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Nucleic acid based biosensors: The desires of the user

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Abstract

The need for nucleic acid based diagnostic tests has increased enormously in the last few years. On the one hand, this has been stimulated by the discovery of new hereditary genetic disease loci following the completion of the Human Genome Project, but also by the presence of new rapidly spreading viral threats, such as that of the SARS epidemic, or even micro-organisms released for the purpose of biological warfare. As in many instances rapid diagnoses of specific target genetic loci is required, new strategies have to be developed, which will allow this to be achieved directly at the point-of-care setting. One of these avenues being explored is that of biosensors. In this review, we provide an overview of the current state of the art concerning the high-throughput analysis of nucleic acids, and address future requirements, which will hopefully be met by new biosensor-based developments.

Keywords: DNA biosensor diagnosis; PCR microarray

1. Introduction

Probably no event has altered clinical analysis of DNA and RNA such as the development of polymerase chain reaction (PCR) [1]. The introduction of this technology permitted an examination of small quantities of material, even as little as a few or single cells, with the result being obtained within hours. This was in stark contrast to methods used until then, such as Southern (for DNA) or Northern (for mRNA) blot analysis, which relied on microgram quantities of pure nucleic acids, and where a result was usually obtained only after a few days or even weeks.

It is therefore little wonder that PCR was readily seized upon by diagnostic or clinical laboratories, and that these in turn introduced many of the "cutting edge" applications, which later found widespread use in the bio-medical research community. Examples of this are single cell PCR (SC-PCR), which was pioneered for pre-implantation genetic diagnosis (PGD) [2], and allele-specific PCR, which permitted the rapid determination of mutant loci, which frequently differ by only a single base change from the normal allele.

The elucidation of genetic loci implicated in hereditary genetic disorders (e.g., cystic fibrosis), or those which were associated with a risk for a particular disease later in life (e.g., the *BRCA1* and *BRCA2* genes and the development of breast cancer), has driven the development of new PCR-based technologies which permit a high-throughput analysis of the numerous mutant alleles associated with such disorders. Of these technologies, probably the most promising is the use of high density nucleic acid arrays [3], also termed DNA Chip Technology, which permits the simultaneous assessment of numerous genetic alleles or loci.

A further technological development of the PCR process, which has found widespread clinical applicability, was the advent of real-time PCR, which facilitated the highly accurate quantitation of nucleic acids [4]. This technology has rapidly been applied to clinical settings, especially in oncology, where it is used for the determination of gene dosage or the associated level of gene expression of genes implicated in the malignancy. It has, however, also been widely used in the examination of infectious diseases, for instance in monitoring the viral burden in HIV-infected

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individuals undergoing a variety of treatment regimens, to assess drug efficacy.

Although the development of PCR, in all its various forms, has completely altered the analysis of nucleic acids for diagnostic purposes, it is to be expected that the next technological revolution will equally transform applications as we know them today. It is to be expected that this new revolution will again be driven by the requirements of the end-user, which amongst others are:

- cheap, rapid, simple
- high-throughput
- ability to be used directly at the point-of-care setting by staff with no laboratory training.

This latter point is gaining in importance as many clinical or other analytical applications increasingly rely on near-instantaneous answers directly at the point-of-care or point-of-entry setting. Examples of these include:

- The rapid identification of infected individuals or carriers for a new viral epidemic (e.g., SARS) directly at the point-of-entry into a country
- Rapid assessment of the fetal genotype in prenatal diagnosis
- Rapid assessment of the oncological status of a malignant lesion directly in the surgery
- Viral load response to drug treatment
- Detection of genetically modified organisms or foodstuffs
- Detection of bacterially contaminants during food processing
- Detection of biological agents used for germ warfare.

These facets will permit much more rapid therapeutic or interventional strategies leading to better and more cost-effective healthcare management. These few scenarios do, however, imply that this next phase cannot be addressed by current technologies, and that new tools will have to be developed, which are as simple to use as current technologies permitting diabetics to monitor their own blood sugar levels in a minimally invasive manner. In as much as such glucose monitoring devices rely on biosensor technologies, it is hoped that the new revolution of nucleic acid analysis will similarly be introduced by novel DNA-based biosensors.

2. Current state of the art of high density DNA arrays

Microarrays are miniaturised analytical systems emerging from traditional biochemical assays as such Southern, Northern, or Western blotting [5,6]. According to these conventional assays, different branches of microarrays evolved, which can be applied in a complementary fashion to achieve a comprehensive global view of genes and gene expression, posttranslational modifications, and protein status. The miniaturisation of the assay systems enables the simultaneous interrogation of either many different events within one biological sample (e.g., a specific cell population under certain stimuli, or the testing of a multitude of samples in parallel for the same event). Both variants provide a high throughput analysis tool, which can be adapted to most different types of experimental questions and customer needs.

Application fields for micro-arrays include diagnostics with genotyping applied to either mutation detection in specific human disease-related genes or identification processes in microbiological tasks, or gene profiling for predictive medicine. In the latter scenario, transcription patterns are correlated with different pathophysiological conditions, prognoses, and disease progressions. Consequently the technology can be used for superior patient-tailored treatment based upon recognition of specific aberrant signalling pathways and expression patterns. A second field encompasses the identification of novel biomarkers and new targets for drug therapeutics as well as monitoring their effects (e.g., efficacy and toxicity) on the organism. The fundamental basis of the microarray technology is a process of hybridisation where two biological molecule pair together in dependence of their complementary sequences or structures: DNA/DNA, cDNA/RNA, and protein/antibody. The application of more novel interactions, such as those with aptamers, affibodies, or glycoproteins for microarray applications, is currently being explored.

Technically advanced robots permit the disposition of probe molecules (oligonucleotides, PCR-amplified cDNA fragments, proteins, and antibodies) in micrometer distances onto the array surface, thereby facilitating the immobilisation of many different probes in a high density array on small surfaces of, for example, 1 cm^2. The oligonucleotide probes can either be synthesised step by step in silico using photolithography (allowing for a density of 10^6 different oligonucleotides per square centimeter) or they are pre-synthesised in vitro and subsequently spotted onto the array surface (10 000–30 000 spots per slide); this surface usually consists of nylon membranes or glass slides coated with various agents providing immobilisation while maintaining probe native structures. The generation of oligonucleotide or cDNA arrays requires highly sophisticated bio-informatics systems as care needs to be taken that the maximum hybridisation potential for every single array probe sample is achieved.

For the analysis of gene expression, also termed gene profiling, the sample RNA is generally converted into stable cDNA, using approximately 5–10 μg of total RNA as starting material. Amplification of this sample material is usually achieved by in vitro transcription or PCR. During this procedure the sample is also labelled with a fluorophore used for the identification of target–probe interactions in the subsequent hybridisation process. Quantitative analysis can either be obtained using a single colour scheme, where the

fluorescently labelled sample is hybridised to different gene arrays, or by the use of multi-colour settings, whereby two samples which are to be compared are labelled with different flourophores and co-hybridised to the same array.

As these high-density arrays permit the simultaneous examination of several thousand loci, new bio-informatic tools have had to be developed in order to facilitate useful data mining, which by careful correlation with the underlying physiological processes will lead to the extraction of biologically important information from this wealth of data.

Although a major application to date of such high density arrays has been for the purpose of gene profiling, great strides have recently been made in applying this technology for genotyping or disease loci or of single nucleotide polymorphisms in order to determine disease associated patterns [7].

3. Current state of the art of real-time PCR

Traditionally, PCR was a qualitative method, which was rendered semi-quantitative by ingenious reaction set-ups, such as competitive PCR where standards of known amount are co-amplified with the sample or quantitative fluorescent PCR.

This deficit has been completely changed by the introduction of real-time PCR analysis [4], which has revolutionized the quantitative analysis of DNA and RNA. In real-time PCR reactions, the concentration of the input template nucleic acid is accurately determined by the accumulated emission of a fluorescent signal during each cycle of the PCR reaction. This system permits the accurate quantitation of sample concentration over a dynamic range of several orders of magnitude. Furthermore, the system has proven to be very reliable and sensitive, being capable of detecting small amounts of target in the order of 1–10 copies. It is suitable for the determination of small differences in copy numbers, as witnessed from our recent description of an assay which permits the detection of fetal aneuploidies [8], where only a 50% increase in target template occurs.

A significant advantage of real-time PCR is that no post PCR handling is required, such as gel electrophoretic analysis of the PCR products, which effectively eliminates the risk of carry-over contamination, a vitally important feature for clinical settings. As the assay can be accessed in real-time, several quality controls can be integrated to monitor the efficacy of the PCR assay, such as final fluorescence, replicate uniformity, slope of the fluorescence curve, and melting curve analysis.

Furthermore, full advantage can be made of the fluorescent signal detection system, in that currently up to four different fluorophores (including a passive loading control) can be monitored simultaneously. In addition the system is very amenable to automation and is constantly being adapted to new formats which permit high-throughput, such as the recently developed 384-well format, which requires very little reagent chemistry (as little as 5–20 μl) and permits very rapid PCR cycles. This implies that by the use of four different fluorophores, 1536 different targets can be simultaneously analysed in a real-time setting, with a result being obtained in as little as 10 min.

It is, hence, no wonder that since its introduction a few years ago, real-time PCR has been used for a myriad of applications including, gene profiling, mutation and SNP analysis, monitoring of viral load, and analysis of cell free DNA concentrations in a variety of disorders [9,10].

It is, hence, to be expected that real-time PCR will continue to spearhead developments concerning the rapid and highly precise analysis of nucleic acids.

4. Biosensors: where do we stand from a user's point of view?

It readily becomes apparent from a quick perusal of the current literature that numerous DNA-based biosensor systems are being evaluated, and that new formats are almost being added on a daily basis. As these are being addressed in much greater depth in other contributions to this volume, we shall only briefly describe a few here and elude to current constraints limiting their immediate or near future use in a clinical or diagnostic setting. In general the underlying physical properties of the various types of sensors use the following modes of detection: fluorescence, electrochemical, or altered surface properties.

One of the most promising approaches for the fluorescent detection of nucleic acids appears to be the use of fibre optic systems, where, in the interim it has become possible to coat pits chemically etched into individual optic fibres with a single sequence specific probe, thereby in essence permitting the analysis of single molecule–target sequence interactions [11]. A highly sensitive CCD camera is used to monitor sequence specific hybridisation on the fibre bundle, which has been arranged into block matrixes, to facilitate large-scale parallel analysis. By using such an approach, it has recently been possible to profile transcript differences in a panel of human cancer cell lines, using starting material from as few as 10 cells.

Electrochemical applications are also being intensively explored, as these are potentially cheaper to operate and maintain than the more complex systems required for the analysis of fluorescent signals. In these approaches the analysis of both nano-particles as well as solid phase array biochips is being explored.

In the former approach, DNA oligonucleotides are spotted in arrays which are flanked by electrodes [12]. Following hybridisation of the target sequence, the combined probe–target sequence is then recognised by gold nanoparticles coupled to specific oligonucleotides, which is used to trigger an electrical signal in combination with a reactive silver nitrate solution. By using this approach,

highly specific sequence detection with femtomolar sensitivity was reported.

In parallel developments, electrical biochip technologies are also being explored [13]. In these systems, ELISA-based enzymatic redox reactions, such as the conversion of the substrate *p*-aminophenyl phosphate to the electroactive *p*-aminophenyl by alkaline phosphatase, are used to register the binding of specific target molecules. Potential applications of this technology which are under investigation include the monitoring of vial infections. An advantage of electrical biochips is that this technology is readily adaptable for a variety of biological targets, such as nucleic acids, metabolites (glucose), or proteins (antigens). Furthermore these devices are highly sensitive, do not require mechanical adjustments, are simple to use, and are readily miniaturised.

Other research avenues include the use of quantum dots [14] and nanomechanical cantilever arrays [15,16]. Quantum dots are generated from discretely sized nanocrystals of cadmium selenide capped with zinc sulfide [14]. The advantage of these dots is that they have unique optical properties, such as the ability to emit single colour signals at different wavelengths depending on the size of the dot. Furthermore, even if the dots are in close proximity of each other, no FRET (fluorescence resonance energy transfer) effect occurs. This implies that highly dense arrays in the nanometer or micrometer scale are possible. The advantage of these dots is that as they very small, they can be incorporated in a vast array of combinations into polymer microbeads. By labelling each of these microbeads with a sequence specific oligonucleotide, this system permits rapid and efficient gene profiling.

Nanomechanical cantilever arrays, on the other hand, make use of physical changes in surface structure, such as the bending of a micro-cantilever upon the specific binding of a target molecule [15,16]. The sensitivity of this system can be significantly improved by the inclusion of a magnetic control system. In this manner, the deflection of the cantilever is enhanced by the application of an external magnetic field, which acts on target sequences bound to the cantilever, which have been tagged with oligonucleotides labelled with magnetic particles.

Although these approaches are all very promising, very few in their current form offer levels of sensitivity permitting the detection of nucleotide sequences without a preliminary PCR step. A further concern for potential diagnostic applications is that most experiments have been restricted to the detection of artificial oligonucleotide mixtures, and the specificity of these detection systems has not be tested under "real-life" conditions where the vast amount of nucleic acids does not correspond to the target sequence.

5. Conclusions

From this overview, it should be clear that significant strides are not only being made with new biosensor technologies for the analysis of nucleic acids, but that considerable advances have been made with more conventional PCR-based approaches. This is evident from new developments in real-time PCR, where results can be obtained in a matter of a few minutes, or from DNA arrays where thousands of alleles can be examined simultaneously.

On the other hand, DNA sensor technologies, while promising, still need to bridge the gap between experimental status and the harder reality of clinical/diagnostic applications. Nevertheless, it is to be hoped that these technologies will lead to the development of new tools, which are simple and cheap enough to be used at point-of-care sites, thereby addressing a real need which cannot be met by current PCR-based platforms.

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