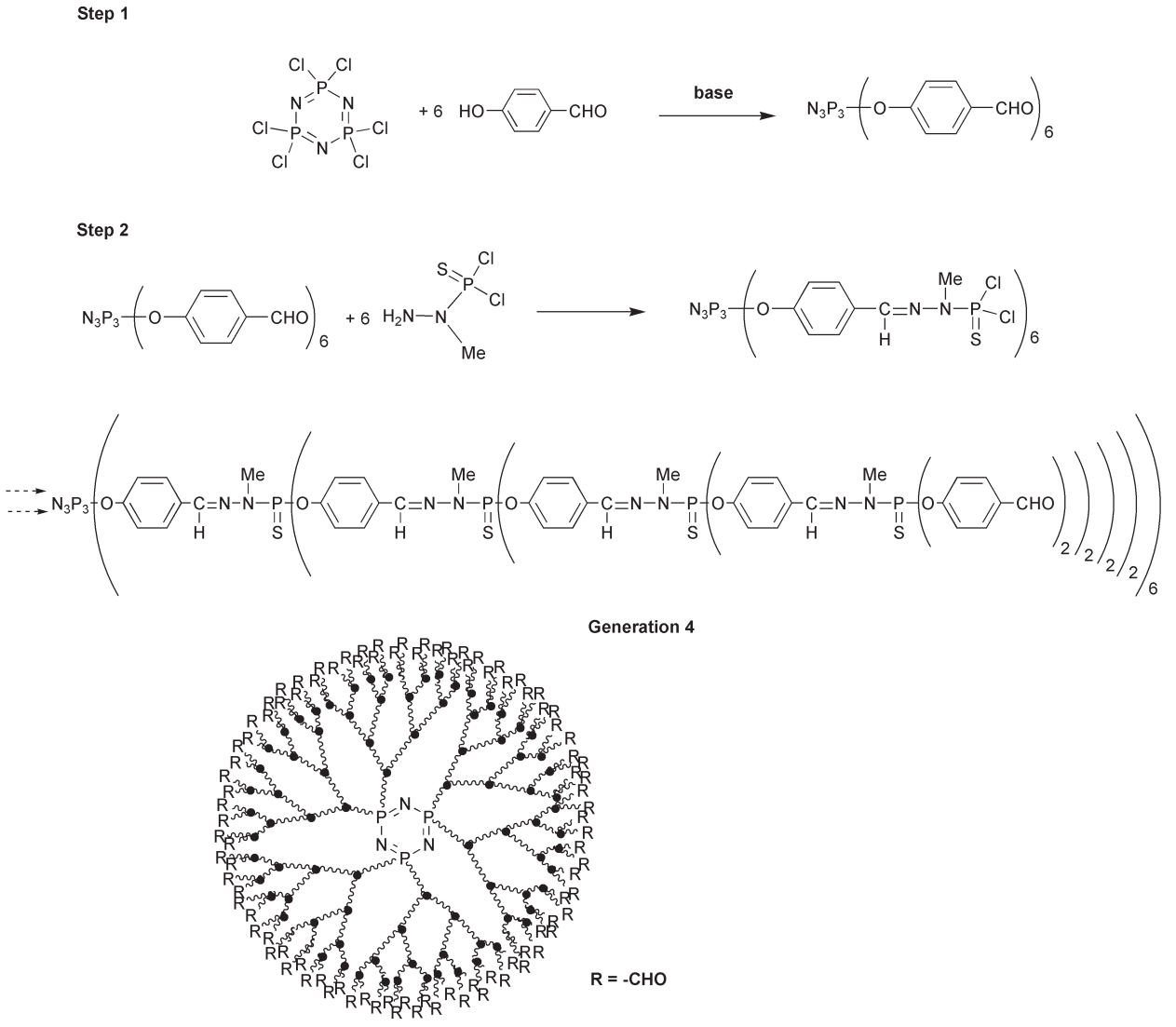
Dendrimers with aldehyde end groups are highly branched functionalized molecules formed by iterative reaction sequences. They have a nanometric spherical structure that originates from a central core linked to external ramifications to give rise to various generations. Briefly, in these dendrimers the core was the hexachlorocyclo-triphosphazene, $N_3P_3Cl_6$, which allows the introduction of six functional groups. The molecule was built by repetition of two steps[24] (Scheme 2) allowing the construction of various generations of dendrimers which only differ by the number of ramifications, and hence by the size and the number of reactive aldehyde groups at the periphery (Table 1).[26] Dendrimers from the 3rd to the 5th generation (G3 to G5) were tested in the present work.

Preparation of the dendrislides

The dendrimers play the role of spacer and linker between the surface of the slides and the nucleic acids. As they possess a large number of aldehyde functions at their periphery it was necessary to introduce a large amount of amine functions during the silanization step. The most common silanization-agent depicted in the literature is the aminopropyltriethoxysilane (APTS) which allows the preparation of amino-derivatized surfaces.[21,27] As described by Benters *et al.*[22] we performed the silanization of glass slides by applying 3-aminopropyltriethoxysilane in ethanol/water (95/5; v/v) for 20 h. This leads to the highest immobilization efficiency of aminosilane, giving the amino-derivatized surface **2** (Scheme 1). These amino-slides **2** were then coated with dendrimers containing aldehyde-peripheral groups. We used aldehyde-dendrimers from 3rd to 5th generation to prepare the dendrislides. The reaction of the active surface of slide **2** with 0.1% of different generation of dendrimers (w/v) was performed for 7 h in dichloromethane. Remarkably, the dendrimer solution after the first incubation can be re-used several times, thus significantly reducing the manufacturing cost of dendrislides.

Binding efficiency

The binding capacity of the activated support is an important criterion for the high density of coupled DNA probes, which allows high signal-to-background ratios and thus to lower



Scheme 2 The first steps for the synthesis of aldehyde dendrimers.

the limit of detection for the hybridization analysis. However, high surface densities and high hybridization signals might not be necessarily related, since steric interference between immobilized probes could hinder access to the target DNA strand, and hence reduce the hybridization efficiency.[28] To investigate the binding capacity of dendrislides, a doubly functionalized oligonucleotide containing a 3′-amino group and a 5′-Cy5 fluorophore was used. For the preparation of the dendrichip, a solution of this oligomer (2 nL) was spotted in triplicate for each concentration, onto the dendrislides (**3**, **4**, and **5**, Scheme 1) and on a commercially available slide functionalized with aldehyde groups. The concentrations of the spotted oligonucleotides were in the range from 0.01 μM to 100 μM. After spotting, the dendrislides were placed at 25 °C overnight; then the reduction of the imine function was performed and the fluorescence intensity was recorded using a fluorescence scanner (Genepix 3000, Axon, photomultiplier (PMT) = 600). As shown in Fig. 1, the dendrislides **3**, **4** and **5** exhibited the higher fluorescence intensity. Saturation of the signal intensity, *i.e.* the maximal signal obtainable with the scanner was reached with 10 μM oligonucleotides. In contrast, the commercial functionalized aldehyde slide showed 1000-fold lower signals with the same concentrations of oligonucleotides spotted, indicating a very weak binding capacity of the activated slide, probably because of the simple linker system grafted on it (lower number of reactive functions on the surface and/or lower accessibility of the DNA target). The binding efficiency was also estimated by the quantification of the covalent binding on dendrislide **4** of a 3′-[^{32}P]-labeled 5′-amino-modified oligonucleotide. The binding capacity of the dendrislides **4** in the presence of 10 μM amino-modified oligonucleotide was found to be approximately 50 fmol mm^{-2}. This value is in the range of recently achieved flat slide surfaces reported in the literature.[29]

Table 1 Number of aldehyde groups and diameter of generation 3 to 5 dendrimers obtained from a N_3P_3 core (the size was determined by high resolution electron transmission microscopy)[27]

Generation	Number of CHO functions	Size (nm)
3	48	6.0
4	96	7.5
5	192	9.0

Limits of detection upon hybridization

The experiments reported above already give an indication that the glass slides functionalized with a spherical dendrimeric structure not only have higher DNA binding capacities compared to the commercially available aldehyde slides, but likely have a better accessibility to the targets. To further investigate this property, and to determine a detection limit of the signal, we performed an hybridization analysis using arrays prepared with a fixed concentration of an amino-modified oligomer

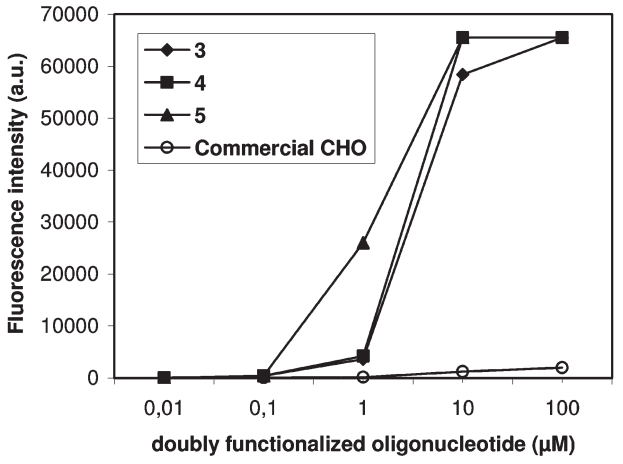


Fig. 1 Binding capacity of dendrislides **3**, **4** and **5** compared to a commercially available aldehyde slide. Single-stranded 35-mer oligonucleotide carrying a 3′ amino function and a 5′-Cy5 fluorophore was spotted on commercially available aldehyde functionalized support (○) and dendrislides (◆: **3**; ■: **4**; ▲:**5**) in concentrations of 0.01 μM to 100 μM. The signal was recorded using a fluorescence scanner (Genepix 3000, Axon, PMT = 600).

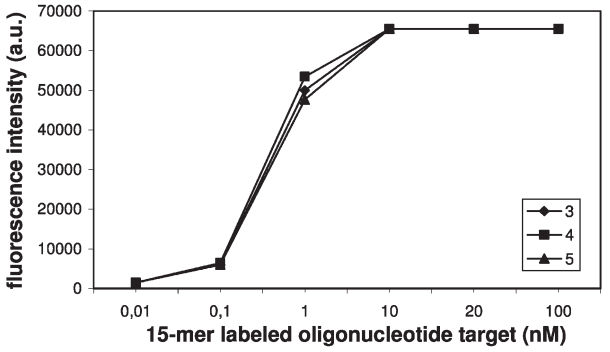


Fig. 2 Detection sensibility of dendrislides **3**, **4** and **5** and signal-to-background curves. The 35-mer amino-oligonucleotide was grafted on the different dendrislides at a concentration of 10 μM. The target Cy5-labeled oligonucleotide was hybridized at various concentrations ranging from 0.01 nM to 100 nM. The signals were obtained with a fluorescence scanner (PMT = 600).

(35-mers, 10 μM, which corresponds to 20 fmol per spot) which were hybridized with increasing concentrations, from 0.01 nM to 100 nM, of a Cy5-labelled 15-mer oligonucleotide target complementary to the probe (see experimental section for sequences of oligonucleotides). Saturation of signal intensities was reached with a concentration of 10 nM DNA target (PMT = 600) and no further increase in the hybridization signal was obtainable at higher target concentrations (Fig. 2). The detection level can be decreased down to 0.01 nM of target. Such sensitivity is particularly interesting for the analysis of low transcripts abundance and for studies using very small amounts of biological material like biopsies.

We evaluated dendrimers from 3rd to 5th generation for their capacity to lower the detection limit after hybridization experiments, and for their ability to enhance the binding capacity of glass slides compared to commercial functionalized aldehyde slides. Our results indicate that, there was no significant difference between the dendrislides **3**, **4** and **5** in terms of detection sensibility. For the following experiments we, therefore, decided to use the dendrimers of generation 4, which have a diameter size of about 7.5 nm and display 96 aldehyde functions at their periphery, since this generation is easier to produce than the next one, due to the iteration process for dendrimer synthesis, and its size is appropriate to keep away the reactive functions from the glass surface. To investigate further the detection limit of dendrislides **4** we have carried out hybridization experiments using the Cy5-labelled target at various concentrations varying from 1 nM to 1 pM. Even at this particularly low concentration the signal intensity is recordable (Fig. 3).

Re-usability

A goal of this work was also to demonstrate the re-usability of the DNA arrays generated by dendrimeric structures. This is interesting because it allows the elimination of the variation between analyses made on two different slides. To this end, arrays produced with oligonucleotides were subjected to successive hybridization/stripping processes. After hybridization of the G4-dendrichips with 2 nM of the Cy5-labelled oligonucleotide target and reading of the fluorescence intensity, the slides were regenerated by ''thermal stripping'' using a phosphate buffer (pH 7.5) containing SDS (0.1%, w/v) for 5 min at 95 °C. After each stripping the slides were scanned to ensure that the removal of the target was complete. The results

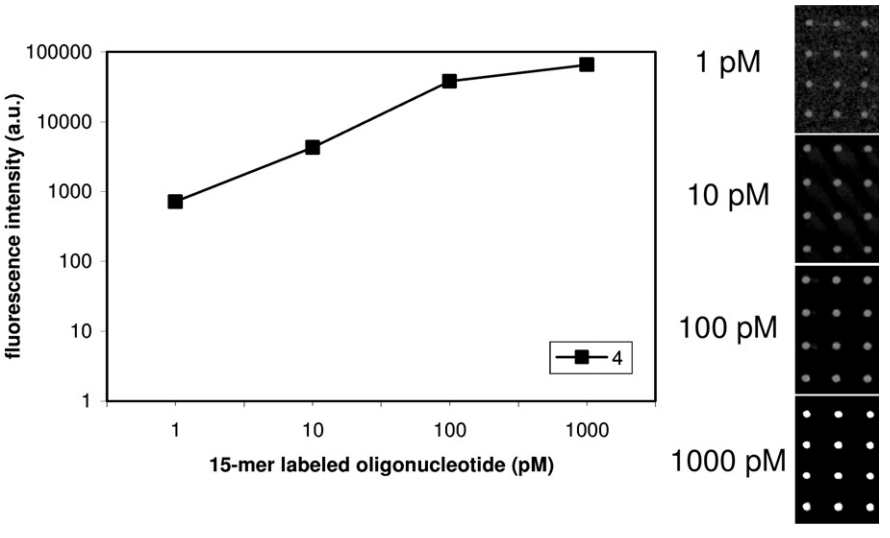


Fig. 3 Detection sensibility of dendrislides **4**. The 35-mer amino-oligonucleotide was grafted on dendrislides **4** at a concentration of 10 μM. The target Cy5-labelled oligonucleotide was hybridized at various concentrations ranging from 1 nM to 1 pM. The images corresponding to the different concentrations were also represented (PMT = 800).

showed that dendrimer-activated slides onto which 35-mer oligonucleotides have been spotted could be re-used at least 10 times without significant loss of signal intensity (Fig. 4). During the hybridization/stripping process the G4-dendrichips showed increased signal intensity after the first cycle of thermal stripping. Then the signal decreases to a plateau, corresponding to the value of the first hybridization.

Detection of single base mutation

Searching for a single mutation in a given gene (Single Nucleotide Polymorphisms, SNPs) is a research field where DNA chip technology should bring a revolution.[30] Though techniques to identify SNPs already exist and most of them rely on the Affymetrix technology, the development of inexpensive cost-effective high throughput methods to identify point mutations in DNA sequences is awaited in the context of medical diagnosis. Search of mutations in a given gene must use dedicated chips that contain all possible sequences of the DNA in which one wishes to seek these mutations. Using our dendrimer-activated support we have investigated a simple case in which four 15-mer oligonucleotides with a single base mutation at position +8 (middle) were hybridized with a 35-mer oligonucleotide probe grafted in four-replica spots on the dendrislide **4**. As indicated in Fig. 5 hybridization is only effective with the oligonucleotide sequence strictly complementary to the 35-mer oligomer, while very weak fluorescent signals were obtained with the three other 15-mer oligonucleotides.

Conclusions

The exponential development of genomics and pharmacogenomics related to the sequencing of microbial, plant and human genome leads academic and private laboratories to use in a massive way, reliable, cheap and easy to use DNA chips. To this end, we have developed a novel dendrimer-activated glass surface to produce very cost-effective biochips. These dendrimer-activated glass slides display most of the suitable criteria that one should expect for accurate and reliable DNA arrays. Since the production of dendrimers and the chemistry for activation of the surface are relatively simple, this process could be adapted at an industrial scale. Moreover, we found that our new dendrislides were still active after three months of storage at room temperature. Since other functions like thiol, epoxy, *etc.* can be introduced at the periphery of the

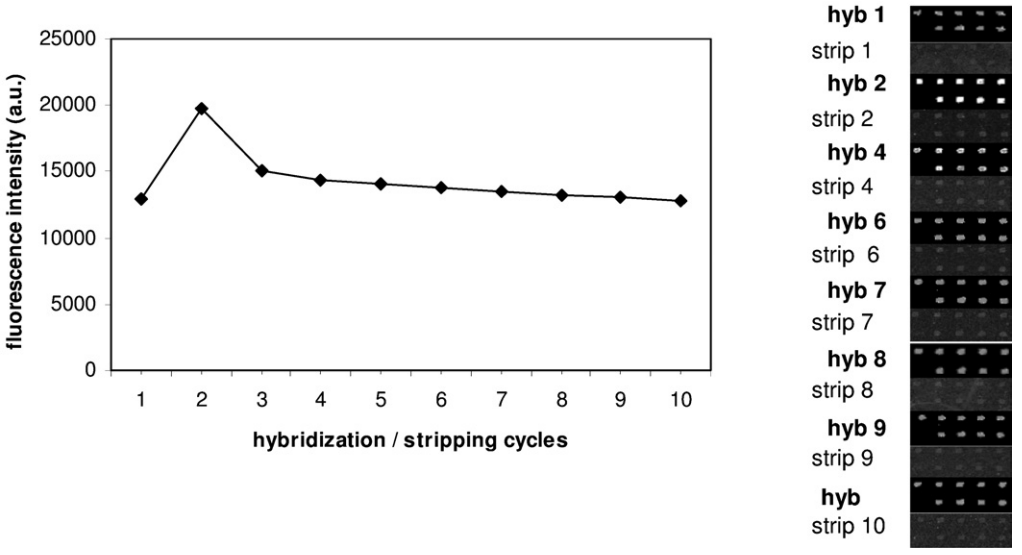


Fig. 4 The dendrichips were prepared by spotting 2 nL of 5′ amino-modified 35-mers oligonucleotide at a concentration of 10 μM. The hybridization was carried out using the target Cy5-labeled 15-mer oligonucleotide at a concentration of 2 nM. Thermal conditions were used for the stripping of the arrays. The images obtained after hybridization/stripping cycles were also represented (PMT = 600).

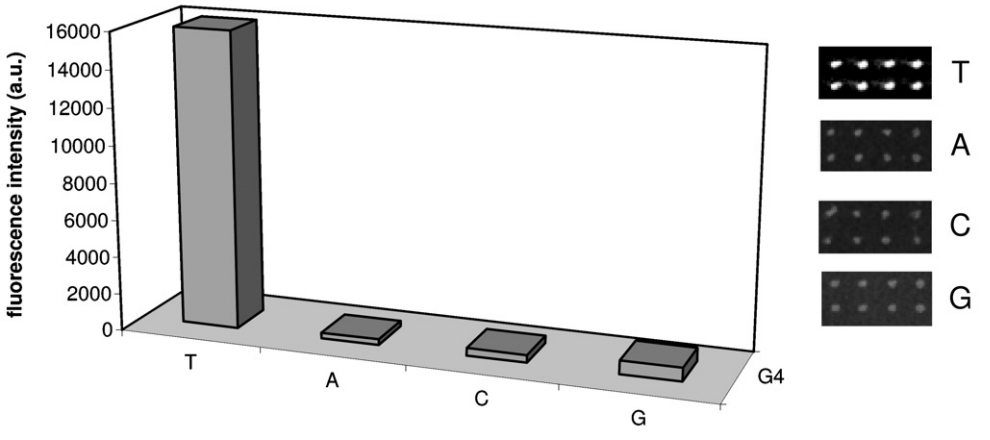


Fig. 5 Mutation studies. The amino-oligonucleotide was spotted on the G4-dendrislide in phosphate buffer 0.3 M at four different positions. Hybridization was carried out using 4 different target labelled oligonucleotides (the mismatched position is at position + 8, in the middle of the sequence, PMT = 600). The target oligonucleotide with T base in the middle of the sequence is the oligonucleotide perfectly complementary to the probe.

dendrimer structure, these polymers are particularly attractive for the immobilization of other macromolecules such as peptides, proteins, antibodies, lipids, *etc.*

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