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SPECIAL ISSUE: Diagnostic and Theranostic Platforms Based on Dendrimers and Hyperbranched Polymers

Multiplexing technology for *in vitro* diagnosis of pathogens: the key contribution of phosphorus dendrimers

Jean Pierre Majoral^{1,2,3*}, Jean Marie François^{3,4}, Richard Fabre³, Alice Senescau³, Serge Mignani⁵ and Anne-Marie Caminade^{1,2}

ABSTRACT After the microbiology based on Pasteur's method and polymerase chain reaction (PCR), the diagnosis company named Dendris has proposed a third-generation of diagnosis enabling the search of a broad range of pathogens with strong sensitivity and specificity. This extraordinary profile was possible thanks to the use of phosphorus dendrimers for which various techniques of deposition on a given support were investigated and described and analyzed in this report.

Keywords: phosphorus, dendrimers, diagnosis, DNA arrays, multiplexing technology

INTRODUCTION

Successful use and reliability of microarrays technology are highly dependent on several factors including surface chemistry parameters and accessibility of cDNA targets to the DNA probes fixed onto the surface. Activated glass slides were commonly used because of their great chemical resistance against solvents, mechanical stability and low intrinsic fluorescence properties. Their performances, i.e., signal intensity, signal-to-noise ratio, spot homogeneity are directly influenced by the amount of DNA probes attached to the surface, the probe length and the accessibility of the labeled targets to the probes [1,2].

While good quality, accuracy and reliability of several microarrays were mentioned, most of them were suffer-

ing from the non-linearity of the structure of the spacer which leads to neither a significant increase of the accessibility of the target to the probes nor of the loading capacity of the support [3–6].

To overcome these major drawbacks, dendrimers (monodisperse nanometric spherical structures), which can be tailored at will, were used as linkers. However difficulties encountered for their multistep synthesis, and low thermal stability of some of these nano-objects prevent from their common use for biological applications [7–12].

In marked contrast, the original contribution of stable phosphorus dendrimers as linkers for DNA based multiplexing technology for rapid and accurate diagnosis of pathogens can be emphasized and will be illustrated in this report through the original methodology developed by the Dendris' company.

PHOSPHORUS DENDRIMERS DEDICATED FOR GRAFTING BIOMOLECULES

The first experiments concerning the design of dendritic bio-arrays starting from phosphorus dendrimers were reported in 1999 using a phosphorus dendrimer of generation 5 built from a trifunctional core and bearing 96 terminal aldehyde groups [13]. This dendrimer was added to quartz or glass slides pretreated by aminopropyltriethoxysilane [14]. Atomic force microscopy (AFM)

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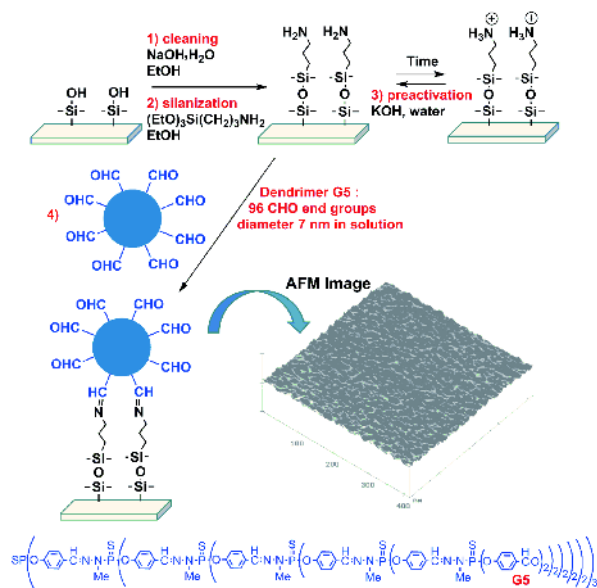


Figure 1 Grafting of aldehyde terminated phosphorus dendrimer on aminosilanzed glass slide.

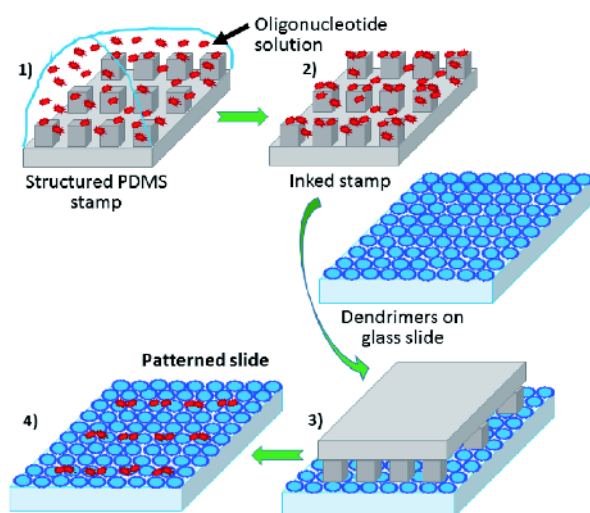


Figure 2 Microcontact printing of DNA involving four steps: 1) inking of the stamp with oligonucleotide, 2) drying under nitrogen stream, 3) contact of the glass slide functionalized by dendrimers with the inked PDMS stamp, 4) transfer of oligonucleotide from the PDMS stamp to obtain the patterned slide.

experiments clearly showed the perfect coverage of the surface of the glass slide (the thickness of the dendrimer layer on the glass slide was found to be 3.7 nm) (Fig. 1). The interaction of this surface with proteins bearing NH_2 units such as human serum albumin (HSA) was also shown by AFM.

Such a methodology was applied later on for the formation of DNA microarrays involving phosphorus den-

drimers obtained from a hexafunctional core and bearing 48 or 96 aldehyde end groups [15], which allow both the direct grafting on aminosilanzed glass slides and in a second step the reaction of the remaining aldehyde groups with aminooligonucleotides [16–17].

Grafting to liposomes oligonucleotides complementary to the nucleotides bound to these DNA chips led to nanocapsules arrays potentially usable for the encapsulation and spotting of proteins keeping their conformation and activity within the liposomes [18].

The phosphorus dendrimer of generation 4 decorated on its surface with 96 aldehyde groups can be also linked to a silica piezoelectric membrane. In this particular case the hybridized complementary oligonucleotide does not bear a fluorescent label but a biotin label which can be recognized by streptavidin-conjugated gold nanoparticles. The modification of the resonant frequencies of these functionalized piezoelectric-membranes was measured *via* an optical beam deflection technique [19].

Another method to create DNA arrays starting from phosphorus dendrimers consists in using stamps for microprinting (Fig. 2). Recent studies reported the use of an automated microcontact printing device using soft-lithography technology to produce low-density multiplexed DNA microarrays. This automatically allows a multiplexed deposition of oligo probes on a functionalized surface by the use of a macrostamp bearing 64 individual pillars each mounted with 50 circular micropatterns (spots) of 160 μm diameter. A fast and efficient washing step in ethanol made this macrostamp reliable and reusable. The low-density microarrays printed on either epoxysilane or phosphorus dendrimer functionalized slides show excellent hybridization responses with complementary sequences at unusually low probe and target concentrations ($1 \mu\text{mol L}^{-1}$ and 1 nmol L^{-1} , respectively). Comparison of analysis of microarrays obtained from mechanical spotting and automated microprinting method highlights the importance of the accessibility rather than the absolute density of the probes in the hybridization response [20].

A sophisticated highly sensitive DNA hybridization was reported using nanotubes of dendrimers formed inside an ordered porous alumina membrane. The nanotubes were obtained by layer-by-layer (LBL) deposition of negatively and positively charged phosphorus dendrimers on the pore walls. After deposition of three bilayers of anionic and cationic dendrimers, a first layer of negatively charged quantum dots (QDs) was deposited. The alternate deposition of polycationic dendrimers with several other layers of three different luminescent QDs offered a

graded-bandgap structure. Then probe oligonucleotides were immobilized, and hybridized with target fluorescent labeled oligonucleotides. An efficient excitation energy transfer occurs by fluorescence resonance energy transfer (FRET) from the outer to the inner surface of the dendrimer nanotubes and allowed detection of hybridization with a very high sensitivity: $2\text{--}5\text{ pmol L}^{-1}$ (Fig. 3) [21,22].

Dendrimer multilayers on gold substrates prepared by LBL assembly technique were characterized and used as substrates for DNA immobilization/hybridization. The multilayers were built using alternatively polycationic and polyanionic phosphorus dendrimers of generation 4: a high loading of DNA probes was obtained through covalent coupling of probe DNA on dendrimer multilayers film. The following hybridization of Cy5 dye labeled complementary target DNA with immobilized probe DNA was detected by surface plasmon field-enhanced fluorescence spectroscopy. The limit of detection of target DNA upon hybridization reached 50 pmol L^{-1} and 30 pmol L^{-1} on 1 bilayer and 4 bilayers, respectively (Fig. 4) [23].

A bilayer formed with a perylene diimide labelled star polymer donor A and a cationic phosphorus dendrimer B (Fig. 5) on a silver substrate was constructed by layer-by-layer approach. Using surface plasmon enhanced fluorescence spectroscopy technique, a time-resolved ultra-sensitive ($10^{-18}\text{ mol L}^{-1}$) and selective detection of DNA targets relying on enhanced optical fields associated with energy transfer were achieved under the excitation at 543 nm . The detection limit is about 8 orders of magnitude better than the achieved one under the excitation at 632 nm , which is ascribed to no energy transfer from the donor to the acceptor under the excitation at 632 nm , resulting in much weaker detection signal in turn (Fig. 6) [24].

A strategy to covalently immobilize biomolecules on AFM tips was also reported on the very classical glutathione-S-transferase (GST)/anti GST protein pair. The AFM tips were first functionalized with amino groups then incubated in a phosphorus dendrimer solution, the dendrimer of generation 4 being decorated with 96 aldehyde groups on its surface. The free aldehyde groups on the surface of the dendrimer are available to react further with amino groups present on every protein and biomolecules. Indeed the dendrimer is acting as a linker between AFM tip and biomolecules. Using these bio-functionalized-dendritips, single molecule force interactions between GST immobilized on a slide functionalized with phosphorus dendrimers (dendrislides), and its cognate antibody immobilized on dendritips were probed by

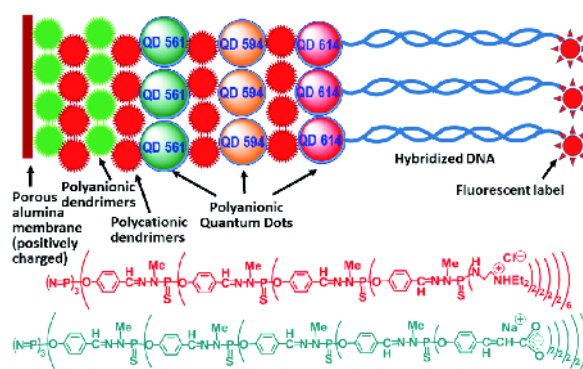


Figure 3 Graded-bandgap structures based on charged phosphorus dendrimers and quantum dots inside an ordered porous alumina membrane.

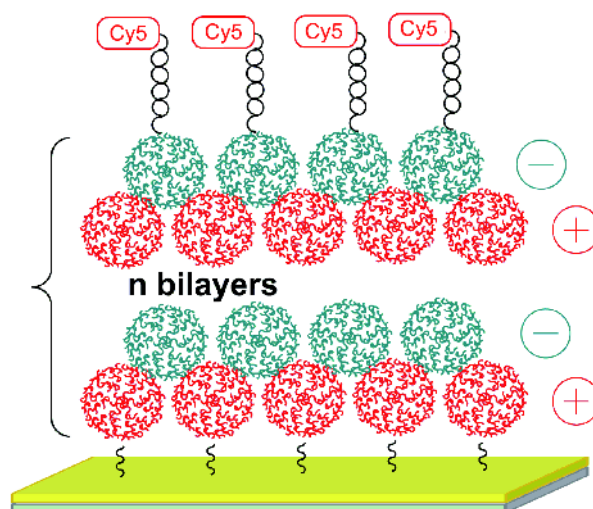


Figure 4 Hybridization of Cy5 complementary target DNA with DNA immobilized on phosphorus dendrimer multilayers (structures of the dendrimers are shown in Fig. 3).

AFM spectroscopy with high specificity [25].

THE DENDRIS WAY

All the technologies briefly presented up to now, appear of interest but suffer from numerous drawbacks and more precisely they are difficult to extend to for example multiplexing and they are quite expensive. Therefore it was a need to propose cheaper, sensitive and reliable DNA microarrays production.

The strategy developed by Dendris is illustrated in Fig. 7. Covalent attachment of the spherical reactive phosphorus dendrimer of generation 4 bearing 96 terminal aldehyde groups on amino-silanized glass slides generates a reactive $\sim 50\text{ \AA}$ layer onto which amino-modified DNA probes are covalently bound. The new

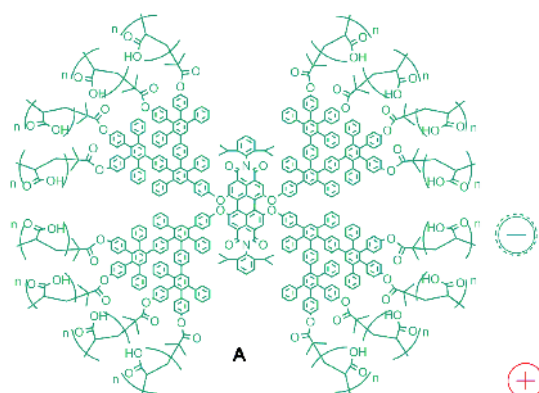


Figure 5 Structure of the anionic perylene diimide labelled star polymer **A** and of the cationic phosphorus dendrimer **B**.

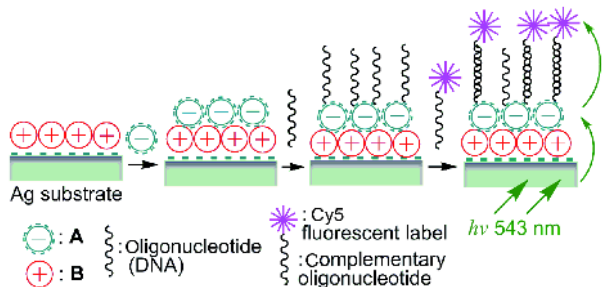


Figure 6 Sensitivity up to 10^{-18} obtained from a bilayer formed with **A** and **B** (Fig. 5) and using surface plasmon enhanced fluorescence spectroscopy technique.

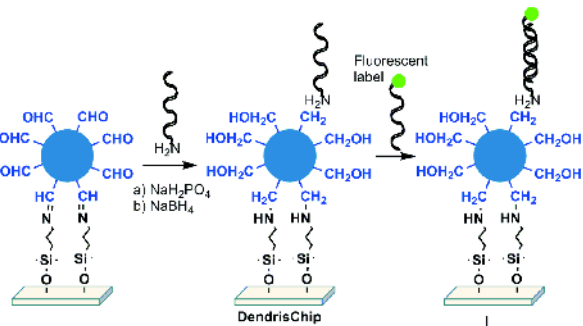


Figure 7 The DNA microarrays technique developed by Dendris.

grafting chemistry leads to the formation of uniform and homogeneous spots. Moreover, probe concentration before spotting could be reduced from 0.2 to 0.02 mg mL⁻¹ with PCR products and from 50 to 5 μmol L⁻¹ with 25 to 70 mer oligonucleotides without affecting signal in-

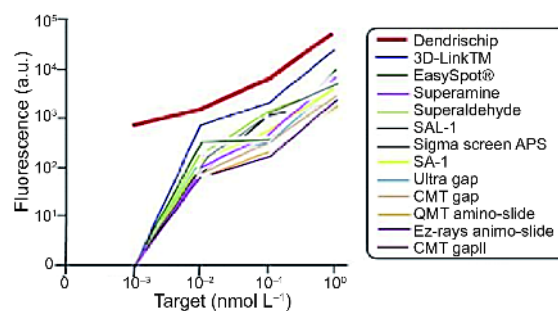


Figure 8 The advantages of DendrisChip[®] compared to other potential competitors. Reprinted with permission from [14].

tensities after hybridization with Cy3- and Cy5-labeled targets. The binding efficiency of these dendrislides was remarkably high, a fluorescent signal being detectable at 1 pmol L⁻¹ concentration of target that is to say 10- to 100-fold higher than arrays made with most of other functionalized glass slides. Interestingly reusability of these dendrislides was possible, based on the fact that the signal intensity of DNA target to oligoprobes was not reduced even after 10 hybridization/stripping cycles [17].

The functionalization of the glass slides with phosphorus dendrimers benefits from three advantages: i) an increase of the density of probes per unit surface and thus in the number of binding sites; ii) a greater accessibility of the target to the probes with hybridization in 3D and not 2D like many other activated surfaces; and iii) a greater spacer from the glass surface reducing the unspecific interaction. Fig. 8 focuses on the advantages of DendrisChip[®] compared to other competitors. The interaction between probe and target is 10- to 100-times higher than with the others.

Since a same clinical symptom diagnosed by a physician can be caused by more than one pathogen, the new paradigm is to consider a syndromic approach of the diagnosis, which basically aims at identifying all potential pathogens responsible for the syndrome. This new approach also coincides with the exponential demand of new *in vitro* diagnosis (IVD) techniques that are more informative by providing more data from a single biological sample, while being more precise, fast and affordable at cost-effective value. The syndromic approach of IVD requires a method, leading to the development and commercialization of multiplex PCR technology allowing parallel identification of up to 22 entities, but they all require expensive equipment.

The polymerase chain reaction (PCR), a technique which is used to amplify any DNA or cDNA is, at present, the most powerful tool of molecular diagnosis due to its

sensitivity and specificity. As the identity of every living species, its molecular characterization, its potential, virulence and pathogenicity, depend on its DNA sequence, the scope of PCR is quite unlimited in human clinical biology, veterinary and plant microbiology. In addition, by avoiding culture, PCR opens the scope of non- or not easily cultivable germs as anaerobic bacteria or viruses

However even if the high specificity related to the very fitted hybridization of the two primers on the targeted DNA is the main advantage to use the PCR, there is a real limitation, as each PCR is unique: one virus, or bacteria is detected at a time. If one wants to detect simultaneously more than one target (multiplex) there are only two ways: enhancing the number of couples of primers to specifically target each of the biological entities, with the risk of interferences, or using the variability inside a given DNA sequences (target) such as the 16S RNA in bacterial genome [26]. The latter one is the most powerful option, but it implies to manage a more complex process able to decipher the slightest differences in the various sequences melted in the postPCR products. Several methods are available, and the simplest one is to use the melting curves produced in the PCR thermocycler by lowering step by step the temperature of the mix from 95°C to 40°C. This melting point is a physical characteristic of the G/C content of the sequence. Sequencing the PCR product is another way to assess precisely its sequence as well as the use of mass spectrometry techniques. These ways are very acute but expensive. Therefore, the Dendris way represents another solution as shown above: the use of DNA chips where post PCR products are hybridized on covalently fixed specific probes of each possible variant of the target.

As we use a piezo electric device to spot probes of 120–150 μm diameter size on glass slide, their number can be very high, but the challenge of Dendris is to transform DNA chips in a rapid, simple, robust and easy-to-use test able to be performed in ordinary clinical labs. Therefore, in practice, we spot hundred to specifically detect and discriminate biological entities linked to a given symptom or application. The contribution of DNA chip to the process after PCR is its ability of multiplexing, enhancing in the same way the productivity of PCR and opening the microbiology to syndromic diagnostic. Table 1 gives a short overview of the advantages of our molecular technology as compared to multiplex PCR and standard microbiological methods.

Based on our technical specificity of dendrimer chemistry reported above, Dendris has developed an affordable

Table 1 Comparative analysis of criteria from the different technologies used in IVD

	Culture method	Mass spectrometry	PCR	Dendrischip ^a
Multiplex	No	No	Yes	Yes
Time of response	48 h	24 h	1–3 h	5 h ^a
Non-cultivable species	No	No	Yes	Yes
Automatization	No	Semi	Yes	Yes
Man power cost	High	Medium	Low	Low

a) Including PCR amplification

and easy-to-use multiplex diagnosis, for syndrome-based diseases and biomarkers validation. The kit is intended for hospitals and medical laboratories with a user-friendly IVD device. In a single test, it provides a rapid and accurate diagnosis, thus improving and accelerating the medical decision making. The IVD solution shall lead to a new standard, which could reduce public health costs. Rapid diagnosis will establish a faster curative action, and determine the best treatment to prescribe in the context of personalized medicine. The β-tester prototype available today from Dendris is depicted in Fig. 9. It includes a dedicated scanner (Dendriscan) to read the DendrisChip[®]. In addition, it contains a simple SoftDiag for data analysis which is based on an algorithmic method.

The statistical method allows to classify and discriminate most of the bacteria species used in the training. Thus the DendrisChip[®] provides an accurate diagnostic and moreover allows questioning about identification assessed by microbial methods.

EXAMPLE OF APPLICATIONS OF DENDRISCHIPS[®]

Our first application has been dedicated to respiratory



Figure 9 β-tester prototype proposed by Dendris.

tract infection which is the most widespread infectious disease, and is caused by a wide range of micro-organisms which are difficult to clinically distinguish due to symptoms similarities. The symptom approach developed thanks to the DendrisChips[®] technology allows rapidly to discriminate 11 bacteria: *Streptococcus pneumoniae* (Spn), *Haemophilus influenzae* (Hi), *Neisseria meningitidis* (Nm), *Moraxella catharralis* (Mca), *Chlamydiae pneumoniae* (Cpn), *Mycoplasma pneumoniae* (Mpn), *Legionella pneumophila* (Lpn), *Klebsiella pneumoniae* (Kpn), *Pseudomonas aeruginosa* (Pa), *Staphylococcus aureus* (Sau) and *Bordetella pertussis* (Bp), responsible for this infection disease.

Our technology has been compared with the still currently largely used microbiological culture. We showed a detection limit in the range of 10^3 cell mL⁻¹ which is in the same range as the culture methods. In addition, the reliability of our DendrisChips[®] technology in the detection of pathogen bacteria was challenged over more than 250 clinical specimens and the results were compared with culture methods. In numerous cases the DendrisChip[®] confirmed the presence of a pathogen suspected but not confirmed by culture in several examples and revealed more information thanks to its ability to detect co-infections. To further evaluate the accuracy of our molecular method, we applied a sensitivity criterion which is defined as the ratio between the number of times a given pathogen has been detected over the total number of sample in which this pathogen was present (i.e., detected by both DendrisChip[®] and microbiological method). This comparative analysis is reported in Fig. 10. Results showed a better sensitivity criterion of DendrisChip[®] than culture method notably for *H. influenzae* (95% vs. 62%) and for *P. aeruginosa* (94% vs. 82%). High sensitivity for *K. pneumoniae* and *M. catharralis* was also obtained with our technology but these values must be taken with caution owing to the low number of clinical samples containing these pathogens. On the other hand, the sensitivity criterion of our technology was apparently very low with respect to *S. aureus* (66% vs. 94%). However, we re-evaluated the microbiological result using the specific PCR assay for this pathogen developed by BD diagnostic and found that only 10 out of the 31 samples positively identified by the microbiological culture were confirmed by the BDmax PCR assay. Therefore, one can conclude that even with *S. aureus*, our technology was as accurate as the culture method. Finally, *B. pertussis*, *L. pneumophila*, *N. meningitidis*, *Chlamydiae pneumoniae* or *M. pneumoniae* were never found by microbiological culture in the 238 clinical specimens.

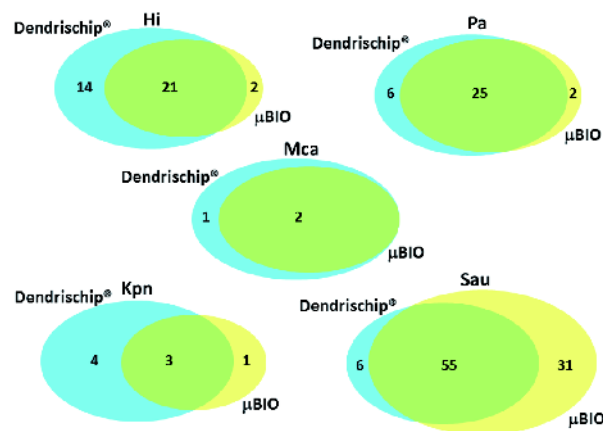


Figure 10 Sensitivity of the DendrisChips[®] technology (sum of blue and green ovals) versus microbiological method (sum of yellow and green ovals). Hi: *H. influenzae*; Pa: *P. aeruginosa*; Mca: *M. catharralis*; Kpn: *K. pneumoniae*; Sau: *S. aureus*. Caution: in the case of Sau, only 10 out of the 31 samples positively identified by the microbiological culture were confirmed by BDmax PCR assays.

The Dendris solution of IVD in respiratory infection is fully compliant with medical practice. This method shall not solely complete traditional diagnostic assays, but overcome limitation of these current assays in providing a rapid, accurate and more informative diagnostic, which could reduce the use of antibiotic therapies.

Other applications that are currently in development by Dendris are in the following area:

Clinical microbiology: According to the World Health Organization (WHO), more than 1 million sexually transmitted infections (STIs) are acquired every day worldwide. Each year, there are an estimated 357 million new infections with 1 of 4 STIs: chlamydia, gonorrhoea, syphilis and trichomoniasis and more than 500 million people are estimated to have genital infection with herpes simplex virus (HSV). The majority of STIs have no symptoms or only mild symptoms that may not be recognized as an STI. In some cases, STIs can have serious reproductive health consequences beyond the immediate impact of the infection itself (e.g., infertility or mother-to-child transmission). Dendris develops a DendrisChip[®] dedicated to the detection of 10 bacteria, virus or parasites known to be transmitted through sexual contact: *Candida albicans*, *Chlamydiae trachomatis*, *Haemophilus ducreyi*, HSV1, HSV2, *Mycoplasma genitalium*, *Neisseria gonorrhoeae*, *Streptococcus agalactiae*, *Trichomonas vaginalis* and *Treponema pallidum*. This application is currently in development and will be available in 2019.

Agro-nutrition and food safety: The search of contamination in alimentary stuffs and breeding, will follow

the same way than clinical microbiology due to the impact on human welfare; but due to the heavy economic constraints in this case evolution will be slower. Thanks to a partnership with academical and analysis laboratories, we have worked on the design of a DendrisChip[®] able to detect the most important bacteria in food safety (*Staphylococcus aureus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Bacillus cereus*, *Salmonella* spp., and 6 serotypes of *E. coli*). This DendrisChip[®] was validated on bacterial strains and tests on food samples are in progress.

Environment microbiology: Dendris is aiming to provide an easy to use method to detect and discriminate legionella species that contaminate water cooling towers. Our actual pre-prototype is actually able to discriminate at least 21 different *Legionella* species among which the four pathogens, namely *L. pneumophila*, *L. anisa*, *L. bozemanii* and *L. longbeachae*

Medical genomics: To identify some markers of acquired or hereditary diseases on a gene, it is necessary to look for the presence of several mutations. It is therefore useful to characterize precisely these mutations by sequencing but, once they are well-known it is easier and less expensive to use DNA chips, best fitted to decipher mixed alleles for instance on biological samples and tumors. So, they are well fitted to the characterization of tumors as for the diagnosis than for the prognosis or the therapeutic choices. The high costs of the new anticancer molecules and the high specificity of their molecular targets command to decipher the good-responders patients from the non-responders. They are well-known as the companion test.

CONCLUSIONS

Today, more than 70% of clinical decisions are based on IVD tests. IVD helps physician to provide the therapeutic and the good management of the patient health. With this decisive role in healthcare, IVD innovation is foreseen to better contribute to health savings and meanwhile is a very important economic sector. That is why many companies keep on investing an important part of their revenue (10 to 12%) in IVD R&D. To address the clinician needs in combination with greater health concern of the patients, the most suitable solutions in IVD shall meet these specific features: i) multiplex rather than simplex (i.e., identify pathogen through a syndromic approach rather than actual mode of single identification by phenotypic or genotypic method), ii) high sensitivity, iii) high specificity, iv) acceptable costs of the whole process (reagents, instrument, and man power), v) non-invasive way.

DNA chips may be the very promising multiplexing tool the biology has a huge and urgent need. DNA chips must progress in the scope of cost, robustness and mainly automation. They have the potential, but they need a large cooperation between biologists, chemists, computer scientists and engineers. The great enhancement performed in that way by Dendris in clinical DNA chips by using phosphorus dendrimers as surface chemistry is a big step forward. In 2018, the main part of medical bacteriology depends on Petri dishes invented in 1887. By paving the way to syndromic diagnosis, to the characterization of non- or ill-cultivable germs and by saving time offered to the clinicians for the right treatment of their patients, the DNA chips post PCR will contribute to the fall of Petri dishes in less than the next ten years. To be a substitute to a centennial process is what we call a disruption.

The solution proposed by Dendris based on the use of phosphorus dendrimers fits perfectly with all requirements summarized above, and forward-looking.

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Author contributions Majoral JP, Caminade AM designed experiments linked to dendrimer technology, François JM and Fabre R conceived the DendriSchip® kit fabrication. Majoral JP wrote the paper which was improved and approved by all the other authors.

Conflict of interest The authors declare no conflict of interest.



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