Soft and Biological Matter

Giuseppe Spoto
Roberto Corradini *Editors*

Detection of Non-Amplified Genomic DNA

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Detection of Non-Amplified Genomic DNA

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Preface

The extraordinary effort in the sequencing of living organism genomes, including the human genome project, is continuously providing new targets for the use of genetic information as specific markers for analysis of samples of biological origin; this process has prompted towards the development of new methodologies for an even more simpler, faster and cheaper detection of nucleic acids to be used for both applicative and research needs in different areas, including clinical diagnosis, environmental monitoring and food control.

Most of the currently available methodologies to detect the DNA base sequences exploit the recognition capabilities of nucleic acids and are based on the hybridization reaction between a probe oligonucleotide carrying a known base sequence and the counterpart present in the unknown DNA target. Most of the above methods require the amplification of the target species as a consequence of the very small amount of DNA material often available and detect the duplex formation by using indicators (labels) and transducers able to generate a detectable signal as a consequence of the specific hybridization event.

The polymerase chain reaction (PCR) is the most widely adopted technique used to amplify a target DNA. It amplifies a single piece of DNA across several orders of magnitude, duplicating millions or more copies of a particular DNA sequence, in order to detect the genetic material more easily. Real-time PCR is presently the golden standard for quantitative DNA analysis.

The PCR amplification of the target sequences is used since it enhances both the specificity and the sensitivity of the assay by increasing the target concentration.

Such enhancement is balanced by the need to use complex reaction mixtures and by the potential contamination of the genetic sample both resulting in ambiguities and errors in the final DNA detection process. Moreover, PCR amplification methods suffer from drawbacks such as the difficulty in amplifying large target DNA molecules (more than about 10000 bases) and the possible amplification of DNA sequences different from the target resulting in non-specific signals.

The concept of amplification has also been extended to other types of schemes, in particular those working under isothermal conditions.

Sequence-specificity is one of the major issues in modern DNA-based diagnostics. Single point mutations are very important markers of alterations linked to genetic diseases and tumour insurgence. Furthermore, the identification of single nucleotide polymorphisms (SNPs), variation of a single nucleotide in the genome sequence, is of great importance in the classification of biological material, allowing to discriminate between individuals, varieties and species. Identification of SNP is one of the major outcome of the modern massive sequencing technologies. Detection of SNP and point mutations is not easily performed by PCR platforms, and specialized techniques are continuously being developed to allow better single-base level recognition ability.

DNA detection is expected to be significantly improved by using simple and economic detection methods which require minimal DNA modifications and provide enhanced signal amplification. In this perspective, the direct analysis of non-amplified genomic DNA is an excellent cost-effective alternative that can be achieved by using ultrasensitive DNA detection protocols, since extra labour and costs from the amplification procedure are reduced.

Scientific literature published over the last 10 years has shown that non-amplified DNA detection can be achieved by adopting different techniques. Such techniques have allowed the commercialization of innovative platforms for DNA detection that are expected to break into the DNA diagnostic market that is expected to be valued at $20.1 billion in 2013 with an annual growth rate of 13.7% between 2008 and 2013. In particular, new diagnostic assays for the PCR-free detection of DNA are expected to introduce a new segment that will compete with the $9.5 billion in 2013 PCR-based diagnostic assay market.

The enhanced sensitivity required when non-amplified genomic DNA is going to be detected has prompted efforts aimed at identify new strategies useful for the ultrasensitive DNA detection. It is clear today that ultrasensitivity can be obtained by combining specific materials with specific detection tools.

Advanced materials play different roles in the ultrasensitive detection. For instance, nanostructures such as metallic nanoparticles have been widely investigated and the specific optical properties of gold nanoparticles have prompted the development of new methods in DNA sensing exploiting the solutions colour change generated by a controlled aggregation of the colloidal nanoparticles or the specific detected optical signal enhancement generated by metallic surface plasmon interferences. Cationic conjugated polymers have been deeply investigated for their ability to produce amplified signal upon interaction with dsDNA.

Probe selection, surface composition and architecture play a fundamental role in the enhanced sensitivity detection of non-amplified DNA. Some of the physical processes which can be used to generate a signal in sensors are strongly dependent on the surface architecture and are greatly improved by the possibility to control distances between the captured DNA analyte and the surface with nanometer precision. This enables to distinguish between short- and long-range effects, which is particularly important in the case of optical detection based on plasmonics. For the generation of strong signals, the advantages offered by specifically designed probes such as peptide nucleic acids (PNA), locked nucleic acids (LNA), or

morpholino oligonucleotides (MO) can be exploited. In particular, synthetic probes offer improved selectivity and specificity in targeting complementary nucleic acid sequences that can be tuned by properly designing their structure. Pushing the analysis down to very low detection limits can be achieved only if the probe-DNA interaction is sufficiently strong to prevent dissociation at very low concentrations; thus enhancing the performances of probes is one of the issues to be controlled in order to design new ultrasensitive methods. PNA and LNA are particularly suited for ultrasensitive detection, due to their high DNA affinity. Cationic polythiophene polymers has been used for the ultrasensitive detection of non-amplified DNA since their intrinsic fluorescence quenches as a result of the planar, highly conjugated conformation adopted by the polymers when complexed with a single-strand DNA probe but increases again after hybridization with the perfectly matched complementary strand.

Optical and electrochemical detection tools are among the most widely investigated for the analysis of non-amplified nucleic acids. Both the methods have been applied to the direct detection of genomic DNA and non-amplified RNAs from bacteria. Optical detection of genomic DNA has gained a new impulse in recent years after the advent of surface plasmon resonance (SPR). The SPR sensitivity in the direct detection of unamplified genomic DNA has been evaluated by using genomic DNA extracted from different living organisms. The limitation suffered by SPR in the parallel detection of different probe/target interactions are overcome by SPR imaging while the ultrasensitivity is obtained by adopting specific metallic nanoparticle signal enhancement protocols.

Biosensors based on piezoelectric crystals have been also used to detect unamplified genomic DNA. The immobilization of oligonucleotide probes on the piezoelectric crystal surface allows the combination of the high sensitivity to mass changes of the piezoelectric transducer with the high specificity of the hybridization reaction between the probe and the nucleic acid target and provides a tool for the label free detection of unamplified DNA.

Finally, next generation sequencing technologies have been greatly improved, thanks to the 1000$ genome challenge, and modern platforms are very close to this goal. Thus redundant information on the genome of specific specimens is also a possibility becoming more and more feasible at the point of care level and certainly the possibility to have the complete screen of genomic information at the nucleotide sequence level is going not only to offer new services, but also to substantially change some of our societies' fundamental issues, such as health and food management.

The brief description of the topic above makes it possible to understand just how heterogeneous the interests and the professional background of the people involved are. In fact, chemists, biologists, biotechnologists and physicists have all devoted their attention to the field of nucleic acid detection using their own cultural background. Moreover, the wide spectrum of information provided has established a multi-disciplinary approach but, at the same time, has expanded the information sources which are to be considered in the field.

For all these reasons a book like this, where topics in the field of detection of non-amplified DNA are reviewed, is the result of multi-disciplinary contributors. The across-the-board relevance of the topics discussed in the book together with the even greater scientific and economic importance of new diagnostic tools for genomic analyses are two fundamental reasons to expect a high level of interest in the readers. The interest shown in the subject by academic departments, colleges and institutes involved in the field would appear to show that the book would also have educational potential.

The evolution of the topic discussed is itself a factor to be considered when evaluating the interest it may generate.

The book starts with two chapter dedicated to the most important fields of application of the DNA-detection technologies, that is health and food, which account for the most important economic issues presumably enjoying the major advantages from the availability of new simplified, fast and reliable methods for massive detection of DNA sequences. **Chapter 1** deals with the most important issues which are presently at stake in medical sciences, from newborn safety arising from pre-natal diagnostics to tumour progression evaluation and early diagnosis, and list the expectations in this field which can be foreseen for the next years.

Chapter 2 reports the enormous progress made possible by DNA analysis in the field of food sciences, with application to food safety, traceability and authenticity. This field, being of huge social and economic relevance, offers the possibility to apply new DNA detection schemes to the development of new and unprecedented services, which will be made easier to perform by methods detecting unamplified genomic DNA without the use of complex apparata.

Of course these desiderata could not be satisfied if new generations of materials and detection schemes would not have been developed by chemists and material scientists. Thus, the next section deals with the materials and detection schemes needed for obtaining ultrasensitive diagnostic techniques, which are a pre-requisite for unamplified DNA detection. **Chapter 3** deals with the use of nanostructured materials such as nanoparticles , nanowires and nanogaps, whose availability paved the way for unamplified DNA detection. **Chapter 4** deals with the molecular probes needed to obtain high fidelity of the DNA capture in sensory systems, and introduces the field of chemically engineered oligonucleotide analogs which allow to reach higher levels of sensitivity and selectivity, such as locked nucleic acids (LNA), morpholino oligonucleotides (MO) and peptide nucleic acids (PNA), together with an explanation of how these molecules can be linked to the sensor and to other reporting groups for ultrasensitive detection. Molecular probes used to directly visualize the genomic DNA by optical methods are also described. **Chapter 5** describes cationic conjugated polymers (CCP) which have demonstrated great potential as DNA-sensing materials and how their optical properties can be changed in the hybridization states. The application of these materials to direct genomic DNA detection is also discussed.

The technical advancements made in signal collection are also responsible for the crossing of the threshold needed to directly detect unamplified DNA. Thus in Part III, a series of contribution dealing with the analytical techniques is reported.

In **Chap. 6**, the advances in DNA detection by optical techniques, including colorimetric, fluorescence, luminescence, surface plasmon resonance (SPR) and Raman scattering assays are reported, with up-to-date description of strategies and technologies for ultrasensitive and unamplified DNA analysis. Electrochemical techniques are another key point in modern DNA genosenors, and in **Chap. 7** the most recent advances in this field are discussed, with a description of how new fabrication techniques and nanomaterials have improved the ability to provide rapid, multiplexed electrochemical DNA detection essential for point of care clinical diagnostics. The third large class of mechanical sensors is described in **Chap. 8** dealing with piezoelectric sensing. In this chapter, the detection principle and the approaches used in DNA-based sensing for microsatellite and target sequence DNA detection will be presented and discussed, together with a useful discussion on sample treatment and probe chemistry. Surface plasmon resonance and related techniques are among the most useful tools for label-free bioanalysis, and this has allowed to more easily achieve PCR-free detection of DNA. In **Chap. 9**, the principles of these techniques and their most recent applications to genomic DNA analysis are discussed.

Modern technologies and surface architectures allow to perform DNA detection in a multimodal approach. **Chapter 10** shows an instructive example of how these elements can be combined for the ultrasensitive detection of DNA samples, using electrochemical and surface plasmon enhanced fluorescence.

For most scientists interested in DNA sensing, the most important issues are the incredible advances in sequencing technologies. In this field also, non-amplificative techniques have become more and more competitive. **Chapter 11** gives excellent and updated information about these next-generation sequencing techniques, and guides the reader into this complicated field, which represents some of the most advanced frontiers of human technologies so far reached.

We are really grateful to all the qualified scientists, who are listed in the list of authors, for their precious contribution to this book, bringing their experiences and know-how and sharing these with our readers. We are also indebted to Dr. Maria Bellantone, Senior Publishing Editor, Springer, for her encouragement, support and patience.

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Part I
DNA Analysis: Current Issues

Chapter 1
Genetic Analyses in Health Laboratories: Current Status and Expectations

Alessia Finotti, Giulia Breveglieri, Monica Borgatti, and Roberto Gambari

Abstract Genetic analyses performed in health laboratories involve adult patients, newborns, embryos/fetuses, pre-implanted pre-embryos, pre-fertilized oocytes and should meet the major medical needs of hospitals and pharmaceutical companies. Recent data support the concept that, in addition to diagnosis and prognosis, genetic analyses might lead to development of personalized therapy. Novel frontiers in genetic testing involve the development of single cell analyses and non-invasive assays, including those able to predict outcome of cancer pathologies by looking at circulating tumor cells, DNA, mRNA and microRNAs. In this respect, PCR-free diagnostics appears to be one of the most interesting and appealing approaches.

Abbreviations

ASO	allele-specific oligonucleotide
EPO	erythropoietin
ErPC	erythroid precursor cells
HbA	adult hemoglobin
HbF	fetal hemoglobin
HPLC	high performance liquid chromatography
IVF	in vitro fertilization
miRNA	microRNA
ODN	oligodeoxyribonucleotide
PCR	polymerase-chain reaction

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RT-PCR	reverse transcription PCR
SCA	sickle-cell anemia
TOP	termination of pregnancy

1.1 Introduction

Genetic analyses in health laboratories should be designed and developed to meet the major medical needs of hospitals and pharmaceutical companies. Basically, genetic analyses involve adult patients [1–5], newborns [6–8], embryos/fetuses [9–12], pre-implanted pre-embryos [13–16], pre-fertilized oocytes [17–21].

In the case of genetic analyses involving adult patients the major issue is the molecular characterization of the disease, eventually linking diagnosis to prognosis and diagnosis to personalized therapy [5]. In this case, genetic testing is performed usually after the onset of the pathology and evidences for clinical symptoms. On the contrary, screening newborns for inherited disorders provides the possibility for pre-symptomatic identification and early intervention to prevent or mitigate morbidity and mortality [6–8]. At present newborn screening can be applied to more than 50 disorders. One example is sickle-cell anemia, an inherited disorder that occurs throughout the world with its highest incidence in areas of Africa where malaria is endemic. There are a number of potentially serious complications associated with the condition, and it is suggested that early treatment (before symptoms develop) can improve both morbidity and mortality. Screening for the condition in the neonatal period would enable early diagnosis and therefore early treatment [7]. Genetic testing on cells from embryos/fetuses allows prenatal diagnosis to determine the genotype of embryos and identify genetic diseases before birth [9–12]. This issue is relevant in several pathologies for which a cure is not currently available. In this case screening tests should be offered to all pregnant women to assess their risk of having a baby with a birth defect or genetic disorder [10]. In this respect, it should be underlined that intrauterine therapy can be offered in some cases in which severe fetal abnormalities are prenatally diagnosed, for which natural history anticipates a fatal outcome or the development of severe disability despite optimal postnatal care [11]. Fetal therapy has therefore developed as an alternative to fatalist expectant prenatal management as well as to termination of pregnancy (TOP). The technological progress in genetic testing at present allows the study of pre-implanted embryos (in this case the diagnosis is expected to help the decision of implanting the embryos) [13–16]. The couples seeking in vitro fertilization, who may screen their embryos for aneuploidy, and the couples at risk for a monogenic disorder but averse to abortion of the affected fetuses after prenatal diagnosis, are likely to be the best candidates to undergo this procedure. Finally, genetic testing can be performed on pre-fertilized oocytes (the objective in this case is to identify the oocyte free of the genetic mutation under analysis) [17–21].

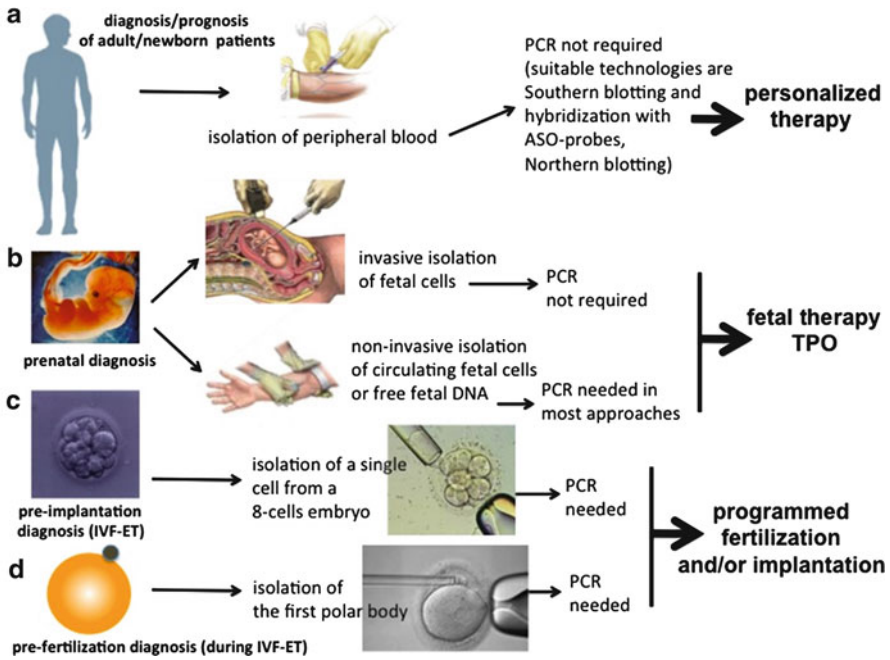

Fig. 1.1 Approaches in diagnosis/prognosis with examples of isolation of biological materials, requirement of PCR steps and biomedical applications. (**a**): genetic testing on adult patients and newborns; (**b**): prenatal diagnosis on embryos/fetuses; (**c**): genetic testing on pre-implanted pre-embryos; (**d**): molecular analyses on pre-fertilized oocytes

Figure 1.1 summarizes genetic testing approaches, pointing out the dependence of these analyses from polymerase-chain reaction (PCR) steps. PCR is required in most of the techniques starting from single cells or low numbers of cells. For instance, PCR and fluorescence in situ hybridization (FISH) are the two common techniques employed on a single or two cells obtained via embryo biopsy during pre-implantation diagnosis [14]. On the contrary, when large numbers of cells are available, several technologies (not requiring a PCR step) can be employed, such as Southern blotting (for genomic analyses) and Northern blotting (for studies on RNA expression) using allele-specific oligonucleotide (ASO) probes. In any case, the major parameters of the molecular techniques supporting these issues (given the fact that the technology should be absolutely reproducible and without the risk of errors) are (a) speed, (b) sensitivity, (c) low-cost, (d) portability. This chapter will give examples of the expectations of the health laboratories in respect to the genetic analyses performed to meet the already mentioned medical needs.

As far as the applications, Fig. 1.1 points out essentially the usefulness of genetic testing for diagnosis and/or prognosis. However, recent studies point out the need of genetic information for the design of personalized therapeutic approaches.

1.2 Genetic Analyses and Personalized Therapies

Personalized therapy is one of the major issues in molecular medicine [22–27]. Examples of personalized medicine involve the choice of the therapeutic drug after considering the genotype (in terms of analysis of the basic genetic mutations as well as of the wide-genome polymorphism scanning) [24] and the phenotype (in terms of transcriptomic and proteomic pattern) [26]. In addition the knowledge of the molecular basis of the disease is expected to help in the development of disease-specific in vivo models as well as disease-specific therapeutic approaches. This issue is very complex in consideration of the fact that even monogenic diseases can be caused by a large variety of genetic mutations. One example is β-thalassemia, a group of hereditary hematological diseases caused by more than 200 mutations of the human β-globin gene, leading to low or absent production of adult β-globin and excess of α-globin content in erythroid cells, causing ineffective erythropoiesis and low or absent production of adult hemoglobin (HbA) [28–35]. Together with sickle-cell anemia (SCA), thalassemia syndromes are the most important problem in developing countries, in which the lack of genetic counseling and prenatal diagnosis have contributed to the maintenance of the high frequency of these genetic diseases in the population [34]. This contributes significantly to driving changes in the distribution of carriers and affected people, in relation to the migration of populations from endemic areas to countries where their prevalence in indigenous populations had been extremely low (USA, Canada, Australia, South America, the United Kingdom, France, Germany, Belgium, the Netherlands and, more recently, Scandinavia). These changes have challenged health professionals and policy-makers in providing equitable access to quality services for the prevention and treatment of Hb disorders in these countries. On the other hand, considering limitations and side effects of the therapeutic approaches and management of the thalassemic patients, novel alternative options for therapy are urgently needed, based on the genetic background of the patient.

1.2.1 Polymorphisms Associated with Fetal Hemoglobin Production

This issue has been the object of several studies in the field of β-thalassemia. For instance, Thein and Menzel [35] reported the progress in the understanding of the persistence of HbF in adults. Three major loci (XmnI-HBG2 single nucleotide polymorphism, HBS1L-MYB intergenic region on chromosome 6q, and BCL11A) contribute to high HbF production. While other numerous loci are expected to be present [36–39], they are supposed to play modest effects. It should be pointed out that the identification of the three major loci has not yet been translated into new therapeutic approaches for HbF reactivation but, in consideration of the fact that they might identify putative binding sites for transcription repressors, an immediate

application is expected to be the prediction of the ability of erythroid cells carrying such genetic variations to produce HbF, which in turn, may improve prediction of disease severity and possible personalized therapeutic strategies [35].

1.2.2 Molecular Approaches for a Personalized Therapy of β-Thalassemia: Mutations Affecting Splicing

A very exciting possibility linking diagnostics to therapeutic choice is the study by Svasti et al., who described a very interesting approach finalized to the repair of β-globin pre-mRNA rendered defective by a thalassemia-causing splicing mutation, IVSII-654, in intron 2 of the human β-globin gene [40]. This intervention was performed using a mouse model of IVSII-654 thalassemia, and based on the use of the delivery of a splice-switching oligonucleotide (SSO), a morpholino oligomer conjugated to an arginine-rich peptide. The SSO blocked the aberrant splice site in the targeted pre-mRNA and forced the splicing machinery to reselect existing correct splice sites. Repaired β-globin mRNA restored significant amounts of hemoglobin in the peripheral blood of the IVSII-654 mouse, improving the number and quality of erythroid cells. This approach is expected to be used in all the several splicing defects of β-thalassemia which produce large amount of uncorrectly spliced RNA molecules deeply interfering with RNA trafficking and translation [41–43]. These studies represent new hopes for specific classes of β-thalassemia patients carrying splicing mutations.

1.2.3 Molecular Approaches for a Personalized Therapy of β-Thalassemia: Stop-Codon Mutations

Another example of the need of genomic characterization of β-thalassemia patients is related to the recently proposed read-through approaches for inducing HbA production in erythroid precursors from patients affected by $\beta^0$39-thalassemia, where the CAG (glutamine) codon is mutated to the UAG stop codon, leading to premature translation termination and to mRNA destabilization through the well-described NMD (nonsense-mediated mRNA decay) [44]. Relevant to this issue, Salvatori et al., after FACS (fluorescence-activated cell sorting) and HPLC (high performance liquid chromatography) analyses, demonstrated that erythroid precursor cells from $\beta^0$39-thalassemia patients are able to produce β-globin and adult hemoglobin after treatment with G418 [45]. This study strongly suggests that ribosomal read-through should be considered a strategy for developing experimental strategies for treatment of β^0-thalassemia caused by stop codon mutations, and might be combined with DNA-based strategies to reactivate HbF [45, 46]. Accordingly, the identification of patients carrying stop-codon mutations might be relevant to design for them a

therapeutic intervention based on the use of read-through molecules. This need does apply also for other pathologies caused by stop-codon mutations, including cystic fibrosis [47] and Duchenne muscular dystrophy [48].

1.3 From Tissues to Single Cell Analysis

Standard genetic testing using the commonly available molecular tools and devices is based on the analysis of large numbers of cells; in this case, however, the results obtained should be considered as an average product and hardly reveal the features and biological parameters of single individual cells. On the other hand, in several fields of applied medicine (for instance in molecular oncology) analysis of the genome and the transcriptome of single cells appear to be of great value. In this case single cells can be isolated from a heterogeneous pool, the genome and the RNA can be extracted and analyzed individually; this "single-cell analysis" is becoming one of the milestones for genetic research in several fields of investigation, such as cancer diagnosis [49–52].

In the field of single cell analysis, movement, isolation and characterization of single cells and cell pairs partnering in heterotypic interactions will contribute to functional studies with unprecedented resolution [53, 54], particularly when either partner belongs to a heterogeneous biological population and/or a scarcely represented cell lineage [55]. Unfortunately, conventional approaches for single-cell handling such as microdissection, micromanipulation and flow cytometry suffer from several limitations. For instance, microdissection is analytical, but does not preserve cell viability and function [56, 57]. Micromanipulation of live cells in suspension may be coupled with microscopical inspection and immunophenotypic tagging, but involves the repetitive, slow handling of one object at a time, and exclusively delivers visual readouts. As for flow cytometry, the ability to detect individual events relies on the serial manipulation of large number of cells (and distinct populations thereof). However, a minimum number of cells (few thousands) in suspension are required [58].

1.3.1 Dielectrophoresis (DEP)

DEP is the movement of particles in non-uniform electric fields [59–61]. Charges in the particle itself are not necessary for the effect to occur. This is due to the fact that when an electric field is applied to a system consisting of particles suspended in a liquid, a dipole moment is induced, due to the electrical polarizations at the interface between the particle and the suspending liquid. If the field is non-uniform, the particles experience a translation force (DEP force), of magnitude and polarity depending not only on the electrical properties of the particles and the medium,

but also on the magnitude and frequency of the applied electric field. This means that for a given particle type and suspending medium, the particles can experience, at a certain frequency of the electrode applied voltages, a translation force directed towards regions of high electric field strength (this phenomenon is called pDEP). Or, by simply changing the frequency, they may experience a force that will direct them away from high electric field strength regions (this phenomenon is called nDEP).

1.3.2 Lab-on-a-Chip Devices and Cell Isolation

The development of advanced analytical and bioseparation methodologies based on micro/macro arrays and biosensors is one of the strategic objectives of the so-called post-genomics. In respect to isolation of pure cell populations, approaches leading to the development of entire laboratories on a single substrate, the so-called "Lab-on-a-Chip", are of great relevance [62–65]. Elsewhere published results (reviewed in [62]) indeed firmly demonstrate that human cell populations of different histotype and differentiation stage could be isolated.

1.3.3 DEP-Based Lab-on-a-Chip Devices

We have recently reported a Printed Circuit Board (PCB)-based chip generating DEP-based cylinder-shaped cages that can entrap cells and move them along the device (Fig. 1.2a, b). This does not require a fluidic system and allows levitation and movement of large numbers of eukaryotic cells [65]. Furthermore, we have reported the development of a DEP-array, constituted of 320 × 320 electrodes and generating over 10,000 spherical-shaped cages able to accommodate single cells [66]. These can be controlled in parallel and two or more cages can be forced to share the same location, making possible in principle to bring in contact, for instance, different cells (Fig. 1.2c–g). These are the most important features of the proposed Lab-on-a-Chip devices: (1) the movement could be programmed at a single-cell level; (2) the chip carries out functions of both actuating and sensing type; (3) single cells can be forced to contact antibody-exposing microspheres. These features allow proposing Lab-on-a-Chip devices for diagnostic applications, cells separation and characterization in the field of non-invasive prenatal diagnosis [29–31]. The development of a dielectrophoresis (DEP)-based Lab-on-a-Chip approach is important to optimize enrichment, identification and genetic characterization of fetal cells in blood of pregnant women for non-invasive prenatal diagnosis. The optimization of prenatal diagnosis for chromosomal and monogenic disorders will be greatly facilitated if obtaining fetal material avoids the risks associated with amniocentesis, chorionic villus sampling and fetal blood sampling.

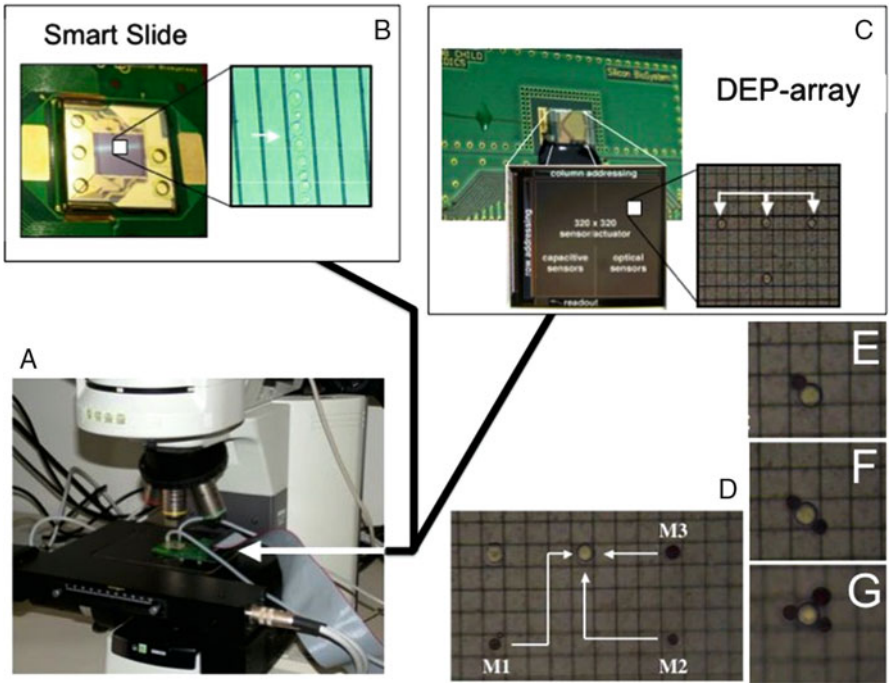

Fig. 1.2 Lab-on-a-Chip based manipulation of small number of cells (**a**, **b**) or single cells and single microparticles (**a**, **c**–**g**). The DEP-based SMART Slide and the DEP-array have been described in Altomare et al. [65] and Borgatti et al. [66]. The schemes of panel (**c**) are modified from Gambari et al. [63]. In panel (**c**) the DEP-based separation of single cells is shown; in panels (**d–g**) an example is shown demonstrating the possibility to target a single cell (see panel **d**) with one (**e**), two (**f**) or three (**g**) microspheres (Modified from Borgatti et al. [66]). This example gives clear evidence of the possibility to isolate/manipulate single cells with the use of DEP-based Lab-on-a-Chip platforms

1.4 Non-invasive Diagnostic Strategies in Prenatal Diagnosis of Genetic Diseases

Current methods for the diagnosis of aneuploidies and monogenic disorders require invasive testing by amniocentesis, chorionic villus biopsy or fetal blood sampling. These diagnostic techniques increase the frequency of fetal loss by about 0.5% [67]. One alternative way for obtaining information on the gestating fetus involves recovery of fetal cells from maternal blood.

In 1969, Walknowska et al. [68] described Y chromatin in blood cells from women carrying male fetuses. Ten years later, recovery of fetal leukocytes from maternal blood by fluorescence-activated cell sorting (FACS) with antibodies against paternally derived HLA (human leukocyte antigen) antigens was reported [69]. Schmorl [70] described deportation of fetal trophoblasts to the lungs of pregnant

women suffering from eclampsia. Trophoblast cells have not found widespread application in diagnostic studies because they are rapidly cleared by the maternal pulmonary circulation and are likely to exhibit confined chromosomal mosaicism [71]. Nucleated red blood cells (NRBC) are the most common cells in fetal blood during early pregnancy. Because they have a relatively short half-life, and because they express hematopoietic plasma membrane antigens, such as the transferrin receptor (CD71), the glycophorin A cell surface molecule and intra-cellular markers (epsilon and gamma globin chains), fetal NRBC have become the targets of choice. In 1990, Bianchi et al. [72] recovered fetal NRBC in maternal blood by using FACS. Fetal origin of the separated cells was confirmed by the presence of Y-chromosome DNA, corresponding to the sex of the fetus. Although the most represented fetal cells, NRBC are rare respect to maternal cells. In maternal blood samples they must be enriched by using antibodies against specific fetal antigens or by physical methods as gradients or electrophoretic mobility-based separations. Antibodies have been used with FACS [73] or magnetic-activated cell sorting (MACS) [74]. These methods exploit antigenic differences between cells. FACS is able to enrich cells with high purity so that slides with sorted cells can be readily scanned manually. It also allows multiparameter sorting and can be adapted for use with intracytoplasmic antigens. MACS, on the other hand, by using magnetic beads coated with specific antibodies, is a faster bench-top technique better suited to process larger cell numbers. Both negative and positive selections can be performed on the same population of cells. Detection levels of fetal cells with these two methods is compounded by small numbers of circulating fetal cells and loss of fetal cells during enrichment procedures. Bischoff et al. [75] reported a simple and rapid density-based progenitor cell enrichment approach. The samples were labeled with a RosetteSep^{TM} progenitor antibody cocktail to remove unwanted maternal white cells (mature T-cells, B-cells, granulocytes, natural killer, neutrophils and myelomonocytic cells).

Cellular fraction collected was analyzed by either fluorescent in situ hybridization (FISH) or real-time PCR for the presence of intact fetal cells and to quantify Y-chromosome-specific DYS1 sequences, respectively. Correct detection rates of the progenitor enrichment approach were 53–89% and fetal sequences were detected in the range from 0.067 to 1.167 genome equivalents per milliliter of blood. This method targets progenitor cells that are not necessarily of the erythroid lineage and may also allow expansion in culture and characterization of the fetal cell types that circulate in maternal blood. Some lymphocytes are long lived and by this approach there is concern that enriched progenitors may be the vestiges of previous pregnancies and do not represent the true fetal genetic status of the current pregnancy [76]. Charge flow separation (CFS) is an antibody independent selection method of fetal cells that relies on the behavior of cells in an electric field and a buffer counterflow gradient [77]. It is an automated rapid method that purifies NRBC from maternal blood and approximately 30% of these cells are fetal. For non-invasive prenatal diagnosis NRBC from fetus must be precisely identified. They could be distinguished from maternal cells by identifying paternal DNA

sequences [78]. This method can be extended using micromanipulation of candidate fetal cells and amplification of chromosome-specific short tandem repeats [79]. It is actually under investigation if recovery of fetal cells with CFS is higher than those obtained with other separation methods. The most important molecular techniques that have allowed genetic analysis of enriched fetal cells are PCR and FISH. The ability of PCR to amplify minute quantities of DNA (even single copies) over a billion fold has been exploited for the prenatal diagnosis of monogenic disorders from maternal blood [80–82]. Chromosomal FISH allows the detection of aneuploidies and chromosomal rearrangements in interfase nuclei. It has been used to detect most of the major fetal aneuploidies within fetal cells isolated from maternal blood [73, 83–85]. Fetal traits so far identified among the separated fetal cells include blood group antigen, the common trisomies, triploidy, polymorphic DNA repeats and some single gene disorders [80, 85–88].

All of these methods result in the enrichment of fetal cells among larger populations of maternal cells, but they do not enable recovery of pure populations of fetal cells. Experimental approaches that combine fetal cell identification with molecular genetic diagnosis in an in situ technique circumvent these limitations and are especially suited for automation [89]. Unfortunately the prenatal diagnosis from maternal blood is not practicable for approximately 30% of pregnant women, because the NRBC are not recovered. It is then important to optimize enrichment identification and diagnostic protocols (Fig. 1.3).

1.5 Non-invasive Diagnostic/Prognostic Strategies in Oncology

Diagnosis and prognosis in oncology is of course a primary goal and in this specific field of investigation, non-invasive approaches avoiding biopsies are of great interest. In most tumors, it is known since a long time that tumor cells are present within the circulatory system of cancer patients as “circulating tumor cells” (CTCs) [90–92]. For this reason not only they represent unique diagnostic targets, but they should be considered important for prognosis. In fact, CTCs are released from primary tumors into blood circulation and then they can initiate new cancer growth at novel tissues/organs. Recent advancements in detection and characterization of CTCs can have a significant impact on early cancer detection. For instance, the quantitation of CTCs can be used for prognosis of the outcomes of metastatic diseases [92]. In another example, the identification of cancer stem cells, a subpopulation of CTCs believed to be tumorigenic, can be used for identifying metastasis and relapse [93]. These assays are based on the use of monoclonal antibodies targeting tumor-associated antigens (TAA). Recently, the efficiency and selectivity of this approach was validated using protocols based on the detection of free nucleic acids in the blood circulation of cancer patients.

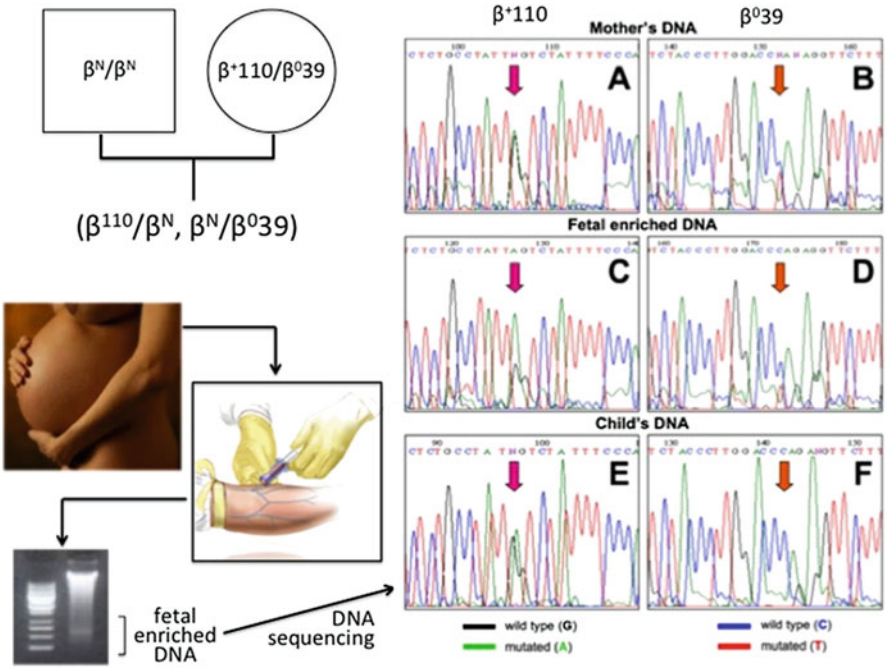

Fig. 1.3 Example of prenatal diagnosis using maternal peripheral blood, isolation of fetal enriched genomic DNA and DNA sequencing (Breveglieri et al., manuscript in preparation). In this example, the sequencing of the mother DNA is shown in (**a**) and (**b**), demonstrating a $\beta^{+}110/\beta^{0}39$ genotype. The sequencing of fetal enriched DNA demonstrates an increase of the $\beta^{+}110$ fraction and a decrease of the $\beta^{0}39$ fraction (panels **c** and **d**), suggesting a $\beta^{+}110/\beta^{N}$ genotype, that was confirmed after birth, as shown in panels (**e**) and (**f**)

1.5.1 Free Tumor DNA as Molecular Target

First of all, it should be underlined that tumor cells from each cancer patient can be isolated and characterized with respect to the genetic changes associated with cancer onset and, more importantly, metastatic progression [94–98]. Regardless of whether these genetic alterations are a direct cause of malignant transformation or whether they are a secondary molecular feature of tumor cells, several reports demonstrated that their analysis can provide useful information for clinical management of the cancer patients. Several molecular genetic alterations including somatic mutations in oncogenes and tumor suppressor genes have been described in melanomas, breast carcinomas, colon carcinomas. Some of these alterations, which are identical to those found in primary tumor DNA, have been observed in extracellular circulating DNA in blood samples of cancer patients. In an attempt to evaluate whether circulating DNA with characteristics of tumor DNA in cancer patients has prognostic value, several studies have reported a direct correlation with poor survival in pancreatic carcinoma, esophageal cancer, non-small cell lung

cancer (NSCLC), melanoma, head and neck carcinomas, and B-cell neoplasias. Cancer patients with circulating tumor DNA may have a different outcome than those patients who do not have this molecular change.

1.5.2 Free Tumor RNA as Molecular Target

In addition to circulating DNA, several studies have demonstrated that tumor cell-derived RNA is present in the plasma of cancer patients [99–104]. This is quite surprising and deserves further validation, since RNA is far less stable even than shared genomic DNA; however free circulating RNA has been found in several cancer patients, including for instance patients carrying breast cancer. One relevant example is the expression of erbB2 mRNA in the plasma analyzed by nested RT-PCR strategy. The available information suggests that cancer patients with erbB2 mRNA in the plasma may have a high malignancy or an aggressive phenotype, and frequently detecting plasma erbB2 mRNA may provide a novel approach for monitoring breast cancer progression or response to treatment.

1.5.3 MicroRNA as Molecular Target

MicroRNAs (miRNAs, miRs) are a family of small non coding RNAs that regulate gene expression by targeting mRNAs in a sequence specific manner, inducing translational repression or mRNA degradation [105–108]. The genes encoding miRNAs are longer than the processed miRNA molecules: miRNAs are first transcribed as primary transcripts or pri-miRNA and then processed to short, approximately 70-nucleotide stem-loop structures known as pre-miRNA in the nucleus. This process is carried out by a protein complex known as the Microprocessor complex, including the nuclease Drosha and the double-stranded RNA binding protein DGCR8 [105]. These pre-miRNAs are then processed to mature miRNAs in the cytoplasm following interaction with the endonuclease Dicer assisted by TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein), which also initiates the formation of the RNA-induced silencing complex (RISC) [108]. This complex is responsible for the gene silencing. MicroRNAs have been so far described to be involved in all the differentiation steps leading to production of highly-specialized cells. For instance, miRNAs have been described to be involved in cell cycle, apoptosis, inflammation, terminal differentiation [105–108]. Recently, it has been demonstrated that miRNAs circulate in a highly stable, cell-free form in the blood (i.e., they can be detected in plasma and serum). Tumor cells have been shown to release miRNAs into the circulation and profiles of miRNAs in plasma and serum have been found to be altered in cancer, suggesting broad opportunities for development of circulating miRNAs as blood-

based markers for molecular diagnostics [96, 100, 109–120]. For instance miR-21 [116], let-7a [113], miR-16 [113] levels in plasma might be employed for early detection of cancer as well as sensitivity to anticancer-based chemotherapy.

1.6 Molecular Diagnosis on Human Embryos

Two important parameters deserve great consideration in procedures related to in vitro fertilization and embryo transfer (IVF-ET), in the case molecular diagnosis of the embryo is required: (a) the choice of the best embryo to be transferred and (b) the fast, reliable and non-invasive diagnosis of genetic diseases. As far as point (a), several investigations on human in vitro fertilization procedures have recently associated the production of soluble HLA-G (sHLAG) molecules by 48–72 h early embryos with an increased implantation rate [121–123]. Previous studies demonstrated that HLA-G antigen modulation creates, by direct and indirect mechanisms, a tolerogenic microenvironment at the feto-maternal interface that is essential for a positive pregnancy outcome. In fact, HLA-G molecules, in both membrane bound and soluble isoforms, inhibit the innate Natural Killer response against cytotrophoblast cells that lack HLA class I and II expression [124]. Moreover, HLA-G antigens affect the adaptative cellular response by inducing the apoptosis of cytotoxic CD8+ T lymphocytes [125], impairing CD4+ T cell functions and preventing dendritic cell maturation [126]. Of course, despite the fact that this and similar approaches allow to select the best pre-embryos to be transferred, no information following this approach can be obtained on the genetic pattern of the analyzed embryo, i.e. whether it carries a gene defect at the homozygous state (point (b)). This is very important for embryo selection, in avoiding implantation of pre-embryos carrying life-threating genetic mutations (such as those causing β-thalassemia). In this case the medical need is a protocol allowing genetic testing on a single cell isolated from 8-cell embryos. The cells in this case are totipotent and, therefore, the pre-embryo from which a single cell has been isolated and genetically characterized can originate, after implantation and fetal development, a fully normal individual. This approach is safe for the diagnostic point of view, and carries low risk for the pre-embryos, as reported in several reviews [13–15].

1.7 Molecular Diagnosis on Pre-fertilized Oocytes

Currently, several oocytes are fertilized during IVF-ET procedures and two or more embryos could be transferred to the uterus in order to increase the chance of a positive pregnancy. However, this approach demonstrates a high risk of multiple pregnancies and perinatal mortality and morbidity. The necessity to overcome these problems has stimulated several studies to obtain pregnancy by a single embryo transfer and for ethical reasons to select the best gametes with a higher

competency in order to avoid the production of surplus embryos. In this context the identification of validated markers to select oocytes represents a fundamental step in IVF-ET [127]. Nowadays the oocyte selection is mainly performed by intra and extracytoplasmic morphological characteristics, but no definitive association between the morphology and the implantation outcome were obtained [128]. Several studies suggested that the embryo developmental potential and protein production at 4–8 cell stage depend mainly on the maternal mRNA [129]. As the oocyte is the keeper of maternal mRNA, the identification of oocytes with a higher production of molecules with a pivotal role in implantation could represent a marker of embryo functionality [130]. In order to understand if the oocytes could be implicated in sHLA-G production during the maturation process, Rizzo et al. have analyzed the culture supernatants of "in vitro matured" oocytes for sHLA-G presence. In order to analyze the sHLA-G production these authors have established a detection system that could reveal low levels of sHLA-G. The results obtained evidenced, for the first time, the ability of the oocyte-cumulus complex to produce sHLA-G antigens and proposed these molecules as a marker for oocyte maturation and consequently with a higher probability to develop in a viable embryo [131]. As far as detection of genetic mutations present in the pre-fertilized oocytes, the molecular analysis concerns the isolation of the first "polar body" (PB), which should be considered to have a complementary genetic pattern in respect to the relative oocyte. Therefore, polar bodies can be used for genetic testing. By cytogenetic analysis of the PB using fluorescent in-situ hybridization (FISH) or chromosome painting, partial or full chromosomal status in the oocyte can be predicted. For example, if the oocyte is derived from a heterozygous β-thalassemia patient and the polar body carries a normal β-globin allele, then the oocyte carries a mutated β-globin allele, with the risk to generate a homozygous β-thalassemia subject if fertilized by a spermatozoon carrying a mutated β-globin allele. Conversely, if the polar body carries a mutated β-globin allele, then the oocyte carries a normal β-globin allele, without any risk to generate a homozygous β-thalassemia subject. This technology has been applied to several genetic diseases and exhibits low risk for oocyte damage and subsequent fertilization rates or embryonic growth to the blastocyst stage [18–21].

1.8 From Complex Protocols and Instrumentation to Low-Tech Technologies

The medical needs in developed countries can be reached employing complex instruments governed by highly-trained researchers. However, even in developing countries, psychosocial and financial factors, inadequate insurance coverage, and the inability to pay for health care services are some of the known barriers to health-care [132]. When prenatal diagnosis is taken as an example, from an economical standpoint it has been demonstrated that these procedures have the potential of saving millions of dollars to healthcare systems. However, several patients do not

have the resources to access prenatal diagnosis in several countries. Developing countries, on the contrary, have completely different needs, most of which concern the identification of the primary mutation of the pathology, the possibility to perform genetic counseling and, possibly, to consider prenatal diagnosis in the case of the life-threating pathologies. However, in developing countries we have to face several problems, which are (a) low-tech facilities; (b) lack of advanced instrumentation in most hospitals; (c) lack of trained technicians; (d) ethical and social issues, making difficult the performance of genetic counseling and prenatal diagnosis. Despite the fact that social issues are difficult to be overcome, the need for advanced diagnostic protocols and tools employing low-tech approaches could be of great interest. Protocols which do not need complex, enzyme-based methodologies are appealing. An example is the deep interest in PCR-free diagnostic approaches [133–139]. Finally, a key point in this context is the portability of the instrument/technology [140] to reach, for example, villages located in geographical positions difficult to be reached, instead of recruiting patients and relatives to hospitals located distally.

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Chapter 2
DNA Analyses in Food Safety and Quality: Current Status and Expectations

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Abstract Food safety and quality are very important issues receiving a lot of attention in most countries by producers, consumers and regulatory and control authorities. In particular, DNA analysis in food is becoming popular not only in relation to genetically modified products (GMOs), in which DNA modification is the "clue" of the novelty, but also in other fields like microbiology and pathogen detection, which require long times for the cultivation and specially in cases in which the microorganisms are not cultivable like some viruses, as well as for authenticity and allergen detection. A new topic concerning "nutrigenetics and nutrigenomics" has also been mentioned, very important but still in its infancy, which could lead in the future to a personalized diet. In this chapter we have described the main areas of food research and fields of application where DNA analysis is being performed and the relative methods of detection, which are generally based on PCR. The possibility/opportunity to detect DNA without previous amplification (PCR-free) will be discussed. We have examined the following areas: (1) genetically modified foods (GMOs); (2) food allergens; (3) microbiological contaminations; (4) food authenticity; (5) nutrigenetics/nutrigenomics.

2.1 Introduction

Methods for the evaluation of food safety and quality have been greatly improved in the last years and received a beneficial input, in particular, from the availability of technologies deriving from molecular biology, which are producing new results and open new perspectives in food science, even giving rise to a new global approach called "Foodomics". "Foodomics" has recently been defined as a new discipline

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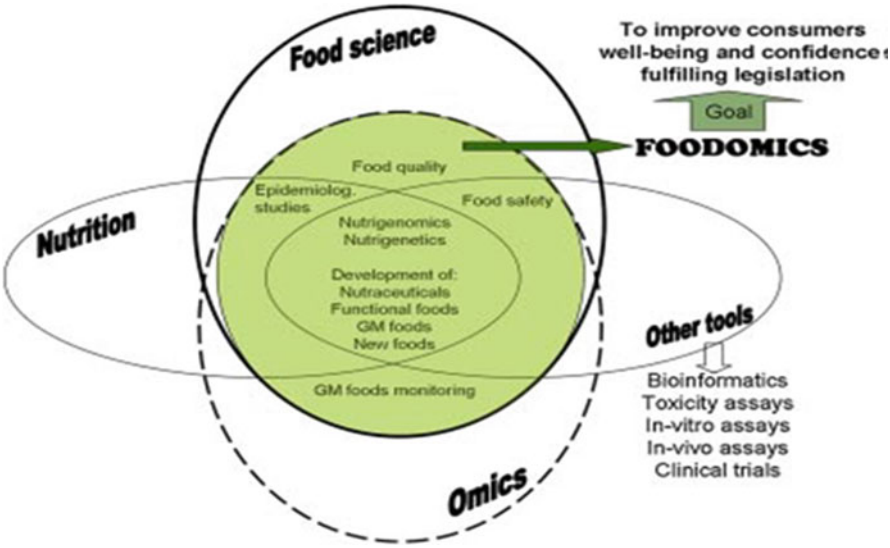

Fig. 2.1 Foodomics: covered areas, tools, and goals [3]

that studies food and nutrition domains through the application of advanced "omic" technologies including the genomic, transcriptomic, proteomic, and metabolomic studies of foods for profiling, authenticity, biomarker-detection related to food quality and safety, development of new transgenic foods, food contaminants, food effects on human health, etc. [1, 2]. Thus, Foodomics is becoming a global discipline that includes all the emerging working areas in which food, advanced analytical techniques (mainly "omic" tools) and bioinformatics are combined. A representation of the areas covered by Foodomics and its main goals can be seen in Fig. 2.1 [3].

This global interest in food also coincides with a clear shift in medicine and biosciences toward prevention of future diseases through adequate food intakes, and the development of the so-called functional foods.

Foodomics involves the use of multiple tools to deal with the different applications included in this field. In particular, research of new and selective methods for fast, reliable and sensitive detection of specific DNA sequences has received a lot of attention in connection with specific topics, such as GMO detection [4], microbial pathogen determination [5], investigation on the presence of undeclared allergenic ingredients [6], authenticity assessments [7].

This chapter does not intend to provide an exhaustive report of the many works published so far on food analysis concerning DNA detection but to provide an overview of the current status of application, of the methods utilized and of the eventual perspectives.

The detection of DNA in foods always starts with its extraction, which must be carefully optimized, since it is strongly dependent on the nature and complexity of

the food matrices and in some cases, such as oils, on the low abundance of DNA [8]. Indeed, extraction and purification procedures of genomic DNA from food strongly depends not only on the matrix, but also on the molecular transformations of food components induced by the different technologies applied and on the eventual additives present in food. High temperatures can give rise to xenobiotics which can interfere with the amplification process, DNA may be degraded or insolubilised [9]. Thus, although several kits are available on the market, no methodology can be considered universal so far [10].

Once the purity of the target DNA extracted has been verified, amplification is usually performed by Polymerase Chain Reaction (PCR) using specific primers in order to amplify selectively the region of interest of the DNA [11]. For routine purposes, the presence of an amplified DNA product of the expected size, normally assessed by gel electrophoresis, is usually considered a sufficient proof of the presence of the sequence of interest. In many cases the identity of the amplified DNA must be confirmed. This is usually performed by "nested PCR", which consists in a further amplification of a shorter sequence within the former strand. Alternatively, recognition of the presence of the target sequence can be performed by hybridisation with specific probes (mostly oligonucleotides) via Watson–Crick base pairing. In place of DNA oligonucleotides, other nucleotide analogues, in particular locked nucleic acids (LNA) [12], morpholino oligonucleotides (MO) [13] and peptide nucleic acids (PNA) [14] and their analogs [15] can be used, since these compounds have shown to improve DNA binding affinity and selectivity. Peptide nucleic acids are particularly suited, since they are robust molecule from the chemical and biological point of view and have shown a high sequence selectivity, thus allowing to detect single nucleotide polymorphisms (SNP) very efficiently [16]. Recognition should be as specific as possible, and must be followed by a change in some measurable properties either in solution or on a sensing surface. This can be achieved by subsequent reactions (mostly enzymatic) or by changes in some physico-chemical properties [17].

End point PCR may be performed at the qualitative level also on devices based on the Lab-on-chip technology, in which sample extraction, amplification and detection occur on a surface functionalised with oligonucleotide probes [18].

Real time PCR is considered the golden method for DNA quantification, it requires more expensive laboratory equipment, but it is extremely accurate and less labour-intensive than other DNA quantification methods. Real-time PCR allows product detection 'in real time'. Amplification occurs in the presence of a target-specific oligonucleotide probe carrying a reporter dye and a quencher group: due to the proximity of the quencher to the reporter, fluorescence is suppressed. The probe anneals to the single-stranded amplified segment within the region flanked by the two oligonucleotides priming the enzyme reaction and, during amplification, the 5′-exonuclease activity of the polymerase cleaves the probe and separates the fluorophore from the quencher, thus switching on fluorescence of the free reporter producing a measurable signal. The increase in fluorescence is proportional to the amount of the specific PCR product. The possibility to use authentic DNA standards is highly desirable for a proper quantification, though not often available.

It is possible to perform multiplex PCR, which requires two or more couples of primers, which must be compatible, i.e. not complementary to each other, not giving rise to duplexes or secondary structures, which may prevent or limit annealing.

Potentials and limitations of molecular diagnostic methods based on PCR in food safety have been reported [19] and will be discussed in connection with the specific topics reported below.

Detection of DNA without previous amplification has been recently achieved by adopting different techniques, described in this book. These techniques require an enhanced sensitivity in order to detect non amplified genomic DNA, which can be obtained with different strategies by combining specific materials with specific detection tools. The possibility/opportunity to detect DNA in food without previous amplification will be discussed in relation to the different subjects reported in this chapter.

In this chapter we'll describe the main areas of food research where DNA analysis is being performed and the relative applications with favorable results or eventual drawbacks which need to be evidenced and overcome. We will examine the following areas: (1) genetically modified foods (GMOs); (2) food allergens; (3) microbiological contaminations; (4) food authenticity. (5) nutrigenetics/nutrigenomics.

2.2 Genetically Modified Organisms (GMOs)

The development and diffusion of genetically modified plants and organisms and their increasing diversification aimed at introducing new characteristics and properties in food and feed creates new challenges for their identification, so that this very complex matter requires not only new methodological and technical approaches but also a particular attention to the legal aspects and general information. According to the latest International Service for the Acquisition of Agribiotech Applications (ISAAA) report [20], 144 GM events representing 24 crops, have received international regulatory approval so far and it is believed that this number is going to increase. The approval and insertion on the market are regulated in different countries by the respective legal frameworks. While in the European Union the legislation is quite severe and requires stringent approval, labelling and traceability of foods and ingredients [21–23], in the USA labelling is only voluntary [24], other countries (Canada, Japan, Australia and New Zealand) have adopted mixed systems of the previous ones [24], others do not have any regulation at all [25]. Thus, a food which is allowed in a country is not necessarily approved in another, causing serious problems for trade.

Real Time PCR is the standard method for the detection of genetically modified organisms (GMOs), since the structure modification of DNA with the insertion of exogenous sequences is the clue to discriminate the GM modified from the wild type genome. Information about the target sequences of GM crops may be obtained through several website: CERA (2011) [26], BATS (2003) [27], GMDD (2011)

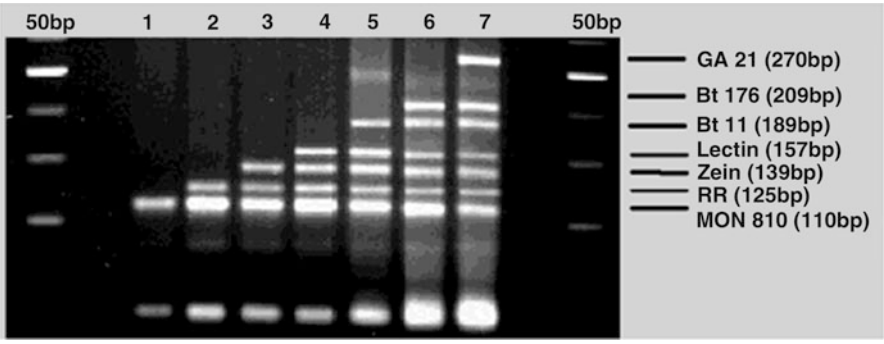

Fig. 2.2 Multiplex PCR for GMO. A mix of the DNA extracted from the lectin and zein wild type and five trangenes containing 20% of each transgene was amplified using, from *lines* 1 to 7, one, two, three, four, five, six, and seven primer pairs, following their relative amplicon length (Reproduced from Germini et al. [33] with permission)

[28], GMO-Compass (2009) [29], CRL (2009) [30], and more general database such as NCBI [31]. The most utilised targets are the gene expression cassettes for herbicide and insect resistance. Therefore, methods to detect genetic traits coding for these characters are mostly utilised together with the promoter DNA (such as the 35S cauliflower mosaic virus promoter, CaMV), terminators (such as the inducible nopaline synthetase terminator iNOS), and genetic markers such as antibiotic resistance. The detection of the 35S CaMV promoter is used for the general screening of the presence of a GM material, regardless of the identity of the crop. Indeed, for the quantitative determination of DNA, specific reference materials are required, which are not often available.

In the last years GMOs carrying a combination of genetically modified traits (i.e. herbicide and insect resistance) have become quite popular so that the detection of a single gene expression cassette is not sufficient to characterise the genetic material, but rather a combination of several targets must be addressed. Thus, new methods are required for the multiple detection of DNA. Several targets can be tracked simultaneously by means of multiple PCR [32]. The various PCR products are distinguished one from another on the basis of their differential migration through agarose gels. A typical procedure for multiplex PCR has been outlined by our group [33], who showed the simultaneous amplification of seven sequences belonging to endogenous soy and Roundup Ready soy, and to five sequences of maize (endogenous maize, Bt11, Bt176, Mon810 and GM25) (Fig. 2.2). This method is qualitative and useful for screening purposes, preliminary to qPCR for the certification procedure.

Confirmatory analysis are often required specially for legal purposes. A general method to address this problem is represented by nested PCR, in which one or more internal traits are amplified via a careful choice of the primers[34]. PCR clamping with PNA probes flanking the sequence targeted by primers have been used in order to assess the identity of the PCR products and the semi-quantitative determination of the GMO content without the need of other instrumentation [35].

Another strategy implies hybridisation of the amplified DNA with specific probes in order to switch on a signal through a sensing platform. Several probes have been reported, mostly oligonucleotides complementary to the target sequence [16]. Chromatographic methods have also been reported for the assessment of the PCR product, based on the different migration time of the PCR product and of the probe-PCR amplicon complex [36]. Fluorescent switching probes such as molecular beacons are very useful for improving sensitivity of the method [37].

A real-time PCR-based approach is the GMO screening platform CoSYPS, which is based on the SYBR® Green real-time PCR method [38] and combines the detection of the presence of major commodity crops (such as soybean, maize, oilseed rape, rice and cotton) with the detection of common genetic recombinant elements (such as the p35S/tNOS elements) and GM-specific elements (such as herbicide-resistance genes and insect-resistance genes).

Another approach for the detection of multiple GM events in a single experiment is the "realtime PCR based ready-to-use multitarget analytical system" recently developed by Querci et al. [39], which is based on the TaqMan® real-time PCR technology and consists of 96-well plates containing lyophilized primers and probes for the individual detection and the simultaneous identification of 39 GM events in seven plant species.

Other technologies have been proposed as a tool for the simultaneous detection of different targets. PNA microarrays [40, 41] have also been shown to be very specific tools, allowing to analyse simultaneously endogenous soy and Roundup Ready soy, and five sequences of maize (endogenous maize, Bt11, Bt176, Mon810 and GM25) with sound results.

Hamels et al. [42] have also developed a multiplex PCR-microarray method for the screening of GMOs.

Currently, only a few publications have reported the use of alternative methods to PCR-based methods for detecting GMOs. One of these is the loop-mediated isothermal amplification (LAMP) method, a technique based on the use of a set of four specially designed primers that recognize a total of six distinct sequences on the sense and antisense strands of the target DNA [43]. LAMP is a highly sensitive isothermal method without need for thermal cycling or any heat denaturation of the template DNA, so that it can be used without a dedicated equipment. Real-time LAMP has been used in the field of GMO detection to target the promoter of the cauliflower mosaic virus 35S, one of the most common genetic elements used in transgenic constructs. The method was tested on a range of different Round-Up Ready soy contents and was shown to be satisfactory for semi-quantitative GMO content evaluation [44]. However, the complex design of primer sets required for each target element may pose very serious problems for the use of LAMP in a multiplex design.

Another technique, which can be used with or without PCR is the Ligase Detection Reaction (LDR), which utilises a thermostable DNA ligase and a pair of probes, which bind to one strand of the DNA target sequence, leaving a nick between them which acts as a template for highly specific ligation by the DNA ligase.

Successive cycles of denaturation-annealing and ligation lead to amplification of the desired fragment [45, 46]. In the PCR/LDR assay, a preliminary PCR step amplifies the target sequence, and subsequently the target signal is detected by LDR. If the ligation primers are labelled with a fluorescent donor and an acceptor group, a ligation event will enable fluorescence resonance energy transfer (FRET) to occur, thus enabling real-time detection. The use of PCR/LDR in combination with universal array capture has been utilized for GMO detection in food, demonstrating high sensitivity and specificity of the method [32, 47, 48]. The simultaneous detection of endogenous genes of soybean and maize (lectin and zein, respectively) and five DNA constructs of genetically modified crops: Roundup Ready soybean and Bt11, Bt176, GA21, and MON810 lines of maize was performed. However, the multiplex PCR/LDR method allowed for qualitative (and semiquantitative) analysis of the GMO content, since the PCR step was unable to amplify the targets proportionally to the amount of the target DNA present.

The Nucleic Acid Sequence-Based Amplification (NASBA) [49] is an isothermal amplification technique that mimics retroviral replication. So it is applied to RNA amplification. In GMO detection, amplification of RNA is very limited due to the low RNA stability in food samples. Thus, NASBA retains a potential for GMO analysis only if adapted to a DNA amplification method. A big advantage in comparison with the PCR technology could be a fast multiplex GMO detection and quantification method if associated with microarray hybridization. This was introduced in GMO screening by Morisset et al. [50, 51], who have developed a novel multiplex quantitative amplification method to be used in combination with a microarray detection named NASBA Implemented Microarray Analysis (NAIMA). In the first step, tailed primers allow for the multiplex synthesis of template DNA. In the second step the transcription-based amplification using universal primers is performed, leading to formation of the complementary RNA product, which is then directly ligated to 3 DNA dendrimers labelled with fluorescent dyes, allowing for signal amplification, and hybridised on an oligonucleotide-based microarray for multiplex detection. Two triplex systems have been applied to test maize samples containing several transgenic lines. The great advantage and further potential of the NAIMA method is its possibility for quantification in a multiplex platform [52].

Several attempts are being studied in order to design platforms for the screening of all authorized and not-authorised GMO in Europe. For this purpose, Waiblinger et al. [53] have established an Excel spread-sheet describing the application of five different screening real-time PCR methods: P35S [54, 55]; T-nos [54]; CTP2-EPSPS [56]; bar [57]; P35S-pat [54] which cover, with the exemption of only three events (maize, LY038, soybean 305423 and cotton 281-24-236 × 3006-210-23), all authorised and non-authorised GMPs in the EU. This is important not only for the GM plants already authorised for placing on the market, but also for upcoming GM crops that might be commercialized in the near future [58]. The screening table will be updated regularly by a network of German enforcement laboratories [55].

The latest progress made in GMO analysis, taking examples from the most recently developed strategies and tools, is reported in a review by Querci et al. [4].

However, in spite of the fact that PCR technology is the method of choice for GMO detection so far, its application in the detection of several targets is limited by the poor multiplexing capabilities of PCR.

Moreover, due to the increasing number of events related to crops and of different crops subjected to genetic transformations, it is expected that in the near future the picture will be even more complex. Traditional approaches, based on the detection of one target at a time, or on a limited multiplexing, no longer will meet the testing requirements. Along with new analytical technologies, new approaches for the detection of GMOs are needed.

The future will be in avoiding PCR and performing the direct detection of GM materials using ultrasensitive PCR-free techniques? Some of these techniques have already been developed, though not yet validated for official purposes. Quartz crystal microbalance (QCM), using oligonucleotide "openers" have been reported in the first "PCR-free" detection scheme [59]. Surface plasmon resonance imaging of DNA targets was also used for ultra-sensitive detection of unamplified DNA according to a sandwich-like test with gold nanoparticles [60]. Using this procedure on genomic DNA extracted from certified standard soy flour, it was possible to detect directly Roundup Ready transgenic soy extracted from certified standard soy flour without any enzymatic amplification [61]. The method allowed to detect 0.1% of transgenic soy, well below the EU legal limit of 0.9%.

Thus, ultra-sensitive methods to detect even few copies of DNA are available, though at the moment quite sophisticated and expensive. Certainly, the advantages to avoid PCR would be tremendous (less steps, less reagents, less possibility of contamination, more accurate quantitative determinations, etc.), but other problems are still to be challenged in order to make these methods suitable for official screening, such as the multiple detection. In any case, the accessibility to correct DNA sequence information, validated methods and reference materials are fundamental prerequisites for setting up an effective strategy.

2.3 Food Allergens

Food allergies might be defined as adverse, immune-mediated (IgE mediated) reactions to foods that can occur in sensitised individuals. The prevalence of IgE-mediated food allergies is estimated to be around 4% of the general population [62] and even higher in infants and children reaching 8% [63]. The severity of the reactions varies from mild symptoms to potentially lethal anaphylactic shocks.

Recently, in order to protect the consumers, the EU introduced several Directives listing the common allergenic ingredients to be declared on the label when present in a given food: Directives 2000/13/CE [64], 2003/89/EC [65], 2006/142/EC [66] and 2007/68/EC [67]. The list, reported in Annex IIIa of the 2003/89/EC [65], and modified by European Directive 2006/142/EC, includes cow's milk, molluscs, crustaceans, hen's egg, fish, tree nuts, peanuts, soy, cereals, celery, mustard, sesame and lupin. Allergies to fruits and vegetables (peach, apple, cherry, kiwi, tomato, spinach, carrot, etc.), less common, are not to be declared so far.

Indeed, of particular concern are the so called “hidden allergens”, i.e. allergenic ingredients accidentally present in food, and thus not declared on the label, which may trigger severe allergic reactions if inadvertently consumed by susceptible subjects.

Allergens are proteins, thus the detection of allergenic ingredients in food is usually performed by methods of protein analysis combined with immunochemical protocols such as radio-allergosorbent test (RAST), enzyme allergosorbent test (EAST), rocket immuno-electrophoresis (RIE), immunoblotting and enzyme-linked immunosorbent assay (ELISA). While RIE e immunoblotting provide only qualitative or semiquantitative results, RAST, EAST e ELISA may be utilized as quantitative methods [68, 69].

At the moment ELISA is the technique mostly utilized for the routine analysis of allergens in foods by Control Institutions as well as by Industry. With this method allergens are detected by complexation with a specific antibody and by measuring the concentration on the basis of a color reaction. Two different approaches are available: competitive and sandwich. Several kits are commercially available mainly using the sandwich approach [70]. ELISA methods offer several advantages, being easy to handle, fast and quite sensitive, though suffering from different drawbacks, since they are very depending on the antibody lot and manufacturer, the food matrix effects, the extraction procedures, cross reactivity and the technological treatments of food, which may alter the structure of the native protein, leading to false positive or false negative [71–75]. Moreover, the availability of certified standard materials is fundamental to perform quantitative determinations [76]. Unfortunately, not many certified allergenic proteins are available on the market; some are produced by IRMM 481 Peanut Test Material Kit, NIST RM 8445 for egg, NIST RM 1549 and NIST RM 8435, respectively for screamed milk powder and whole milk, and NIST RM 2387 for peanut [77]. Limits of detection (LOD) and of quantification (LOQ) are generally quite low, though sometime not defined properly.

Mass spectrometry techniques for detecting proteins are becoming popular, although not yet suitable for quantitative determinations and for routine analysis [78]. In most cases identification of the protein is made through enzymatic digestion and identification of peptides by LC/MS/MS [79–82].

Whenever direct methods fail if applied to complex food mixtures or to severely processed foods, in which proteins may be heavily modified and, therefore, not detectable by antibodies directed to recognize their native forms, indirect methods, based on the detection of DNA sequences specific for a given allergenic ingredient may be used. Indeed, DNA detection is more feasible in these cases, DNA being more resistant to drastic thermal treatments, so that, in general, methods for DNA detection are considered more robust.

Amplification of DNA for allergen detection is usually performed in the two canonical ways: End point PCR (or qualitative PCR), which allows to detect the presence of a specific DNA sequence, but is not quantitative, and Real time PCR (quantitative PCR or qPCR), which allows the detection in real time of amplified DNA and also its quantitative determination, by utilising an adequate reference material. Although methods for DNA detection for all foods listed in Annex IIIa

of the 2003/89/EC [65], and modified by the European Directive 2006/142/EC and following exemptions have been reported in the literature [6], the methods of DNA detection most intensively studied in the last years are for wheat, peanut and hazelnut, followed by sesame and soy. Not always the DNA sequences studied are located on the genes expressing the allergen. In these cases the analysis will not establish the presence of the allergenic protein but rather the fact that the food contains (or has been in contact with) the allergenic ingredient (i.e. the hazelnut). This message is already important for the allergic patient.

In some cases a multiplex PCR may be used, for the simultaneous detection of different DNA sequences of the same organism or of different organisms. These systems are very convenient allowing to save time and resources, but they must be carefully designed and validated. Not many are the "multiplex" protocols reported in the literature for the detection of allergens, in particular those based on multiplex Real time PCR [83]. Amplification of different genes codifying for different allergenic proteins of the same organism (i.e., Cor a 1, Cor a 8, Cor a 14 for hazelnut) requires a careful experimental design and a critical selection of the target sequences for obtaining equivalent amplification yields [84]. In general, the amplification of a specific single sequence, both in End point PCR and in Real time PCR, is considered more reliable on account of the higher specificity and sensitivity [23].

In order to favor identification of the DNA of an organism codifying for an allergen, it can be useful to increase the yield by using target sequences which are repeated in the genome [85]. Alternatively, in the case of low amount of DNA present in food (for instance in oils) a very specific confirmatory analysis is usually mandatory. Peptide Nucleic Acids (PNA), thanks to their very specific DNA binding properties, their chemical and enzymatic stability and the possibility to be used in connection with several detection methods, are ideal candidates for the task [16]. The possibility to use PNAs as confirmatory probes post-PCR analysis has been first demonstrated in combination with HPLC/FLD for hazelnut [86]. In this case a PNA probe complementary to a sequence of Cor a 1 was injected together with an amplified fluorescently labelled DNA strand in HPLC: the formation of a PNA–DNA duplex was visualized by a shift of the retention times of the complexed DNA. Successively, the post-PCR confirmation was tackled with a different technique, i.e. by using PNA microarrays, demonstrating that multiple allergen detection is feasible by this platform [87]. In this case PNA array platforms were devised with two different PNA probes complementary to the gene regions coding for Cor a 1 (the major hazelnut allergen) and Ara h 2 (the major peanut allergen). Then, a duplex PCR was developed and the PCR products were deposited on the plate, giving rise to fluorescent signals whenever complementarity was present.

A mixed technique "PCR-ELISA" has been also proposed, by combining the two standard techniques, allowing to obtain semi-quantitative results [88].

Although methods for DNA detection are considered specific and robust and specially useful for the identification of "hidden allergenes" in food, however, in some cases, PCR may be misleading: DNA may be degraded at acidic pH, at high temperatures (i.e. roasting of hazelnuts and peanuts) leading to under-evaluation

of the real allergen content [89]. A comparative analysis of PCR/ELISA methods was performed and the impact of heat treatment on peanut kernels as well as the impact on a peanut-containing food matrix was investigated. It was shown that heat treatments had a detrimental effect on the detection of peanut with either type of method and that both types of methods were affected in a similar manner. Moreover, the DNA methods are not related to the specific gene expressing the specific protein of a food which triggers a particularly severe allergic reaction, but to the general genomic asset of the organism. Commercial kits concerning most foods reported in the European Regulation 142/2006 EC are available on the market (cereals, crustaceans, molluscs, eggs, fish, peanuts, soy, cow's milk, almonds, hazelnuts, walnuts, pistachios, celery, mustard, sesame and lupin). Other protocols for the detection of DNA of allergenic foods, not listed in Annex IIIa of the 2003/89/EC [65], and modified by the European Directive 2006/142/EC, such as peach, tomatoes, carrots, etc, have been reported in the literature. Fast SYBR Green DNA melting curve temperature analyses and duplex PCR assays with internal control have been developed for detection of these allergenic vegetables and have been tested on commercial foods. Spiking food experiments were also performed, assessing that limits of detection (LOD) of 1 mg/kg for carrot and tomato DNA and 10 mg/kg for celery DNA have been reached [90].

The quantitative analysis of DNA by PCR is strongly dependant on the availability of reference materials and on the knowledge of the genomic sequences. Only a few standard materials are commercially available at the moment (NIST, IRMM), although not yet certified. The limits of detection (LOD) and of quantification (LOQ) are often not reported in the publications and on the commercial kits. However, since in most cases the correlation between the amount of DNA and of the allergenic protein present in the food has not been determined, the quantity of DNA cannot be utilised in risk evaluation so far. Thus, the results obtained by PCR and by ELISA are not immediately comparable in most cases.

The European Committee for Standardization (CEN) has recently issued new standards for the detection of food allergens in order to uniform the allergen analysis in foodstuff across Europe: the EN 15634-1: 2009 "Foodstuffs – Detection of food allergens by molecular biological methods" reports the requirements for the specific amplification of target nucleic acid sequences and for the confirmation of the identity.

More recently, the European Standard EN 15842 (2010) has provided general considerations and validation of the methods (immunoassays, nucleic based and chromatographic) to be utilized for the detection of allergens in food.

As far as the applicability of PCR-free methods to the detection on food allergens, the situation is more critical and complex than for GMOs. In fact, allergens are proteins and the preferential methods of detection are primarily related to proteomics, especially Mass Spectrometry [3]. However, DNA evaluation is complementary or an alternative to protein determination whenever the protein is modified, following technological treatments, or difficult to extract or present in a very low amount. The presence of DNA of a particular species/commodity testifies unequivocally that the food is contaminated or has been in contact with or contains

the ingredient which may cause adverse reactions to susceptible individuals. In these cases the availability of ultra-sensitive PCR-free methods may be very useful, being able to detect even small traces of genomic DNA. An example has already been provided in the previous chapter on GMO, where it has been shown that it was possible to detect DNA from a certified sample of flour containing Roundup Ready soybean down to 41 zeptomolar concentrations, corresponding to 11 copies of DNA bases [61]. The finding is relevant in this case, since soy is an allergenic product to be declared on the food label.

Finally, the PCR-free methods available at the moment cannot be used on a routine basis to prevent exposure to potentially harmful ingredients, however they can already be very useful in particular cases when other methods fail or are equivocal, for confirmatory purposes, for instance for legal controversies.

2.4 Microbial Contamination

Foodborne illness represents a major public health issue. The Centers for Disease Control and Prevention (CDC) has recently estimated that the total effect of contaminated food consumption in the United States amounts to 47.8 million episodes of illness, 127,839 hospitalizations, and 3,037 deaths. Among these cases, CDC has calculated that each year 31 major pathogens in the United States (US) caused 9.4 million episodes of food-borne illness, 55,961 hospitalizations and 1,351 deaths. In the US, most (58%) illnesses were caused by Norovirus, followed by non-typhoidal *Salmonella* spp. (11%), *Clostridium perfringens* (10%), and *Campylobacter* spp. (9%), whereas leading causes of hospitalisation were non-typhoidal *Salmonella* spp. (35%), Norovirus (26%), *Campylobacter* spp. (15%), and *Toxoplasma gondii* (8%). Again, the leading cause of death were non-typhoidal *Salmonella* spp. (28%) followed by *T. gondii* (24%), *Listeria monocytogenes* (19%), and Norovirus (11%) [91, 92].

According to the most recent report produced by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), a total of approximately 339,000 confirmed cases of zoonoses were reported in 2008. Campylobacteriosis remained by far the most frequently reported zoonotic disease in humans (190,566 confirmed cases) followed by salmonellosis (131,468 confirmed cases). Moreover, 5,332 food-borne outbreaks were reported by the 25 reporting Member States during the same year. Overall, 45,622 human cases, 6,230 hospitalisations and 32 deaths (case fatalities) were related to the reported outbreaks.

In the EU *Salmonella* remained the most commonly known causative agent of food-borne outbreaks being responsible for 35.4% of all reported outbreaks. As in 2007, food-borne viruses were the second most common causative agent of food-borne illness, accounting for the 13.1% of all reported. *Campylobacter* caused 9.2% of all reported outbreaks.

Considering that the source of illness often comes from public settings such as restaurants, cafes, schools and kindergarten and taking into account that for several foodstuff the contamination event may occurs at numerous stages along the food chain, it is clear that the availability of diagnostic tools specifically developed for food safety is crucial to control and prevent food-borne illnesses and out-brakes, protecting the consumers and reducing the loss of money related to medical care and elimination of commodities.

Microbiological testing plays a relevant role in food production and processing and represents a growing field at retail and consumer level. The increasing awareness about food safety and the requirements of guidelines and laws at national and international level are strongly boosting the technical innovation in food microbiology. Culture-independent techniques, molecular tools for genomics and proteomics and sensor based approaches may play a pivotal role in diagnostics, community profiling and gene expression studies in food and food-related ecosystems in the next future. Even now, some of them, such as PCR-based techniques, are fundamental tools for food safety investigations.

A wide range of culture-based methods is currently available to detect common food-borne pathogens. These analyses are generally based on several sequential operations: a cultural enrichment (often composed of pre-enrichment and selective enrichment sub-steps), a selective and differential plating, a confirmation phase and a strain identification process. Usually, the whole process can take from 4 days to a couple of weeks. Although culture-base methods are quite reliable, they have often turned out to be time consuming and strongly labour intensive. The increasing need to reduce the time for detection led to the replacement of the selective and differential plating step with molecular-based assays such as PCR, which has currently become a gold standard for the detection of food-borne pathogens.

PCR-based methods potentially represent appropriate tools to study microbial communities in food-associated matrices and processes [93]. However, nowadays, these techniques are mainly employed for the detection, the identification and the quantification of microbial pathogens or for monitoring specific beneficial population like fermenting microorganisms.

Methods focused on nucleic acid amplification like PCR are often coupled with some degree of cultural enrichment as pre-treatment step and they offer some fundamental advantages over culture-based microbial analysis: (i) they are faster and less labour intensive. PCR takes from 5 to 24 h depending on the method used and the reasonable level of automation available reduce the needs for manual operations, thus simplifying experimental protocols; (ii) they are more sensitive and more specific. Being based on selective amplification of small amounts of DNA fragments, PCR is often more reliable and informative than culture-based approaches; (iii) the detection of sub-populations is possible even in the absence of a selective enrichment medium. Targeting specific genome sequences allows to detect only desired microbial strains even in the presence of other dominant populations, which otherwise equally grow in not selective media. Moreover, it is possible to set up quantitative assays. In order to be compliant with food safety requirements, accurate quantification is crucial for food microbiology applications,

specially in food pathogens diagnostics. A wide range of real-time quantitative PCR (qPCR) assays are available for this purpose. Compared with end-point PCR, these approaches do not require post-amplification processing and offer template quantification over a wide dynamic range (7–8 log).

It is possible to selectively detect living cells vs dead cells. Since genomic DNA is present in dead cells for long time, conventional PCR does not allow to discriminate between viable and not-viable microorganisms. To overcome this limitation, quantitative reverse transcriptase PCR (RT-qPCR) assays were developed. These kind of assays use RNA as primary target instead of genomic DNA, thus allowing to detect only the actively transcribed genes. With PCR it is possible to detect viable but non-cultivable (VBNC) microorganisms. In certain conditions PCR provides good results even without pre-enrichment steps and in some cases microorganisms, that cannot grow in standard growth media, can be detected and identified via PCR. Considering that numerous microorganisms, including pathogens, are able to enter in VBNC state, these molecular tools are particularly useful to obtain a more accurate analysis of microbial composition or microbial processes, specially in foods.

Several PCR-based assays allow to monitor the amplification of more than one target in the same reaction tube (multiplexing capability). This approach leads to reduced costs and time-to-results and gives the possibility to introduce internal amplification controls (IACs) and normalization strategies during gene expression analyses.

Several commercial kits and detection methods are currently available. The most commonly employed reporter systems in food microbiology are two: one is based on aspecific DNA binding dyes acting as fluorophores, such as SYBR® Green, and another focused on the hydrolysis of peculiar probes, like TaqMan®, due to the 5′-nuclease activity of certain DNA polymerases. The first one is relatively inexpensive, flexible though relying exclusively on primer specificity; moreover, the only specificity evaluation consists of the indirect measurement of post-amplification melting curves. The latter is more expensive but it ensures monitoring of the specific amplicon and allows multiplex analysis.

Since PCR is a mature technology, several commercial kits, well-defined protocols and numerous scientific papers are available for food safety applications (see Table 2.1). Moreover, it must be pointed out that ISO standards have also been defined for detection of food-borne pathogens by means of PCR, providing guidelines and standardized approaches (ISO/TS 20836:2005; ISO 22174:2005; ISO 20837:2006; ISO 20838:2006; ISO 22118:2011; ISO 22119:2011).

It is possible to couple PCR techniques with other technologies and methods such as Surface Plasmon Resonance (SPR), Quartz Crystal Microbalance (QCM), Enzyme-linked ImmunoSorbent Assay (ELISA) , Fluorescent In-Situ Hybridization (FISH).

In order to simplify the operative protocols or to increase sensitivity and specificity, several PCR-free amplification strategies for nucleic acids detection have been proposed and developed so far. Although PCR-based methods are currently recognised as gold standard in nucleic acid detection, isothermal amplification

technologies such as Nucleic Acid Sequence-Based Amplification (NASBA) [106], Loop-Mediated Isothermal Amplification (LAMP) [107, 108] recombinase polymerase amplification (RPA) [109], Helicase-dependent isothermal DNA amplification (HDA) [110, 111], or Rolling Circle Amplification (RCA) [112] are increasingly gaining popularity as effective alternatives for specific diagnostic purposes. These diagnostic tools are generally based on the employment of enzymes and primers that allow the amplification of DNA or RNA in isothermal conditions, eliminating the needs for complex thermal cycling instrumentation. Some of these methods are extensively investigated in clinical, environmental and food diagnostics. For example, NASBA has been used for food safety purposes to detect Norovirus [113–115], *Campylobacter jejuni* [116, 117], *Lysteria monocitogenes* [118]; Hepatitis A [119] and LAMP assays were developed to detect *Salmonella* spp. [120–126], *Listeria monocytogenes* [127, 128], *Legionella* spp. [129], *Escherichia coli* [130], *Clostridium perfringens* [131], *Vibrio vulnificus* [132, 133], human Norovirus [134], *Fusarium graminearum* [135], and *Campylobacter coli* [136]. Although quite recent, isothermal amplification methods are rapidly gaining ground in the diagnostics area because they present some advantages over the PCR-based approaches: the majority of the isothermal methods provide shorter time-to-result thanks to massive amplification processes and no needs for complex post-amplification operations. In some cases, such as in LAMP applications, the whole analysis takes around half an hour to be completed [107].

The robust amplification platform, such as in some NASBA applications, brings up to 10^{14}-fold amplification in less than 90 min (a couple of order of magnitude more than PCR), thus helping to increase the sensitivity of the whole detection method [137]. Some applications of these methods are usually scarcely labour intensive and do not require highly trained operators to perform the analysis. Another extremely important aspect consists on the ability of some isothermal systems to directly target RNA molecules instead of double stranded DNA, without any additional step in the procedure such as in NASBA. This helps to identify microorganisms that have an active transcription machinery (often corresponding to live vs death condition) even in a highly concentrated DNA background. Moreover, targeting RNA can help to increase sensitivity when the targeted gene is intensively expressed and enable the easy detection of viruses with a RNA-based genome, like Norovirus. Compared to PCR-based strategies, also the need for dedicated instrumentation is less demanding and in some cases completely absent.

All these reasons make the isothermal technologies more suitable for integration with miniaturized and integrated systems with "lab-on-a-chip" formats or other microfluidic devices [138–146] and with "rapid diagnostic methods" like lateral flow devices [147–155]. These emerging diagnostic approaches are at their initial development stage but they are undergoing to a rapid evolution in the field and some of these technologies should provide the next generation of rapid, low cost and easy-to-use diagnostic kits for laboratory, *in field* or *point-of-care* applications.

However, despite the advancements in techniques based on nucleic acid amplification, there are still some strong limitations specially in the field of food safety applications. Since food industry includes a wide spectra of production and

Table 2.1 Detection of pathogens in different food matrices and experimental conditions

Target	Technology	Matrices	Time/enrichment	Limit of detection
Salmonella [94]	Cultural enrichment method vs real time PCR	Ice cream	PCR ca. 3 h cultural enrichment method 5 to 7 days	10^3 CFU/ml
Salmonella [95]	Multiplex and quantitative real-time PCR	Cheddar cheese, raw and turkey	48 h of selective enrichment + analysis	6.1×10^1 CFU/ml
Salmonella [96]	TaqMan quantitative real-time PCR	Raw milk, chicken meat, salmon	16 h enrichment + analysis	5 CFU/25 ml (raw milk), 2.5-5.0 CFU/25 g (chicken and salmon)
Salmonella [97]	TaqMan quantitative real-time PCR	Chili powder and shrimp	48 h (24 for pre-enrichment and 24 for selective enrichment)	0.04 CFU/g
Salmonella [98]	TaqMan quantitative real-time RT-PCR (qRT-PCR)	Spinach, tomatoes, jalapeno peppers and serrano peppers	24 h pre-enrichment	40 copies of invA mRNA per reaction
Campylobacter jejuni and Campylobacter coli [99]	TaqMan multiplex real-time quantitative PCR	Chicken	3 h	1.18–5.30 log CFU/ml
Campylobacter, Salmonella spp. [100]	Multiplex real-time quantitative PCR	Chicken skin rinses	/	3.0×10^3 CFU/ml
Escherichia coli O157:H7 [101]	RT-qPCR (Scorpion probe)	Artificially contaminated milk natural samples of raw milk, pasteurized milk, ice cream, kulfi (frozen dessert), paneer (soft cheese), infant foods	/	10^3 CFU/ml

Escherichia coli O157:H7 and Listeria monocytogenes [102]	Real-time quantitative PCR – (Molecular Beacons)	Milk and milk products	With and without enrichment (6 h)	1 and 3 log CFU (with and without enrichment)
Listeria monocytogenes [103]	Real-time quantitative PCR – (SYBR Green)	Collard green, cabbage, lettuce, mixed parsley and spring onion bunches, Chinese cabbage, arugula, chicory, wild chicory, spinach and watercress	48 h total time of analysis	1–5 CFU/50 g
Listeria monocytogenes [104]	TaqMan quantitative real-time PCR	Artificially contaminated raw milk, salmon, pate, green-veined cheese naturally contaminated fish, meat, meat products, and dairy products	24 h	7.5 CFU/25 ml milk, 9 CFU/15 g salmon, 1 CFU/15 g pate and cheese
Hepatitis A virus and norovirus GI and GII [105]	Multiplex real-time PCR	Different food surfaces (lettuce, strawberry, raspberries and green onions)	Concentration using electrostatic binding	10^1 viral particles for HAV and norovirus GII, and 10^2 viral particles for norovirus GI

processing methods and food contaminations can often occur at every stage before food consumption, the needs for suitable diagnostic tests all along the food chain are far to be fulfilled. In several processes, food might be tested for contaminations at the level of raw materials, during processing or stocking, after packaging or at retail and sometimes consumer level. Obviously, all these potential levels of control require different diagnostic approaches: in some an accurate quantitative test represents the best choice, such as in the case of foods that have to pass mandatory requirements. However, these kind of tests are often expensive, take long time and need dedicated laboratories to be accomplished. Sometimes short time-to-results is the key factor to be preferred in order to quickly move big commodity stocks or introduce an *in line* check point along a food process. On the other hand, at retail and consumer level, the demand for easy to perform, rapid and cheap diagnostic tests is rapidly increasing. The nucleic acid amplification methods developed in the last decades are focused on quite complex laboratory based procedures and appear to be not flexible enough to fit the vast majority of the above mentioned settings and conditions.

Although less cumbersome and time-consuming compared to culture-based methods, PCR methods still require well trained operators, are relatively expensive and rely on complex equipments for thermal cycling. Moreover, they need a dedicated area in the laboratory in order to limit accidental contaminations. Some of these drawbacks might be minimised using the proper "new generation" isothermal technology. For example LAMP allows to greatly reduce the instrumentation requirements since the amplification occurs without thermal cycling steps, at constant temperature (65°C) and a wide range of solutions for the detection step have already been developed, moving from fluorescent instrument-based detection to low cost approaches based on naked eye visualization or real time monitoring using an inexpensive turbidimeter. LAMP, however, is based on the interaction of six specially designed primers and sometimes the difficulties in primers definition may discourage from using this technique [108].

As previously mentioned, these methods may open a new era in nucleic acid diagnostics however there is currently no single method that meets all the requirements of microbial diagnostics devoted to food safety. For a comprehensive description and comparison of the most employed isothermal methods see Gill and Ghaemi [156].

Other factors that limit the employment of nucleic acid-based amplification methods common to PCR, as well as the isothermal approaches, are intrinsic to the employment of enzymes during the amplification steps. The activity of these enzymes is dramatically affected by the presence of inhibitory compounds commonly present in foods that might be co-extracted and concentrated from the usual procedures of extraction and concentration of nucleic acids from complex food matrices.

Beside the development of affordable nucleic acid detection methods, it must be pointed out that, concerning food diagnostics, one of the most critical phase involves all the operations performed upstream the analysis, during sample preparation. Thus separation and concentration procedures need to be optimised more carefully to obtain rapid, direct, and quantitative methods [157].

When real food samples have to be analysed, all the methods targeting nucleic acids depend on some degree of cultural enrichment in order to be reasonably affordable. Food samples often consist of heterogeneous and complex matrices, containing numerous components that are incompatible with the majority of the analytical methods, such as fats and complex carbohydrates. A second relevant issue comes from the volume usually obtained from food manipulations that is often much bigger than the volume that can be employed in a nucleic acid-based detection method. In this conditions, the targeted nucleic acid is often under the detection or the quantification limit of the chosen method.

In order to circumvent these critical issues, numerous approaches have been developed with the aims to purify and concentrate the microbial targets combined with the removal of matrix-associated components that act as inhibitors for downstream processes. These operations have been collectively defined pre-analytical processing, or "upstream" sample processing [5].

Beside the culture-based and the nucleic acids-based amplification methods, numerous biosensors have been developed for microbial and food safety applications. Numerous techniques have been exploited such as SPR, QCM, Surface Enhanced Raman Scattering (SERS), Evanescent Wave and a wide spectra of other optical and electrochemical applications [158, 159], but only a few of them have been used to target nucleic acids. However, tremendous limitations to the use of these technologies arise from the lack of effective pre-analytical processing methods designed to be integrated *ad hoc* in sensors for food safety [160].

Thus, according to Dwivedi and Jaykus, the pre-analytical sample processing still represent the "crucial unresolved step for successful application of biosensor-based technology in food microbiology" [5]. The aspects described above fit in the same way once transposed to the development of amplification-free methods for nucleic acids detection, that currently have no examples in food-borne pathogen diagnostics.

Despite the wide range of diagnostic methods, further efforts must be made in order to fullfil all the requirements of food analysis, specially for microbial pathogen diagnostics. Among the most innovative strategies, the direct amplification-free detection of nucleic acids appears to be one of the most promising approach. However, there are some critical unresolved issues that need to be faced independently from the detection method.

2.5 Authenticity

"Food traceability", under the EU legislation, is the possibility to track any food, feed, food-producing animal or substance that will be used for consumption, through all stages of production, processing and distribution [161].

Requirements for traceability have been introduced in national regulations as an effective mean to contribute to product safety and consumer confidence. In recent years, food traceability has become a matter of great importance in relation not only to safety and quality issues, but even more significant for fresh and typical

food products. In the latter case, in particular, the problems of authenticity and of adulteration recognition have become of paramount importance so that it is requested to verify the identity of products, under compositional and geographic aspects, in order to protect both producers and consumers from frauds.

The possibility to confirm the authenticity of traded foods is vital to guarantee the integrity of the food supply chain. Using a variety of advanced chemical, biochemical and molecular biological techniques, many laboratories are now able to assess the origin of food and feed [7].

Although proteins and other chemicals have been used to identify food products, DNA markers are the best-suited for identification of biological samples. This is because of the remarkable durability of DNA, even in hostile environments which are encountered during many processing steps. The use of DNA markers as diagnostic tools for food authenticity, origin, and traceability of variety/type composition of complex food matrices has been investigated in an increasing number of projects worldwide. However, some processed food contains highly degraded DNA and/or polymerase chain reaction (PCR) inhibitors, both of which may affect the subsequent PCRs used for the amplification of diagnostic DNA sequences. These effects may be overcome by modifying the DNA extraction process and the PCR design and conditions. It is sometimes possible to overcome the inhibitory effects by extensive dilution of the DNA extract; however, this may not be an option when the amount of DNA in the sample is limited. In these cases, a sensitive method for the detection of small amounts of highly degraded DNA is necessary. Therefore, as first step, it is important to be able to optimize the DNA extraction method, which should be adapted to the nature of the food matrices.

Here we report some examples concerning the most frequently used foods, which need traceability inspections.

2.5.1 Meat

Rapid progress in farm animal breeding has been made in the last few decades. Advanced technologies for genomic analysis in molecular genetics have led to the identification of genes or markers, in particular genome sequencing and SNPs maps, which can be useful to trace farm animals back to their source breed, to detect eventual frauds and to validate typical productions.

In the studies reported in the literature, DNA tracts from meat belong to mitochondrial DNA extracted from the muscle tissue. Mitochondrial DNA (mtDNA) is practically the only reliable genomic target for PCR in processed meat products, and its haploid nature and strict maternal inheritance greatly facilitate genetic analysis. The use of mtDNA as a target for PCR enabled to obtain results in highly processed products from which nuclear DNA is often unavailable for reliable and reproducible analysis [162].

The PCR reaction can be associated to a multiplex single base extension (SBE) assay, which makes use of diagnostic single nucleotide polymorphisms (SNPs)

for the identification of a particular type of meat. For example, this methodology was successfully applied to the discrimination between Asian and European *Sus scrofa* lineages [163]. The test was robust, sensitive and accurate in a wide range of meat foodstuffs (fresh or frozen or smoked, dry-cured and complex mixtures such as patè and sausages) allowing accurate detection of pig genetic material and identification of maternal ancestry. As it comes out from these studies, the success of the amplification methodologies with cooked-meat is strictly dependent on the design of short PCR amplicons.

Recently, gold nanoparticles have been used to detect swine-specific sequences and nucleotide mismatches in PCR-amplified and non-amplified mitochondrial DNA mixtures in order to authenticate the species [164]. The entire assay (hybridization plus visual detection) was performed in less than 10 min. The LOD (for genomic DNA) of the assay was 6 μg ml^{-1} of swine DNA in mixed meat samples. This is a really very promising study, which could be applied in the future for species assignment in food analysis, mismatch detection in genetic screening and homology studies between closely related species. It is also a rare example of first attempts to detect non-amplified DNA for food authenticity.

2.5.2 Fish

Also in the case of fish which has undergone different treatments such as canned fish, it is not possible to amplify PCR products of a large size, because the thermal treatment generates DNA fragmentation. However, it is important to have at one's disposal a method that can be used routinely, allowing the easy amplification of DNA and providing a reliable species identification. The best situation is the amplification of a small fragment which contains enough information to enable the differentiation of all the species studied.

The presence of additives used in seafood industry as spices or sauces may attenuate or inhibit DNA amplification. Moreover, the different kinds of sauces added produce differences in the quantity and quality of extracted DNA as this molecule is very sensitive to acid and alkaline agents. To this purpose, it is worth highlighting the pickled products, in which the low pH produces high DNA degradation.

In a recent study on the amplification of a fragment of mitochondrial DNA by means of PCR, combined to the Forensically Informative Nucleotide Sequencing (FINS), a method which allowed the genetic identification of the most important commercialized species of horse mackerel and related species [165] was developed. The main advantage of this method is that, besides including a high number of species (20 species), it is based on the use of a DNA fragment with a size below 250 bp and can be applied to all kinds of processed products, including those that have undergone intensive transformation processes. The possible applications of this method are the following: normative control of raw and processed products, particularly aimed at verifying the authenticity of imported species; traceability of

different fish batches along the commercial chain; correct labelling; protection of the consumer's rights; fair competence among fish operators; and control of fisheries.

Moreover, PCR-RFLP (Restriction Fragment Length Polymorphism) amplification methodology has been successfully applied for the characterization of fish species in processed food products labelled "anchovy" [166]. This methodology combines PCR amplification to the digestion of DNA fragments which show variations in homologous sequences.

The same amplification method combined to the use of lab-on-a-chip capillary electrophoresis for endpoint analysis enabled accurate sizing of DNA fragments and identification of white fish species at a level of 5% (w/w) in a fish mixture [167]. The method was applied to a range of products and subjected to an inter-laboratory study carried out by five U.K. food control laboratories. One hundred percent correct identification of the single species samples and ca. 70% identification of nine mixture samples was achieved by all laboratories. The results indicated that fish species identification could be carried out using a database of PCR-RFLPprofiles without the need for reference materials.

The technique is also proposed as a qualitative test for screening raw, frozen, and lightly processed samples for the presence or absence of fish species. As such, this method is extremely useful as it has the potential to detect a wide range of fish species following the development of suitable PCR-RFLP profiles or can be applied as a rapid mean of comparing samples.

2.5.3 Milk and Dairy Products

The dairy sector has taken "the viewpoint that traceability/product tracing as a tool is not only relevant in the context of food inspection and certification systems, but may also be applied for industry-driven business reasons and is mainly concerned with food safety aspects" (Bulletin of the International Dairy Federation 412/2007; IDF Guiding Principles for Traceability/Product Tracing).

In particular, molecular markers are becoming useful tools to trace the whole chain: they provide a diagnostic approach for food authenticity that can benefit the farmers, the dairy industries and the traders. Species-specific PCR has shown to be a suitable method to control food authenticity because a specific target sequence can be detected even in matrices containing a pool of heterogeneous genomic DNA, such as milk or other dairy products.

Molecular markers can be found in DNA of different grass species and forages commonly fed to animals, or DNA coming from the microbial environment.

In a recent study [168], using a PCR approach, the rbcL marker, a chloroplast-based gene, was selected to amplify plant DNA fragments in raw cow milk samples, collected from stock farms or bought on the Italian market. rbcL-specific DNA fragments could be found in total milk, as well as in the skimmed and the cream fractions. Upon sequencing of the PCR amplified fragments, a nucleotide composition

of the chromatogram was obtained which reflected the multiple contents of the polyphytic diet. The unambiguous characterisation and correlation of the amplified DNA present in milk with that present in the commercial fodder or in a specific pasture area can allow traceability and/or authenticity assessment specific for the food chain of dairy products. These new tools open the possibility that, in the future, many type of cheeses requiring the Protected Designation of Origin (PDO) certification could be validated by searching the plastidial DNA fragments present in the milk used for their production.

By a different approach, analytical support for providing authentication of the geographic origin of alpine milk productions [169] was obtained by studying milk bacterial composition, by using intergenic transcribed spacer–PCR fingerprinting. This work showed for the first time that the composition of the bacterial communities is related to the altitude of pastures strongly enough to allow the distinction of milk origin in terms of altitude, by means of the adopted ITS-PCR (Internal Transcribed Spacer) fingerprinting protocol, followed by multivariate statistical analysis of the resulting band patterns. Although further research is required to better elucidate the relationship between bacteria and environment (type of flora, climate, altitude), this new analytical approach and its findings can be of significant interest for the manufacturing, protection, traceability and promotion of the PDO Fontina cheese and other alpine dairy productions.

The same methodology has been also applied to distinguish italian Protected Designation of Origin (PDO) water buffalo Mozzarella cheese from different producers on a molecular basis in relation to the place of manufacturing within the production district [170]. Microbial DNA was isolated from the governing liquid of the cheese, the whole microflora ribosomal 16S–23S internal transcribed spacers ITS-PCR fingerprinting was amplified by means of an original primer pair. Although further analyses are required to better investigate the capabilities and limitations of the method, and in particular, the possibility to univocally identify samples and their places of production, different PDO water buffalo Mozzarella cheeses were identified by using the described protocol. Moreover, the bacterial populations present in this cheese was shown to be substantially stable over time, as the electrophoretic profiles obtained did not show major changes over 1 year.

Recently it was demonstrated for the first time that cow's milk contains large amounts of microRNAs (miRNAs) and that the unique expression profile of milk-specific miRNAs can serve as a novel indicator and possible new standard for the quality control of raw milk and milk-related commercial products, such as fluid milk and powdered formula milk [171]. A total of 245 miRNAs were identified in raw milk. By using TaqMan probe-based miRNA quantitative RT-PCR, seven miRNAs that have a relatively consistent expression throughout the lactation process were identified. The expression profile of these milk-specific miRNAs can serve as an ideal biomarker for discriminating poor-quality or "manipulated" milk from pure raw milk, as well as for the quality control of commercial milk products, such as fluid milk and powdered formula milk. Very interestingly, these findings provide a basis also for understanding the physiological role of milk miRNAs.

2.5.4 Coffee

Coffee is one of the most important world food commodities, the commercial trade consisting almost entirely of Arabica and Robusta varieties. The former is considered to be of superior quality and thus attracts a premium price. Methods to discriminate between the two coffee cultivars could be useful for unveiling either deliberate or accidental adulteration. Spaniolas et al. have described a molecular genetic approach to distinguish Arabica from Robusta coffee beans [172]. A PCR-RFLP (Restriction Fragment Length Polymorphism) was used to monitor a single nucleotide polymorphism within the chloroplastic genome. Samples were analyzed with a Lab-on-a-Chip capillary electrophoresis system. Coffee powder mixtures were analysed with this technique, showing a 5% limit of detection. The plastid copy number was found to be relatively constant across a wide range of bean samples, suggesting that this methodology can also be employed for the quantification of any adulteration of Arabica with Robusta beans.

This methodology, analogously to the previous studies on fish, is very easy to use, and the analysis performed by a Lab-on-a-Chip technique, is straightforward and relatively fast, approximately 60 min per 12 samples after the restriction digest step. This would mean that this methodology may be suitable for the routine analysis of coffee samples. A similar approach could be employed for the analysis of roasted beans and even ground coffee, providing that DNA suitable for PCR-RFLP could be extracted from these commodities. There is a strong possibility that during the process, either through roasting or other treatments such as decaffeination, degradation of DNA can occur. However, a previous study [173] has shown that DNA suitable for PCR analysis can be extracted from beans subjected to various levels of roasting and from commercial coffee powder, although with low yields and reproducibility.

2.5.5 Olive Oil

Olive (*Olea europaea* L.) is one of the oldest agricultural tree crops spread worldwide and is an important source of oil with beneficial properties for human health, due to its content in polyphenols. In spite of its economic and nutritional importance, few data are available in the literature related to the genetics of olive in comparison with other fruit crops. Available molecular data are especially related to the application of molecular markers to the analysis of genetic variability in *Olea europaea* with the aim of developing efficient molecular tools for defining the olive oil quality and traceability [174], as required for PDO extra-virgin olive oil. At the moment, DNA analysis is more than a promising approach to distinguish the different cultivars from which the oil is produced, since it is less influenced by environmental and processing conditions in respect to other methods (i.e., metabolites). However, although methods of extraction of DNA from olive oil have been developed by several authors, all studies confirmed that DNA extraction from extra-virgin olive oil is a critical point, since DNA is present in very low amount.

DNA extracted by olive oil was studied by means of different amplification and molecular markers techniques. Pafundo et al. [175] traced the cultivar composition of monocultivar olive oils by Amplified Fragment Length Polymorphism (AFLP) markers, suggesting that DNA extraction is the most critical step affecting the procedure. The same group [176], starting from AFLP amplified in olive oil, developed some Sequenced Characterised Amplified Region Markers (SCAR) that amplified successfully on DNA extracted from olive oil.

By using Simple Sequence Repeats (SSR, also referred to as microsatellites) analysis, it was also demonstrated that microsatellites are useful for checking the presence of a specific cultivar in a PDO oil, thus verifying the identity of the product [177]. However, the marker profile of the main cultivar in the oil was only obtained: no signals were detected for the minor cultivars eventually present.

Consolandi et al. [178] reported the development of a semi-automated SNP genotyping assay to verify the authenticity of extra-virgin olive oils. The authors developed a Ligation Detection Reaction (LDR)/Universal Array (UA) platform by using several olive SNPs. They found that 13 accurately chosen SNPs were sufficient to unequivocally discriminate a panel of 49 different cultivars.

Finally, in a recent study [179], the effect of storage on the degradation of DNA purified from oil was investigated. Interestingly, a negative correlation between storage time and quality–quantity of recovered DNA was observed. In fact, 1 month after the production of the oil the degradation increased, making traceability efforts rather hard.

Considering all these investigations, it is possible to note that DNA-based olive oil traceability is a topic of great interest, but a cautionary note on the use of DNA markers for origin testing is mandatory, as reported also by Doveri et al. [180]. Their observations were based on a non-concordance between the genetic profiles of olive oil and of the fruit. The authors suggested that this could be due to the contribution of pollen donors in DNA extracted from the paste obtained by crushing whole fruits. Moreover, standardization of the genetic profile for both trees and oil should be made for each territory to account for eventual variations due the climate. Thus, care must be taken in the interpretation of DNA profiles obtained from DNA extracted from olive oil for assessing the origin and authenticity of the products.

An interesting application of DNA analysis is the possibility to detect adulteration of olive oil with other chipper oils. In our group, a method based on PNA-microarrays allowed to verify adulteration of olive oil with hazelnut oil by detecting the presence of DNA from hazelnut [16].

Although many efforts have been made in the last years, genome studies in *Olea europaea* L. are currently behind those of other crops. Several groups have started to work on the olive genome sequencing (i.e., OLEAGEN genomics project, Fundacion Genoma, Spain, www.chirimoyo.ac.uma.es/oleagen/) and, thanks to the rapid development of the new sequencing technologies, soon the complete sequence of olive genome will be available. This will allow to identify new sequence polymorphisms, which will be very useful for the development of new cultivar-specific molecular markers (e.g., SNPs) and for the implementation of more efficient protocols for tracking and protect olive oil quality.

2.6 Nutrigenetics/Nutrigenomics

Sequencing of human genome and of other organisms has determined a definitive revolution in the biomedical sciences and has provided a strong impulse to the development of the so called "omic sciences" (genomics, transcriptomics, proteomics, metabolomics, etc.). Integration of these sciences with informatics provides the basis for functional genomics, which aims at clarifying the role of single genes and how they interact with each other, with reciprocal influence. This complex network of genetic information applied to nutrition sciences leads to nutrigenetics and nutrigenomics, with the primary objective to investigate the interaction mechanisms between nutrients and genes and their role in the development of diseases for the general population and also for the single individual, opening the way to personalised diet. The development of concepts and research on genetic variations and dietary response has been named nutrigenetics [181] (e.g., individuals responding differently to the same diet by attaining different levels of serum cholesterol and blood pressure because of genetic variations), while studies on the role of nutrients in gene expression are defined as nutrigenomics (e.g., polyunsaturated fatty acids suppressing fatty acid synthase (mRNA) gene expression).

Relevant research projects have started in order to define the mechanisms by which genes influence nutrient absorption, metabolism and excretion, taste perception, and degree of satiation and the mechanisms by which nutrients influence gene expression [182]. Moreover, some important food industries have started programs to develop novel foods based on nutrigenetic/nutrigenomic concepts.

It is well known that the response of blood cholesterol concentration to diet is genotype-dependent [183–185] and therefore general recommendations such as to increase the uptake of polyunsaturated fatty acids in order to reduce the plasma level of cholesterol and consequently the risk of developing cardiovascular disease are not equally relevant for all individuals [186].

Despite the high complexity of these researches, it has been possible, at list in some cases, to identify correlations between several genetic variants, diet and development of cancer diseases. A number of epidemiologic studies and animal experiments suggest that omega-6 fatty acids increase the risk of certain cancers, whereas omega-3 fatty acids decrease this risk. But the correlation between the consumption of omega-6 and development of breast cancer has only been seen in subjects carrying a polymorphism of the protein activating the 5-lipoxygenase (ALOX5AP-4900 A > G) [187]. Another example is given by studies on the protective role of omega-3 fatty acids against the risk of developing prostate cancer only in correlation with specific genetic variants [188, 189]. Other examples about dietary factors and genetic interactions are reported in a recent review by Simopoulus [190].

As far as the effects of nutrients on gene expression (nutrigenomics), particularly relevant are the effects of dietary cholesterol and fatty acids. Dietary cholesterol exerts a strong inhibitory effect on the transcription of the gene for β-hydroxy-β-methyl-glutaryl (HMG)-CoA reductase [191]. Polyunsaturated fatty acids (PUFAs)

suppress the hepatic mRNA production of fatty acid synthase for lipoproteinemia in adult, according to the degree of fatty acid unsaturation. Thus, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) contained in fish oils are more effective than arachidonic acid (AA) (which has 4 double bonds) [192, 193].

Different types of carbohydrates (a rye pasta diet characterised by a low postprandial insulin response and an oat-wheat-potato diet characterised by a high postprandial insulin response) were shown to have a different effect on gene expression in subcutaneous adipose tissue in persons with metabolic syndrome [194]. The insulinogenic index improved after the rye-pasta diet and not after the oat-wheat-potato diet, indicating that different carbohydrates modulate gene expression and metabolism in patients with the metabolic syndrome.

The studies on the interactions gene-food components are in their infancy and performed with the methods and technologies derived from the "omic" sciences, as already partially described in this chapter.

A recent paper by Masotti et al. [195] reports how the microarray technology can be a promising tool in nutrigenomics, for the global evaluation of gene expression profiles in tissues and for understanding the factors controlling the regulation of gene transcription. These Authors show that oligonucleotide and cDNA microarrays represent powerful tools for a rapid and high-throughput gene expression evaluation, since they allow a quantitative assessment of the relative cell or tissue concentration of the specific messenger RNA (mRNA) that is directly related to the level of expression of that particular gene. The mRNA transcript present in the cells or tissues is extracted and is used to create a complementary labeled strand of DNA (cDNA), which can be hybridised with a complementary probe, previously spotted on a glass slide or nylon substrate containing a known set of gene sequences (genome). The intensity of the color, quantified by a fluorescent scanner after the hybridisation process, is directly related to the amount of target mRNA and reflects the expression level of that particular gene. In this way, it is possible to determine which gene is up-regulated or down-regulated as compared to the control sample.

This technology, however, has certain drawbacks. In fact, in order to obtain reliable and reproducible results, several parameters of the experimental protocol (array production, RNA extraction, cDNA labeling and hybridisation, and data analysis techniques) should be optimised.

The MicroArray Quality Control Consortium (MAQC) has recently evaluated the performance and reproducibility of the most common platforms and generated a set of data that justifies their use for gene expression profiling, both in basic and applied research leading to clinical diagnostic tools [196].

In conclusion, Nutrigenomics analyzes the physical and chemical characteristics of food components, as well as the structures and polymorphisms within the genome, with the aim to identify a pool of biomarkers that can predict the beneficial or adverse effects of dietary nutrients or components on the general population or on individuals, thus being used as predictive or screening tools to promote health and prevent disease.

However, it is only by combining information from the different sciences and appropriate bioinformatics, that it will be possible to understand all aspects and implications of nutrition on health and its adverse toxicological effects.

2.7 General Conclusions and Perspectives

The development of affordable and robust quantitative assays for DNA in food represents a fundamental task in food safety and quality. The definition of reliable and harmonised strategies for nucleic acids quantification represent a big challenge both for scientists and international organisms involved in food safety regulation. One of the major limitations in this process is represented by the employment of techniques for the detection of nucleic acids that often are organism- and matrix-dependent. These are among the reasons that explain why the definition of common norms and guidelines takes very long times requiring a huge amount of experimental work. After several years, international organisms have defined some minimal requirements for PCR but the debate on which are the best quantification practices for PCR applications is still going on at international level. Moreover, a similar process needs to be developed for the new generation of isothermal methods and for emerging pathogens like foodborne viruses.

The development of direct, amplification-free approaches for the detection of nucleic acids represents an opportunity to improve the area of quantitative diagnostics for food analysis. The possibility to remove enzymes from the process, exclusively relying on direct nucleic acids binding should minimize the effects of the matrix on the final results. On the other hand, this approach may present other issues to be faced: one of the most relevant should be the definition of reference materials for calibration curves. Absolute quantification for Real Time PCR is usually performed by means of plasmid DNA, RT-PCR products or synthetic DNA/RNA oligonucleotides. These products behave similarly to the targeted genes in terms of amplification efficiency because they are designed to be similar (sometimes identical) in length and in sequence composition. Whenever amplification-free nucleic acid methods are supposed to be designed to detect direct signals from a genomic DNA portion or a transcribed RNA, this aspect should be taken into account: since genomic DNAs and cellular RNAs are often bigger and highly structurated molecules compared to the oligonucleotides usually employed for absolute quantification, it is possible to obtain very different signals. However, if this issue will be successfully minimised, quantitative direct nucleic acids detection will open a completely new era in food diagnostics, nutrigenetics and nutrigenomics.

Another point concerns the concomitant needs for effective pre-analytical methods to be easily integrated with the detection methods, which seem to grow slowly and often not considered for an integrated development. This issue becomes more and more relevant for the direct nucleic acids detection methods that are theoretically able to move from sample preparation directly to the detection step, without any additional process. Since the pre-analytical treatments represent the bottle-neck

of numerous analytical processes because are still highly time-consuming and labour intensive, the pre-analytical processing must be contextually investigated and developed with the methods focused on amplification-free detection of nucleic acids. Otherwise, even the most efficient method will not fully accomplish the needs in the food analysis field, reducing the impact of these innovative techniques on real diagnostics.

Moreover, the possibility to integrate all the concepts previously described might lead to the development of automated, miniaturised and portable devices that will help rapid detection of foodborne pathogens and process monitoring all along the food-chain, strengthening the chance to develop a "food diagnostics from farm-to-fork". Methods that rely on direct detection of nucleic acids appear to be particularly suitable for this purpose.

Since foods have complex and heterogeneous matrices, the next-generation methods should be able to detect targeted sequences in a robust, accurate matrix-independent way. This means nucleic acids coming from the same sample should be directly detected giving comparable results, regardless of the methods employed during the concentration/purification steps.

Finally**,** the development of customised methods for food diagnostics, covering all the critical steps in food safety and quality, will enable to face the actual limitations in contexts like *in-line* processes or *on-site* settings. Optimising the time and costs required for a single analysis, along with easy-to-perform procedures will open interesting opportunities in food diagnostics.

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Part II
Advanced Materials for Ultrasensitive Analysis

Chapter 3
Engineered Nanostructures for the Ultrasensitive DNA Detection

Dong-Kwon Lim, Amit Kumar, and Jwa-Min Nam

Abstract Coupled with nanotechnologies, a wide variety of DNA sensing methods have been developed to achieve ultrahigh sensitivity and selectivity without the aid of enzymatic amplification procedures or complicated assay procedures. Structurally engineered nanomaterials have several useful aspects including their unique optical properties depending on size, shape, composition and structural details and electrical properties, which have been translated into various signal transduction modes. However, the most important challenge in DNA detection assay to compete with or complement the polymerase chain reaction (PCR) is matching the sensitivity of PCR, which can detect 10–100 copies in whole sample via various non-enzymatic amplification strategies. Here, we introduce recent advances in engineered nanostructure-based DNA detection methods that show potential for PCR-like sensitivity and can address the existing issues of conventional DNA detection assays. The basic principles, advantages, and limitations of engineered nanostructure-amplified DNA detection methods will be introduced and discussed.

3.1 Introduction

Immediately after invention, the polymerase chain reaction (PCR) was readily recognized as a powerful tool for amplifying a trace amount of DNA of interest to detectable amount and this method completely changed the landscape of DNA research and detection. The versatile PCR methods are now routinely used in medical and biochemical research; particularly, in cloning for DNA sequencing, DNA-based phylogeny, functional analysis of genes, the identification of genetic fingerprints (used in forensic sciences and paternity testing) and diagnosis of

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hereditary and infectious diseases. However, PCR-based methods require numerous enzymatic target amplification steps and complex instrumentation, resulting in limited multiplexing capability and high susceptibility towards contaminations [1, 2]. Recently, many sophisticated metal nanostructures with strong electromagnetic response, have been used for enzyme-free optical DNA-biosensing up to single molecule detection capability, hence, presenting a better alternative of PCR [2]. This chapter provides an overview of the state-of-the-art DNA-sensing nanostructures, and these nanostructures are categorized as (1) nanoparticle probes for solution-state analysis, (2) nanostructures for DNA detection on solid surface, and (3) nanochannel/nanopores for DNA sequencing. In these methods, detection signals have been obtained and analyzed in various ways, including optical (e.g., fluorescence, Raman scattering, white light, surface plasmon resonance, vibrational spectroscopy, etc.), electrical, mechanical, and magnetic methods [2–4]. Here, we show how these engineered nanostructures, coupled with powerful signal amplification strategies, were designed, synthesized and used for highly sensitive DNA detection that can potentially replace or complement conventional DNA detection platforms such as PCR.

3.2 Nanoprobes for Solution-Based DNA Analysis

A variety of engineered nanoprobes have been developed and investigated for DNA analysis in solution to obtain amplified signals with various signal transduction modes (e.g., optical, electrical, mechanical, and magnetic signals) [3, 4]. Mainly, it is desirable to gain higher sensitivity, selectivity, multiplexing capability, and wide dynamic range using such inorganic/organic functional nanomaterials [1, 3, 4]. Currently, a wide range of nano-sized materials with different physicochemical properties have been used to achieve these purposes. The microscopic images of plasmonic nanosphere, nanorod, nanostar, bipyramids, core-shell, and nanowires composed of gold or silver, quantum dots (QDs), silicon nanowire, single-walled carbon nanotubes (SWCNTs), and graphene are shown in Fig. 3.1. Plasmonic nanoparticles not only show strong scattering signals when illuminated with white light, but also are sensitive to changes in size, shape and interparticle distance. Surface plasmon resonance (SPR) wavelength could be tuned from the visible range to the NIR range by simply changing their shape from spherical to rod, star, bipyramids or nano-shell (Fig. 3.1a–c) [5–7]. Due to the narrow emission spectrum, excellent photo-stability, and small size of quantum dots, it has been extensively studied as promising labeling materials for highly sensitive and stable probes that can replace conventional organic fluorophores. These nanostructure-dependent plasmonic and fluorescent properties could be used as basic sensing principles and signal amplification strategies. Finally, field effect transistor devices enabled new functional materials such as silicon nanowire, carbon nanotube, and graphene to be used and extensively studied for DNA detection (Fig. 3.1d–e) [8, 9].

In this chapter we describe how these nanostructures can be combined in different architectures and methods for the ultra-sensitive detection of DNA.

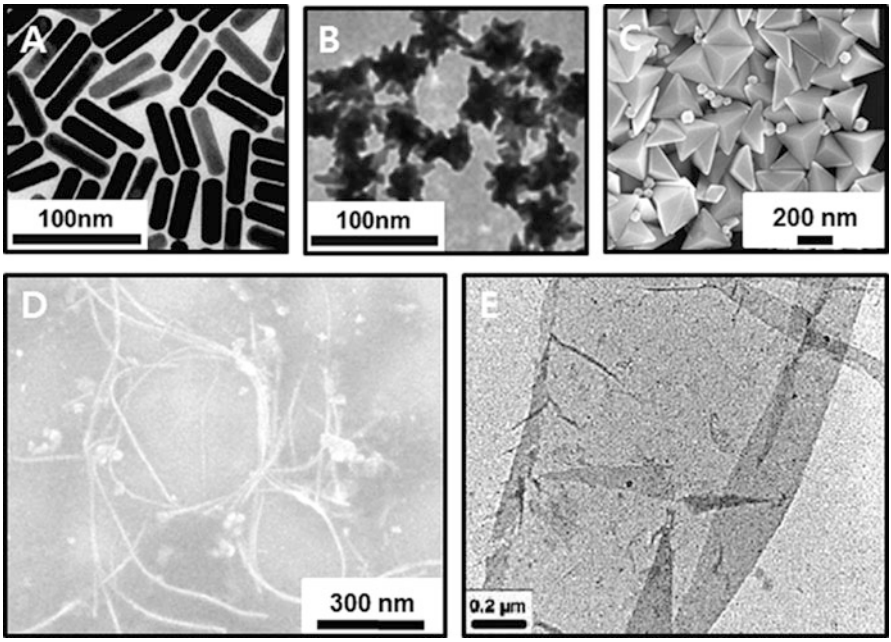

Fig. 3.1 Microscopic images of gold nanoparticles carbon nanotubes and graphene sheets with different shapes. (**a**) gold nanorods, (**b**) gold nanostars, (**c**) gold bipyramids, (**d**) STEM images of the PEG_{1500N}-[13] C-SWNT sample, (**e**) TEM image of a partially folded water-soluble graphene sheet [5–9] (Reprinted with permission from the American Chemical Society: (**a**) Ref. [5], Copyright (2008); (**b**) Ref. [6], Copyright (2008); (**c**) Ref. [7], Copyright (2011); (**d**) Ref. [8], Copyright (2005); (**e**) Ref. [9], Copyright (2008))

In 1996, Mirkin and coworkers reported interesting colorimetric response of DNA-functionalized gold nanoparticles upon hybridization with complementary oligonucleotide sequence. In this so called cross-linking method (Fig. 3.2a), there was a significant red shift in plasmonic band of gold nanoparticles [10–12], and the solution color changed from red-wine to blue, depending on the amount of target DNA (above ~nM amount) under certain salt conditions (Fig. 3.2b, c) [11, 13, 14]. The aggregated gold nanoparticles were dehybridized upon increasing the temperature above the melting temperature (T_m) of DNA or under reduced salt concentrations [14]. Mismatch in oligonucleotide sequence triggered distinct change in a sharp melting point curve due to the cooperative binding of densely modified DNA on each particle, which enabled to detect single nucleotide polymorphism [13]. In addition, the simple color changes could be permanently recorded by spotting assay solution on the C_{18}-coated thin-layer chromatography plate, and the sensitivity was greatly increased from ~nM range to ~fM range when illuminating the spots with white-light [15, 16], coupled with silver enhancing method [17], further combined with Raman scattering [18] or electrical sensing method [19].

The surface based assay will be discussed in more details in the next section. Silver nanoparticles also have great potential to be used as a DNA probe. Due to

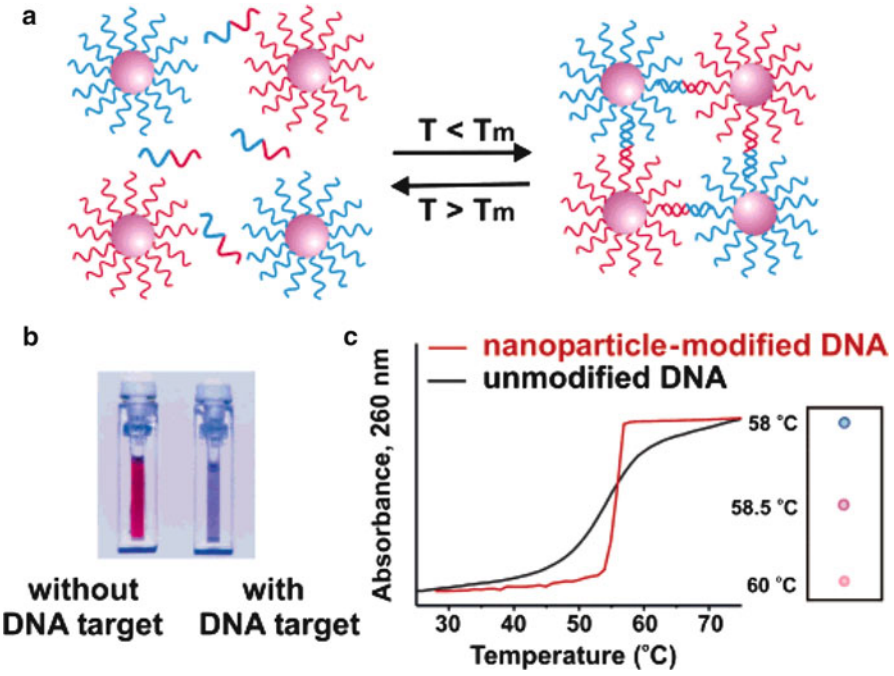

Fig. 3.2 Plasmonic color change in oligonucleotide-modified gold nanoparticle solution via target DNA detection. (**a**) Schematic description of a colorimetric assay with DNA-modified gold nanoparticles. (**b**) *Color* change with or without target DNA. (**c**) UV-Vis spectral change with or without target DNA [10] (Reprinted with permission from the American Chemical Society: Ref. [10], Copyright (2005))

the larger extinction coefficient of silver nanoparticles, a limit of detection (LOD) in the order of 40 pM has been reported, which is roughly 50-fold better than gold nanoparticles [20]. However, unstable surface chemistry of silver nanoparticles often easily induces nonspecific aggregation in solution state analysis [21]. To address this issue, DNA-modified silver-gold core-shell (Au@Ag) nanoparticles [22], DNA-embedded Au-Ag core–shell (Ag@Au) nanoparticles [23], and multi-thiol anchored oligonucleotide-modified silver nanoparticles [21] were synthesized and utilized in solution state DNA analyses. Other than conventional crosslinking methods [13, 14], assays based on electrostatic interactions with unmodified metal nanoparticles [24, 25] have been successfully applied for DNA detection in solution state, as shown in Fig. 3.3. One of the methods by Rothberg and Li [24] is based on different behaviors of single stranded DNA and double stranded DNA towards the stabilization of gold colloidal solution under high ionic strength. In other example [25], conjugated polyelectrolyte was used with unmodified gold nanoparticles to detect target DNA. This method is much simpler and sensitive (down to ~fM) with wider application scope as compared to conventional crosslinking methods, however, their limited multiplexing capability is further needed to be improved.

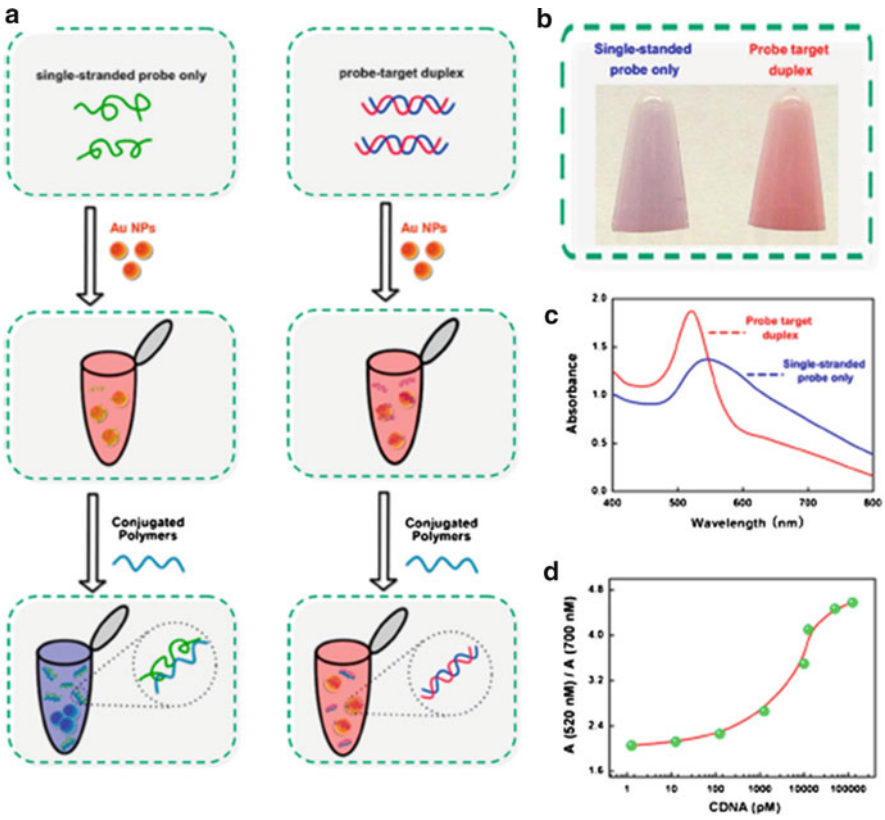

Fig. 3.3 Colorimetric change of gold nanoparticles with target DNA (noncrosslinking). (**a**) Schematic description of noncrosslinking-based colorimetric assay. (**b**) *Color* change with or without target DNA. (**c**) UV-Vis spectral change with or without target DNA. (**d**) Change in absorbance while increasing the concentration of target DNA [25] (Reprinted with permission from the National Academy of Sciences of the United States of America: Ref. [25]. Copyright (2010))

Mirkin and coworkers introduced ultrasensitive detection methods for DNA and proteins based on bio-barcode DNA-functionalized gold nanoparticles and magnetic separation scheme [26–31]. A typical bio-barcode assay utilizes two different particles that are each designed to bind a target molecule. One of the probes is magnetic particles with target capture elements, and the other is gold nanoparticle probe with target capture elements and a number of barcode DNA strands. The functionalized gold nanoparticle (barcode probe) carried with it hundreds to thousands of barcode DNA molecules which served as surrogates for the target. After target binding, the released barcode oligonucleotides from gold nanoparticles were measured with various signal transduction methods including light scattering with *in-situ* silver enhancement (Fig. 3.4a–c) [26], PCR for additional amplification (30 aM) [27], on-chip [28], scanometric analysis [29], fluorescence intensity measurement (if fluorophore molecules are linked to barcode probes) [30] and colorimetric detection [31].

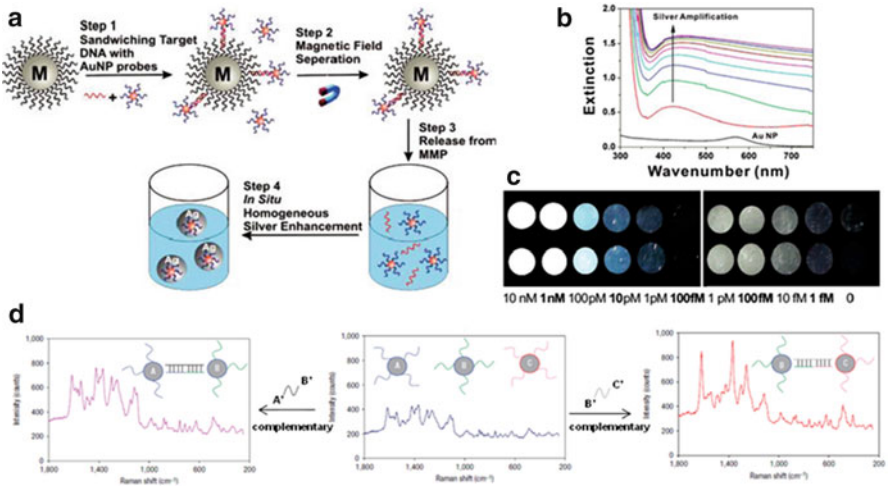

Fig. 3.4 Ultrasensitive DNA detection methods in solution. Bio-barcode assay with silver staining and UV-Vis spectrophotometer (**a**–**c**). (**d**) Solution-based SERS detection using dye-coded silver nanoparticles [26, 32] (Reprinted with permission from the American Chemical Society: (**a**–**c**) Ref. [26]. Copyright (2007). Reprinted with permission from Nature Publishing groups: (**d**) Ref. [32]. Copyright (2008))

Surface enhanced Raman scattering (SERS) methods also showed great potential for DNA analysis in solution which depends on size, shape, assembly, and interparticle distance of plasmonic nanoparticles [32–40]. Controlling hot-spots between noble metal nanoparticle junctions and placing reporter molecules in the hot-spots were major parameters for generating greatly enhanced Raman scattering in solution and solid surface. Raman tags are promising sensing labels in detecting multiple targets simultaneously because they offer differentiable fingerprint signals from various Raman-active molecules. However, generating target structures with a high accuracy and in a large scale and signal reproducibility have been critical issues in this research area [33–36]. Relatively higher SERS intensity was observed in solid state surface analysis comparing to solution state, owing to the reduced molecular movements, higher density of reporter molecules per unit illuminated area at a time on solid surface, and availability of ultrahigh sensitive detection scheme in surface analysis. However, solution-dispersed SERS particles have potential as cell and *in vivo* imaging probes as well as drug carriers, which are not possible with surface-based SERS detection formats. Many research efforts have been made to increase sensitivity and demonstrate multiplexing capability in solution state analysis. In 2008, Graham and coworkers reported dye-coded silver nanoparticles modified with probe DNA to detect target DNA-specific SERS signals in solution (Fig. 3.4d) [32]. The target DNA acted as linker to assemble the Ag nanoparticles into an aggregate plasmonic nanostructure, exhibiting SERS with signal in a range of ∼2.5–7.5 nM. In a different report, similar strategy was applied with different Raman reporters (e.g., malachite green), showing a LOD in the order of 25 nM [37]. Most recently,

Johnson and coworkers reported ∼pM level of sensitivity for oligonucleotide sequence derived from the West Nile Virus, using magnetic nanoprobes with Raman reporter molecule-adsorbed gold nanoparticles [38]. Quenching the SERS signal ("off") upon detecting the target DNA with a probe DNA hairpin is another SERS-based detection method [39]. Recent study showed that SERS-based DNA detection method can be integrated on a microfluidic system, which opens the way to future "lab on a chip" device system [40].

Apart from utilizing surface plasmon resonance (SPR) properties of noble metal nanoparticles, super-paramagnetic nanoparticles (e.g., iron oxide) have been investigated and demonstrated as sensitive probes for solution state DNA analysis [41]. The increase of hydrodynamic radius could greatly affect the magnetic susceptibility of nanoparticles in solution. However, relatively lower sensitivity and expensive instrumentation are the major limitations of this method. In an interesting solution-based method, magnetic nanoparticles acted as magnetic relaxation switch (MRS) to detect DNA-DNA interactions with high sensitivity and selectivity [42]. The oligonucleotide-functionalized iron oxide nanoparticles were aggregated in the presence of target DNA and the T_2 relaxation time of the surrounding water molecules decreased. The observed change in T_2 relaxation time was proportional to the concentration of target strands, and the detection limit was in a sub-fM range. Since the magnetic changes are also detectable in turbid media and in whole cell lysates, the MRS could be useful for *ex-vivo* DNA analysis.

The distance-dependent fluorescence quenching effect of dye molecules presents a potential basis for sensitive DNA detection using nanoparticles. The methods involving fluorescence resonance energy transfer (FRET) or metal enhanced fluorescence (MEF) have been reported to show high sensitivity and dynamic detection ranges (from ∼nM to sub pM). When CdSe/ZnS core/shell nanocrystal-DNA conjugates were used, limited dynamic range (10–50 nM) and detection sensitivity (2 nM) were observed [43]. Gold nanoparticle-DNA and carboxytetramethylrhodamine-DNA system [44] and gold nanoparticle and DNA-fluorescein system [45] also showed similar dynamic ranges and sensitivities. More complex but sophisticated nanostructures involving Fe/Au core/shell nanoparticle-DNA and methylene blue in solution, enabled to further improve the sensitivity (∼pM), selectivity, and dynamic range [46]. Here, the decreased amount of intercalated methylene blue by the formation of double-stranded DNA on the Fe/Au shell after target detection was responsible for the decline in fluorescence intensity. MEF-based DNA detection was demonstrated by the Lakowicz group. They reported a 12-fold fluorescence signal enhancement when DNA-conjugated fluoresceins were attached to Ag nanoparticles rather than being dispersed freely in a solution without Ag nanoparticles [47]. Gunnarsson and coworkers reported a single-molecule readout scheme using total internal reflection fluorescence microscopy (TIRFM) technique and demonstrated 10 fM sensitivity for oligonucleotides [48]. In this strategy, they used rhodamine-labeled lipid vesicles (these 100-nm vesicles were also modified with cholesterol-DNA-duplex structures). As lipid vesicles were immobilized to silicon dioxide surface via target DNA hybridization, the number of surface-bound vesicles was counted as a function of time using the TIRFM.

Solution state analysis is well suited for electrochemical signal transduction mode. Therefore, various signal amplification methods have been developed utilizing distinct electrochemical characters (e.g., electrocatalytic property) of nanomaterials and stripping voltammetry in solution state. Large surface area of nanomaterials and well-established bioconjugation methods enable the use of nanomaterials as efficient carriers of nucleotides, antibodies and smaller nanoparticles and as signal amplification substrates for electroanalysis. Stripping voltammetry is an electrical method for the quantitative determination of specific metallic ion species and other electrochemical analytes. Due to the preconcentration step of the desired analytes on the surface of the electrode by electrodeposition or adsorption, more than 2 orders of magnitude amplified signals can be detected in a stripping voltammetry. Another sensitive electrochemical assay utilized electrocatalytic reduction of hydrogen peroxide by avidin-hydrazine [49]. This assay employed a conducting polymer-linked dendrimer and hydrazine. Gold nanoparticle was used as a substrate that carries numerous polymer-linked dendrimers, and this high loading facilitates the signal amplification. After the human IgG and target DNA were captured by antibodies and DNA probes, respectively, analytes were detected by adding hydrogen peroxide to the electrolytic solution. The detection limits for human IgG and DNA were 1 fM and 50 aM, respectively.

In summary, the engineered nanostructured materials have been utilized as useful and sensitive DNA sensing platforms with several types of signal transduction modes such as naked eye, fluorescence, white-light, SERS, and electrochemical signal detections. A variety of principles have been applied to each sensing method such as inter-particle distance dependent plasmonic shift, fluorescence quenching or enhancement, light-scattering properties, electromagnetic enhancement in SERS, catalytic properties for electrochemical sensing, etc. However, some efforts are still needed in order to further enhance the sensitivity, high throughput capability, and multiplexing capability, and signal reproducibility in real samples without the need of expensive and sophisticated instruments. Therefore, new classes of nanomaterials that can address these issues are always in need in this research field for practical applications.

In Chap. 6, other types of nanostructures (e.g., carbon nanotubes and graphene) and their use in optical detection of DNA are described.

3.3 Engineered Nanostructures for DNA Analysis on Solid Surfaces

Conventionally, DNA analysis on solid surface is performed with glass surface arrayed with DNA, which is allowed to hybridize with complementary sequence, and then imaged with fluorescent molecules used as labeling materials [43]. Bio-functionalized inorganic nanomaterials also have shown great potential as new labeling materials for *in-vitro, ex-vivo, and in-vivo* detection technologies. With the

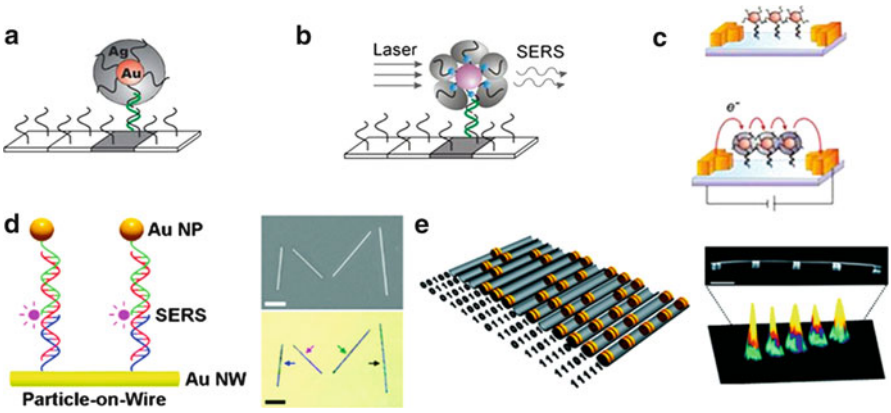

Fig. 3.5 Various DNA analytical strategies with nanostructures on surface. (**a**) Silver-enhanced scanometric, (**b**) SERS, and (**c**) electrical signal transduction mode, (**d**) particle-on-wire and (**e**) nanodisk-on-wire (Reprinted with permission from the American Chemical Society, (**a**–**c**) Ref. [10]. Copyright (2005), (**d**) Ref. [34]. Copyright (2010), (**e**) Ref. [54]. Copyright (2007))

development of theoretical studies and surface patterning technologies, engineered nanostructures fabricated on 1, 2-dimensional solid surfaces generated sensitive signal changes such as localized surface plasmon resonance, SERS, white-light scattering, electronic signal transduction, quenched or enhanced fluorescence, etc., after the target DNA binding event in heterogeneous state. In spite of non-specific binding and complex washing procedures which is essential in solid surface based analysis, the main advantages of DNA analysis on solid surfaces are high throughput, high sensitivity, stability, feasibility of mass production, and cost-effectiveness.

Biotinylated DNA and streptavidin-modified gold nanoparticles have been widely investigated to identify hybridized target DNA on DNA microarray surface. Gold nanoparticles could be detected by scanning with a flat-bed scanner, and the scattering signals could further be amplified by silver enhancing method [17] (Fig. 3.5a).

Also, gold nanoparticles could be illuminated with white-light scattering [15], which is now a commercialized platform [50]. In 2002, Mirkin and coworkers reported a sensitive (20 fM), multiplexed SERS-based nucleic acid detection method, combining silver enhancing method with Raman spectroscopy [18] (Fig. 3.5b). In this assay, multiplexing was achieved using Raman dye-encoded particles with different probe sequences corresponding to different target DNAs. In spite of a limited multiplexing capability, electrical signals have great potential to detect biological molecules including DNA in a rapid, portable, operationally simple, and cost-effective manner. In an array-based nanoparticle platform, target complementary capture DNAs were immobilized between electronic gap and subsequently hybridized with DNA-AuNPs via target DNA recognition. The immobilized AuNPs were then treated with silver staining, giving a sensitive (∼fM) and selective

electrical "on" signal [19] (Fig. 3.5c). Fan and coworkers reported a highly sensitive electrochemical method for the detection of DNA, based on a sandwich detection strategy involving capture probe DNA immobilized on gold electrodes and reporter probe DNA labeled with gold nanoparticles [51]. Another method is involved a real-time DNA detection and utilized single-stranded DNA-modified nanoparticles and micropatterned chemoresponsive diffraction gratings, interrogated simultaneously at multiple laser wavelengths [52]. This chemoresponsive diffraction grating signal transduction scheme permits experimentally simple, real-time observation of DNA hybridization on surfaces with ∼1 pM sensitivity. Fluorophore-dye doped silica nanoparticles also have been explored as alternative labeling material, generating increased fluorescence signals during DNA detection with high sensitivity [53].

After early stages of conceptual discovery of DNA analysis on surface as shown in Fig. 3.5a–c, more sophisticated nano-patterned structures have been explored to obtain improved sensitivity and reproducibility using optical (Raman, fluorescence, etc.) or electrical transduction modes. In one study, a sandwich type of nanoparticle-linked nanowire complexes were assembled by target DNA hybridization with Raman reporter molecules placed in the junction [34] (Fig. 3.5d). These multiplexable assemblies exhibited greatly amplified Raman signals. Mirkin and coworkers used dispersible arrays of nanodisks, prepared by on-wire lithography and functionalized with Raman-active chromophores, for DNA detection in a multiplexed format with high sensitivity (∼100 fM), as shown in Fig. 3.5e [54].

Recently, Nam and coworkers reported SERS-active gold nanodumbbells linked with DNA, synthesizable in a high yield, and these nanogaps structures exhibited single-molecule detection capability repeatedly [55] (Fig. 3.6). In this method, they were able to control the nanogap between gold nanoparticles by varying the thickness of silver nanoshell in order to finely tune the SERS signal. They found that controlling inter-particle nanogap is extremely important for highly sensitive and reproducible single-molecule SERS detection. More recently, they also synthesized nanobridged nanogap Au particles with 1-nm interior gap. With the aid of DNA on Au particle surface and specific reaction conditions, highly uniform 1-nm gap was generated inside a particle, and this interior nanogap was responsible for strong, uniform and quantitative SERS signals [56]. When these NNPs were analyzed on the surface, ∼90 % of analyzed particles displayed detectable Raman signals. Another simple method to fabricate dimeric Ag nanostructures in solution state is shown in Fig. 3.7a–d [35]. In this method, by controlling the concentration of electrolytes in Ag colloidal solution, the dimeric Ag nanoparticles were dominantly produced and the structures were fixed with additional silica coating (Fig. 3.7b).

Two different surface-based nanogap fabrication methods have been shown in Figs. 3.8 and 3.9, respectively. Using sequential angle evaporation technique, <5 nm gap between silver nanospheres on the surface were uniformly generated (Fig. 3.8) [36]. Vertically oriented, <10-nm gap was also achieved *via* anisotropic etching procedure on the surface (Fig. 3.9) [33].

In the above-mentioned methods, relatively uniform nanogaps in the large area were possible to generate, but the next imminent challenges remain to locate the

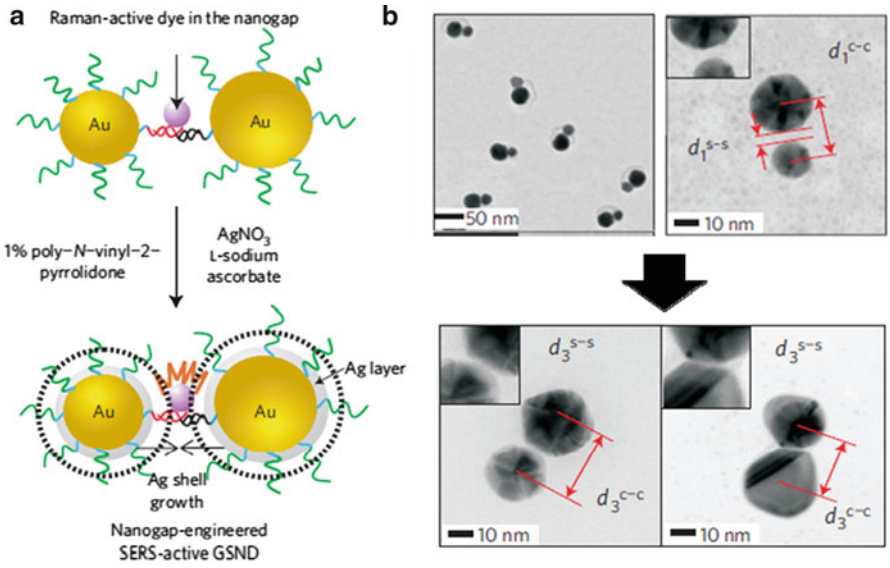

Fig. 3.6 Single DNA detection with gold-silver core-shell nanodumbbells. (**a**) Synthetic scheme with two different oligonucleotide-modified gold nanoparticles and additional silver shell formation for inter-particle gap engineering for single-molecule SERS. (**b**) TEM images of synthesized gold nanoparticle dimers (*upper*) and silver shell-coated nanoparticle dimers (*lower*) (Reprinted with permission from Nature Publishing Group, Ref. [55]. Copyright (2009))

target molecule of interest in the fabricated hot-spot regions and to synthesize targeted nanostructures with high structural reproducibility in a large scale.

The conductivity-based electrical DNA sensors, which were based on silver enhancing procedure, were further improved by the use of DNA-linked–CNT nanowire motifs and enzymatic metallization-based amplification [57]. In this method, a DNA detection limit of 10 fM was achieved with the ability to discriminate single, double, and triple base pair mismatches (Fig. 3.10a). Gao and coworkers reported nanogap sensor arrays for highly sensitive electrical detection and quantification of DNA [58]. The sensing process is based on bridging the nanogap upon hybridization of the two termini of a target DNA with two different surface bound capture probes, followed by a metallization step (Fig. 3.10b). Using this method, a minimum of 1.0 fM DNA was detected via two-order-of-magnitude enhancement in conductance. Above-mentioned methods validated the importance of engineered nanostructures for generating amplified optical and electrical signals, applicable for biomolecule detection with high sensitivity.

Xiao and coworkers demonstrated that engineering of biofunctionality can address many limitations of electrochemical biosensors [59]. Their electrochemical SNP (E-SNP) sensor is based on a single, redox-labeled DNA strand that incorporates three stems, which is different from redox-labeled single-stem molecular beacon [60] and double-stem pseudoknot [61] electrochemical sensors (Fig. 3.11a).

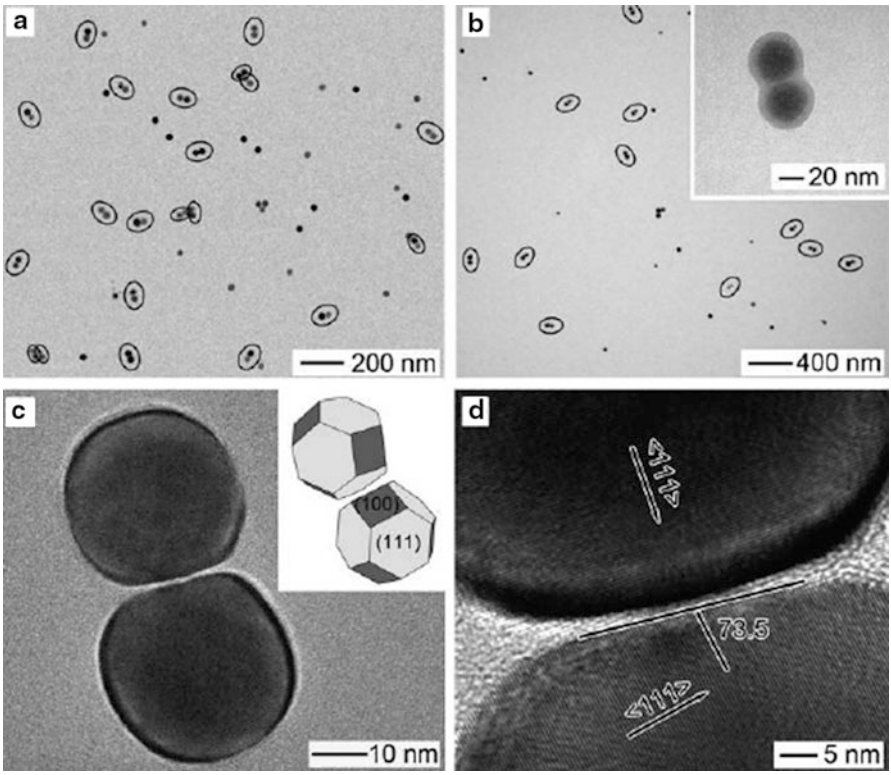

Fig. 3.7 Salt-induced silver nanoparticle grouping into dimers and fixation using silica shell formation. TEM images of synthesized silver dimers (**a**) and silica-coated dimers (**b**). (**c**) HR-TEM image of Ag dimer and its lattice plane (*inset*). (**d**) Magnified image of Ag dimer junction (Reprinted with permission from the American Chemical Society, Ref. [35]. Copyright (2008))

When complementary target oligonucleotides (17 base pairs) were present, 3′-modified MB can be placed in a close contact with Au surface, eventually, increasing the faradic current (335 ± 7 %). The signal changes were large enough to detect a single base mismatch [59].

Semiconductor silicon nanowire-based field effect transistor (SiNW-FET) has been used as a biosensing platform for the real time detection of nucleic acids [62]. Based on the change in conductance of DNA-modified SiNWs, specific target DNA sequence was detected with high sensitivity (∼1 fM). Using this method, simultaneous and selective detection of two pathogenic strain virus DNA sequences (H1N1 and H5N1) of avian influenza was achieved (Fig. 3.11b).

Based on the similar concept, GaN nanowire(GaNNW)-based extended-gate field effect-transistor (EGFET) biosensor was developed and this platform dramatically improved sensitivity (∼aM range) and dynamic range (10^{-19}–10^{-6} M) for short oligonucleotides (15 base pairs) under label-free in situ conditions [63]. The wide bandgap semiconducting nature of GaN, surface-sensitivity of

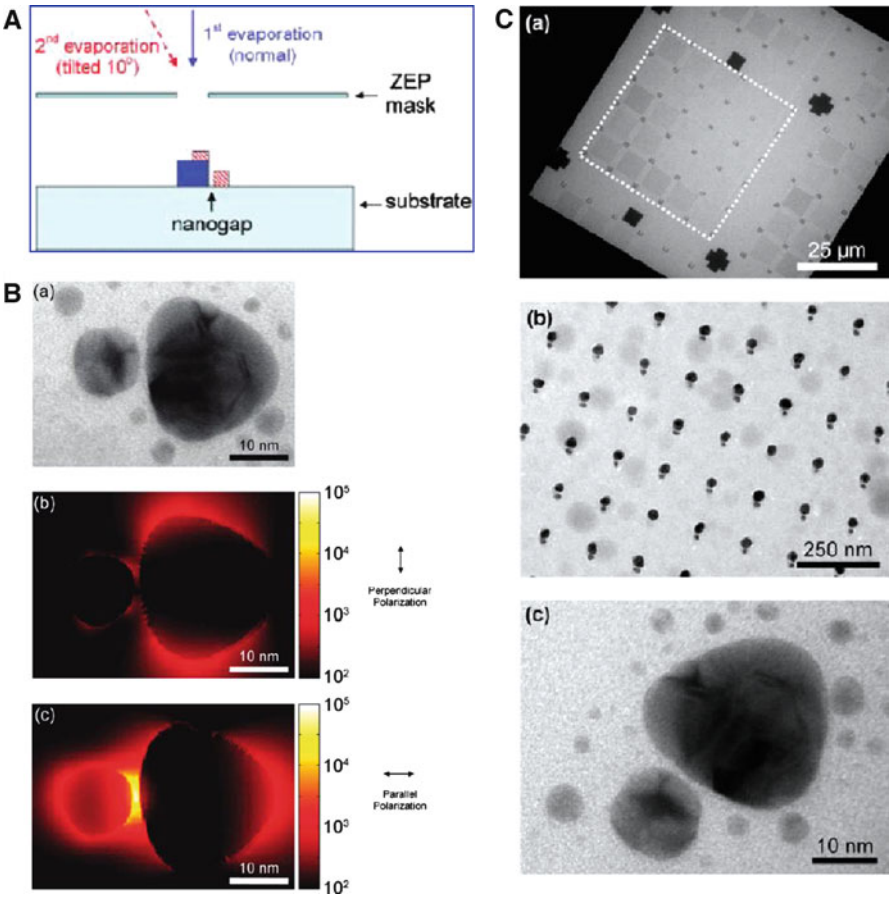

Fig. 3.8 Fabrications of nanoparticle dimer array on surface using angle evaporation technique. (**A**) Scheme for angle evaporation technique. (**B**) HRTEM image (**a**) and simulated polarization dependence in perpendicular (**b**) and in parallel (**c**). (**C**) low resolution SEM image (**a**), TEM image (**b**) and HRTEM image (**c**) of arrayed surface (Reprinted with permission from the American Chemical Society, Ref. [36]. Copyright (2010))

the NW structures, and high transducing performance of the EGFET-design were responsible for improved sensitivity and specificity in this method, as demonstrated by the detection of single base-pair mutation in the human p53 gene. As evident from above discussion, a wide variety of engineered nanostructures have been fabricated with different materials, often displaying higher sensitivity and specificity for target DNA than solution-based methods. Although highly limited for *in-vivo* applications, surface-based nanostructure platforms offer the high throughput potential and relatively simple transduction modes such as electrical transduction mode and it will not be long to see commercialized version of DNA sensors for the widespread use for *in-vitro* clinical DNA analyses.

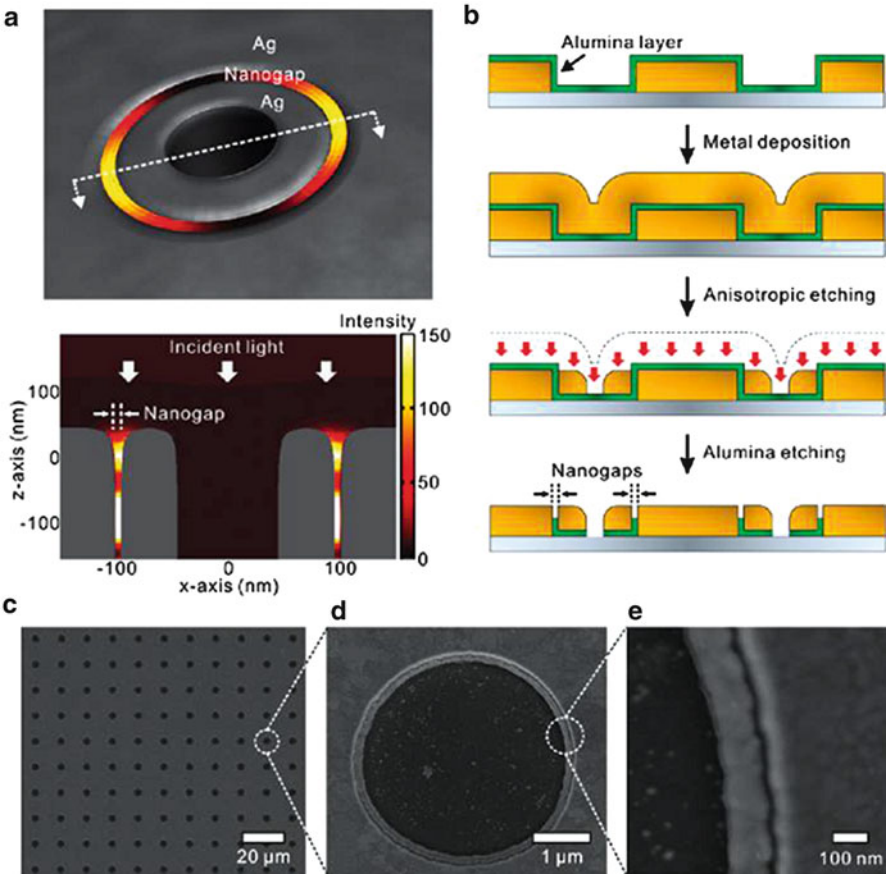

Fig. 3.9 Fabrication schemes for vertically oriented plasmonic nanogap using anisotrophic etching technique. (**a**) Calculated electromagnetic distribution profile, (**b**) Schematic description of fabrication procedures, (**c**) SEM images of patterned surface, (**d**, **e**) Magnified images (Reprinted with permission from the American Chemical Society, Ref. [33]. Copyright (2010))

3.4 Engineered Nanopores and Nanochannels for DNA Analysis

Nanopore or nanochannel is a nanometer-sized path through a thin layer, which selectively allows specific biomolecules (e.g., DNA) to pass, based on their size, shape and chemical nature, typically in a 'one-by-one' mode. During the process, individual molecules are detected and analyzed via optical or electrical signals. These engineered three-dimensional nanostructures along with microfluidics and nanowells have been extensively investigated as promising candidates for real-time, efficient genome-wide next generation DNA sequencing methods [64–66]. In this section, we will discuss the DNA sensing technologies developed using

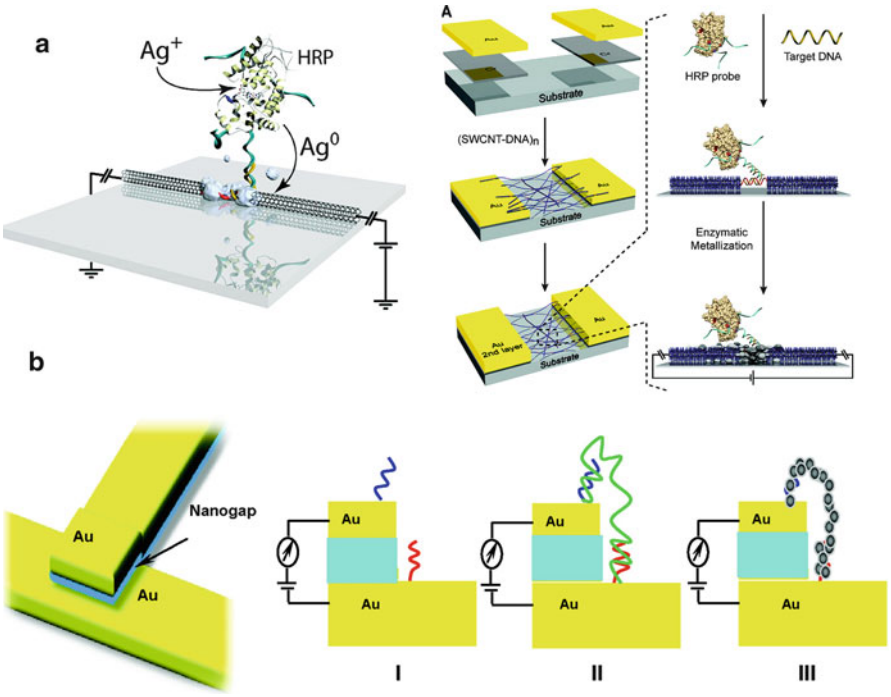

Fig. 3.10 Electric nanogap device**.** (**a**) DNA-CNT nanowire network for DNA detection, (**b**) nanogap sensor arrays for ultrasensitive DNA analysis (Reprinted with permission from the American Chemical Society, (**a**) Ref. [57]. Copyright (2011), (**b**) Ref. [58]. Copyright (2009))

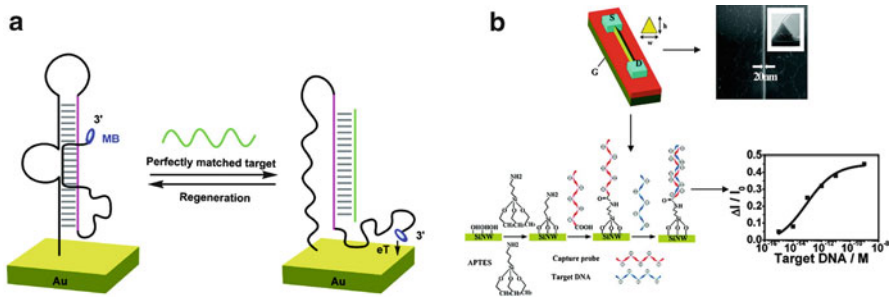

Fig. 3.11 Triple-stem DNA probe based electrochemical sensor for single nucleotide polymorphism detection (**a**) and silicon-nanowire-based CMOS compatible FET nanosensors for the ultrasensitive electrical detection of nucleic acids (**b**) (Reprinted with permission from the American Chemical Society, (**a**) Ref. [59]. Copyright YEAR (2009), (**b**) Ref. [62]. Copyright YEAR (2011))

microfluidic channels and nanochannels. Nie and coworkers reported the use of color-coded nanoparticles and dual-color fluorescence coincidence for real-time detection of native biomolecules and viruses in a microfluidic channel [67]. They

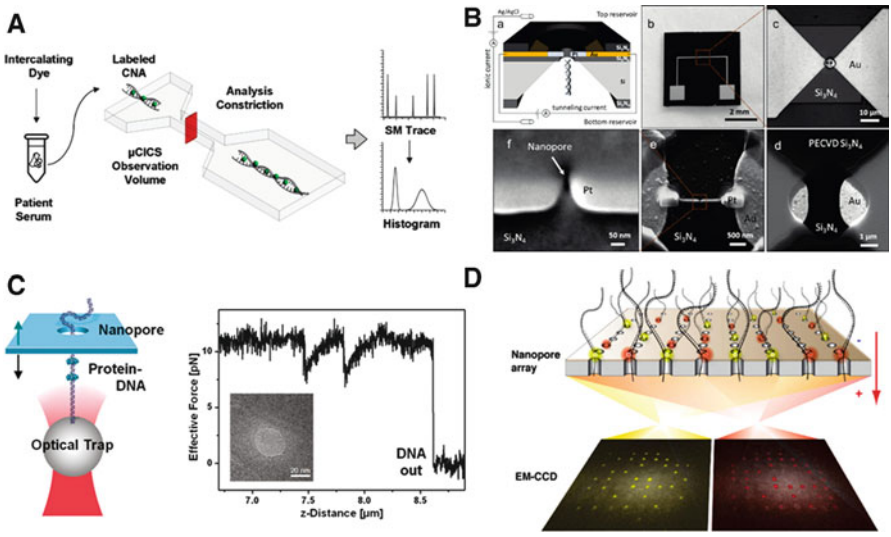

Fig. 3.12 Microfluidics and nanopore-based DNA detection and sequencing technologies. (**A**) Microchannels for small fragment DNA [68]. (**B**) Schematic of the nanopore/nanogap device integrated into an electrochemical cell. The tunneling junction is located at the exit of the nanopore. DNA is inserted in the *bottom* reservoir and electrophoretically driven through the nanopore and the tunneling junction [73]. (**C**) Optical trap for effective DNA stretching [75]. (**D**) Multiple nanopores for optical detection of two *color*-coded oligonucleotides [76] (Reprinted with permission from the American Chemical Society, (**A**) Ref. [68]. Copyright YEAR (2010), (**B**) Ref. [73]. Copyright YEAR (2010), (**C**) Ref. [75]. Copyright YEAR (2011), (**D**) Ref. [76]. Copyright YEAR (2010))

demonstrated that individual molecules of interest can be detected and identified in complex mixtures without target amplification or probe/target separation at the target concentration of as low as 20–30 fM. Recently, Wang and coworkers reported single molecule analysis of circulating fluorescently-labeled DNA (CNA) in human serum using microfluidic cylindrical illumination confocal spectroscopy and fluorescence burst size analysis as shown in Fig. 3.12A [68].

For the development and widespread use of new DNA sequencing technologies, critical issues such as cost, reliability, and high-throughput capability should be addressed. Fast spectroscopic response, easy chemistry and multiplexing capability are other important desired features for the next-generation DNA sequencing technologies. Development of software and bioinformatics tools for data analysis and sophisticated instruments are also needed to obtain reliable and accurate DNA sequencing results. However, the most important requirement is the development of engineered nanostructures such as nanowells [69], nanochannels [70–72], and nanopores [73] where DNA polymerization or efficient stretching can occur often in a single-molecule level. At the same time, engineered nanostructures play a critical role in reducing background signal, thereby, making possible to detect fluorescence signal per single polymerization reaction. An example is provided by Zero-mode waveguides (Pacific Biosciences, Menlo Park, CA, USA), with which illumination can be restricted to a zeptoliter-scale volume with a surface-tethered polymerase

such that incorporation of single nucleotide (with a fluorescent label on phosphate group) can be monitored with very low background signals [69].

Single, sub-20-nm wide and centimeter-long nanofluidic channel, fabricated by a nanoimprint mold method, can successfully stretch small DNA fragment by liquid flow in the nanochannel [74]. Kim and coworkers reported that polystyrene-*block*-poly(methyl methacrylate) copolymer having dicarboxylic acid end group(PS-*b*-PMMA-diCOOH)-coated nanochannels having a pore size of ∼15 nm and depth of ∼80 nm can successfully differentiate single-nucleotide polymorphisms (SNPs) in 18 base-pair oligonucleotides [72]. Due to the ultrahigh density ($\sim 6 \times 10^{11}/\text{in}^2$) and short nanochannel length, very high resolution was achieved therein.

Recently, Edel and coworkers fabricated a device that combines a nanopore with a tunneling junction [73]. In such a configuration, the nanopore was used to "unfold" the DNA strand to a linear configuration, while a tunneling junction, precisely aligned at the pore opening, detects sequence-specific changes in the tunneling current, as shown in Fig. 3.12Ba. The experimental platform that was composed of a 5 mm × 5 mm silicon chip (Fig. 3.12Bb) with Si_3N_4 and Au layers microelectrodes with a 2 μm gap (50 nm thick gold with 10 nm chromium adhesion layer) was fabricated by a conventional optical lithographic technique on the front face of the wafer. An additional 300-nm thick layer of Si_3N_4 was deposited by plasma enhanced chemical vapor deposition (PECVD). Windows (5 μm × 5 μm) centered at the microelectrode tips were opened in the PECVD Si_3N_4 layer (Fig. 3.12 Bc, d). The additional Si_3N_4 layer reduces the membrane capacitance and faradic currents by minimizing the electroactive area. Furthermore, the additional Si_3N_4 layer helps improve the mechanical strength of the membrane. A window in the LPCVD nitride on the back face of the wafer was opened by reactive ion etching (RIE), followed by a wet etch of the silicon in KOH solution, resulting in a 70 nm thick free-standing Si_3N_4 membrane (40 μm × 40 μm) aligned to the microelectrodes (Fig. 3.12Bc, d). The solid state Si_3N_4-membrane (20 nm thickness) was milled with He ions, rendering nanopore openings with typically 20–60 nm in diameter. The membrane was integrated into the fluidic cell so that the two sides of the membrane were only electrically contacted via the nanopore. Translocation dynamics of a single protein molecule attached to double stranded DNA has been studied using the nanopore force spectroscopy (Fig. 3.12C) [75]. In a different approach, Meller and coworkers reported the use of multiple nanopores and optical sensing method (Fig. 3.12D) [76]. Here, they used two color readout platform, which was coded for four DNA base, for example, 1,1 for A, 0,1 for T, 1,0 for G , 0,0 for C, and then specific hybridization to the 0 and 1 sequences was followed. The converted DNA and hybridized molecular beacons were electrophoretically threaded through a solid-state pore.

3.5 Conclusion

Improving sensitivity is the foremost important issue in bioassays without deteriorating other factors such as assay cost, time, complexity, dynamic range and reproducibility. It would be beneficial to develop a nucleic acid detection assay

with PCR-like sensitivity and without the need for enzyme and temperature control-based target amplification. This way, problems of using enzymes such as extra steps and complex instrumentation to control the conditions could be avoided. The development of new functional nanomaterials for amplification methodologies could improve or replace ELISA method for protein targets and PCR for nucleic acid targets. As discussed above, new 1,2,3-dimensional nanostructures have been extensively investigated for this purpose owing to their important physiochemical properties, and some of them are already commercialized and utilized in many laboratories for research purpose and as clinical diagnostic tools. However, the composition, size, shape and other chemical and structural features such as nanogap of nanostructures still required to be finely controlled for more accurate and sensitive DNA analysis with multiplexing capability. Recently, nanopore- or nanochannel-based DNA sequencing methods have great potential in this field, but these strategies face many challenges including selective recognition of individual nucleotide and multiplexing capability (parallel recognition) along with assay cost and time.

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Chapter 4
Advanced Molecular Probes for Sequence-Specific DNA Recognition

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Abstract DNA detection can be achieved using the Watson-Crick base pairing with oligonucleotides or oligonucleotide analogs, followed by generation of a physical or chemical signal coupled with a transducer device. The nature of the probe is an essential feature which determines the performances of the sensing device. Many synthetic processes are presently available for "molecular engineering" of DNA probes, enabling label-free and PCR-free detection to be performed. Furthermore, many DNA analogs with improved performances are available and are under development; locked nucleic acids (LNA), peptide nucleic acids (PNA) and their analogs, morpholino oligonucleotides (MO) and other modified probes have shown improved properties of affinity and selectivity in target recognition compared to those of simple DNA probes. The performances of these probes in sensing devices, and the requirements for detection of unamplified DNA will be discussed in this chapter. Chemistry and architectures for conjugation of probes to reporter units, surfaces and nanostructures will also be discussed. Examples of probes used in ultrasensitive detection of unamplified DNA are listed.

4.1 Introduction

The possibility to perform detection of specific nucleic acid sequences in biological samples and to correlate the information obtained with specific genetic traits is one of the most powerful tools available in this so called "post-genomic era" [1]. Tracking DNA and RNA instead of other analytes, such as proteins, carbohydrates, lipids or other metabolic products, has the advantage to be accessible through a

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rational design of probes. Oligonucleotide probes have DNA structure and can bind to target sequences via formation of the same base pairing occurring in the genetic material, i.e. Watson-Crick hydrogen bond scheme and several other simple and well-established interactions. Genosensors are often realized by coupling a set of specific oligonucleotide probes with transduction devices able to transform the molecular recognition into a physical measurable signal, mostly electrochemical, optical, mechanical or magnetic [2, 3].

Since the recognition event is the central part of the biosensing process, an ideal probe is that which binds to the target sequence with high affinity, recognizes only a specific target sequence and does not bind in the presence of mismatched bases; however, this goal is met only under appropriate conditions and by careful design of the probes, and still differences of only one base in the target sequences, corresponding to single nucleotide polymorphisms (SNPs) or point mutations, are still the most difficult to detect, so that discrimination at this level is one of the ultimate goals of genosensor design. In fact both SNPs and point mutations have great relevance in diagnostics. Mutation at a single base level are often found in connection with pathologies: hereditary diseases such as thalassemia [4] and cystic fibrosis [5] are correlated to single point mutations, and point-mutated genes (e.g. p53 or K-ras point mutation) are frequently associated to tumor onset [6, 7].

SNPs can be used as molecular markers to discriminate between species, breeds, varieties and even individuals [8], and in several applications, SNP-detecting devices can be used as tools for identification of raw materials in foods [9].

In order to achieve best performances in DNA recognition in terms of affinity and sequence specificity not only for analytical purposes, but also in the design of new drug candidates, chemists have designed and synthesized a large number of DNA mimics. Structures of oligomers bearing natural nucleobases in combination with unnatural backbones have been proposed in the last two decades, and some of the best performing structures have been used as new tools for improving the performances of genosensing devices. The optimization of probes is a crucial element especially when ultrasensitive techniques are used for enabling detection of nucleic acids at very low concentrations. Some of the most important classes of DNA analogs relevant for ultrasensitive detection will be discussed in the present chapter.

Furthermore, a series of chemical tools aimed to the modification of the molecular probes with reporter groups or sensor surfaces have been developed in the last decades, and the possibility to have probes “decorated” with the proper combination of elements has become very important in the design of specific tests. More recently conjugation with nanostructured materials such as liposomes, nanoparticles, nanotubes or graphene has become more and more important, due to the suitability of these components for optical and electrochemical detection. The use of these tools will be discussed thoroughly in another chapter of this book [10] and only chemical linking of probes to these objects will be taken into account here. Finally, examples of probes used in ultrasensitive detection of unamplified DNA will be presented.

4.2 Oligonucleotides (ON) and Modified Synthetic Analogs

The availability of synthetic oligo(deoxyribonucleotides) (ON), mostly synthesized by phosphoroamidite chemistry described by Caruthers, [11] (now commercially available from many sources) is one of the factors that make possible a large variety of applications from biotechnology to nanotechnology and is particularly useful in the design of genosensors. A careful quality control of commercial products and eventual purification steps are advisable for best performances in analytical systems.

Proper choice of DNA probes is necessary in order to design a genetic test for specific purposes and in combination with specific devices, but some limitations are intrinsic in the chemical nature of DNA. Thus, oligonucleotide mimics are currently under development in many laboratories and some of them are only available in the inventors' lab; however, best performing structures have become commercially available and can be purchased from specialized firms. Among them, most interesting are locked nucleic acids (LNA), peptide nucleic acids (PNA) and morpholino oligonucleotides (MO), which will be discussed more in detail, but also other structural variations of DNA have proven to be very effective in DNA recognition and have potentiality to become new commercially available tools. In this paragraph the performances of these probes will be discussed.

4.2.1 Performances of Probes in DNA Analysis

In order to have control of the sensing devices, the characteristics of DNA probes should be taken into account, especially for the design of ultra-sensitive techniques. Supramolecular interactions occurring in solution can be evaluated using existing models, since thermodynamics of DNA:DNA interactions have been extensively studied; surface-attached probes have however different models for their interactions and differences in affinity and selectivity from the solution data are likely to occur; this led many scientist to specifically study the probe-target interaction on solid surface and to evaluate surface effects which can be rationalized. Finally, the multiple functionalization of nanostructures, such as nanoparticles, nanorods, nanotubes give rise to binding processes which, although occurring in solution, are governed by a surface-like environment in which multiple interactions can occur. The following paragraph will discuss these issues in terms of affinity and selectivity of DNA probes.

4.2.1.1 Affinity

The possibility to perform detection of a specific DNA sequence of interest is given by the formation of a duplex with the target sequence (T) by the probe (P):

$$T + P \rightleftarrows T : P$$

The stability constant of the process is given by:

$$K_{ass} = [T : P] / [T] [P] \tag{4.1}$$

and the dissociation constant is the reciprocal of this constant. i.e.

$$K_d = 1/K_{ass} \tag{4.2}$$

the latter parameter is useful in analytical applications since it is correlated to the possibility of duplex formation under very low concentration of the target sequence [12]. If an excess of P is used, the dissociation constant represents the concentration of P at which half of the target is bound to the probe. Dilution of a 1:1 complex have a dissociation curve such as that reported in (Fig. 4.1a) for a 10^{15} M^{-1} constant. For oligonucleotide analogs, unfortunately, most of the studies on DNA binding report melting temperature (Tm) as a measure of affinity. Tm is the temperature at which, under given conditions, a DNA:DNA duplex is half dissociated, and therefore is not a direct measurement of the stability constant, but only of its temperature dependence (Fig. 4.1b). However, thermodynamic parameters can be inferred from melting profiles using several models [13].

Thermodynamic data for oligonucleotides are available in several databases and different models for predicting the DNA:DNA duplexes stability on the basis of the sequence have been proposed. Prediction of $\Delta G°$ values can be done using these models [14]. As an example, using the Santa Lucia model, a 10mer duplex of sequence 5′-GTAGATCACT-3′ at 1 μM in 0.1 M NaCl can bind its complementary sequence with a Tm of 23.47 °C and a $\Delta G° = 36$ KJ $mole^{-1}$, which corresponds to K of 2.4×10^6. Therefore, using DNA probes of this length, with analytes largely below micromolar concentration is not likely to succeed.

By doubling the sequence, the $\Delta G°$ is more than doubled, leading to a K of 1.1×10^{15} as calculated by the web tool available at http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::melting (for a library of Tm prediction website see http://protein.bio.puc.cl/cardex/servers/melting/sup_mat/servers_list.html).

A different case is given when the probe is immobilized onto a surface.

In this case, the concentration of the probe can be substituted by its density on the surface and T:P is formed only on the surface itself. In this case the simple equation (4.1) can be used for the evaluation of stability constant on the surface, provided that 2D concentration (i.e. probe density) is used.

Alternatively, a Langmuir model can be used by considering each probe as an “active site”. A complete model for thermodynamics as well as kinetics of oligonucleotide hybridization on surfaces can be found in the work of Erikson et al. [15].

It should be noted that models are affected by deviation due to the surface localization of the probes, which forces the target molecules to remain on the surface. In the case of DNA molecules, which are negatively charged, a negative cooperative effect can be present, since the occupation of one active site by a DNA molecule would disfavor the binding of another DNA molecule on an adjacent site

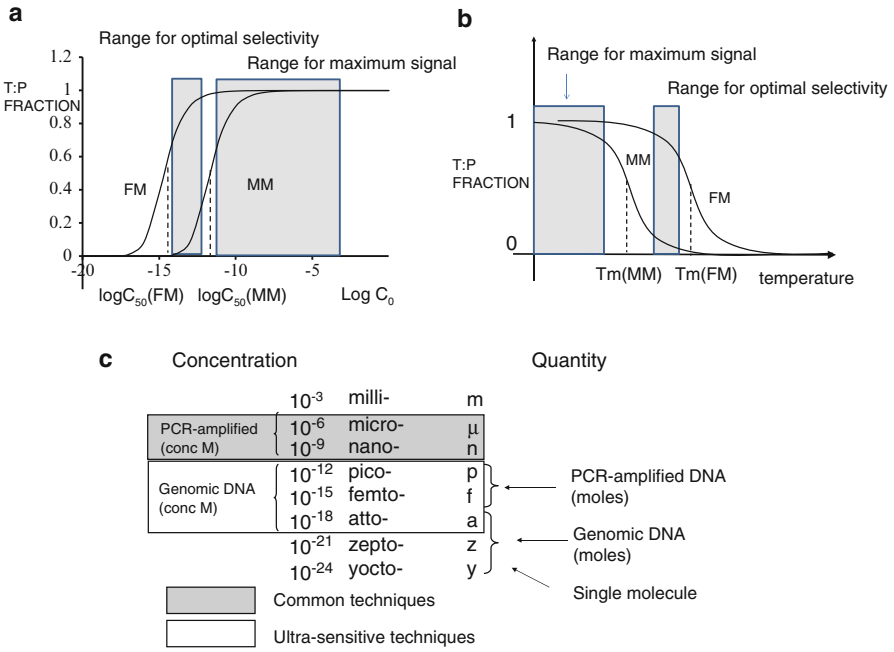

Fig. 4.1 (**a**) Effect of dilution on T:P fraction in the case of a full matched (*FM*) duplex and in the case of a two order of magnitude less stable mismatched (*MM*) sequence; (**b**) effect of temperature on T:P fraction for full-match and mismatched duplexes.; (**c**) ranges of concentration for detection of PCR-amplified or non-amplified DNA

by electrostatic repulsion. This effect depends on probe density and on the ionic strength of the aqueous solution, but also on the nature of the probe itself. This issue has been studied in detail for SPR sensors by the group of Knoll and hybridization efficiency was shown to depend on the probe density and reached nearly 100 % for 15mer DNA fully complementary targets at a probe density of $\sim 1.2 \times 10^{12}$ molecules/cm^2 [16]; thus this coverage has been taken as reference by successive works in order to avoid these negative effects.

A third case is that of modified nanoparticles bearing a large number of probe molecules. Modeling of this situation can be done using the concept of multivalence developed by several authors and described in different review articles [17]. Also in this case negative cooperativity can be present for DNA. A simplified model can be used by considering the total concentration of probes as the concentration of nanoparticles multiplied by the mean number of probes per nanoparticle. Again, this model is valid only if the probe density on a single nanoparticle is sufficiently low to prevent cross-talking.

As a term of reference, in Fig. 4.1c the concentrations of DNA targets in analytical applications are depicted. In normal applications in which a PCR protocol is used, the concentration of the target is at least in the nanomolar range and

therefore stability constants of 10^9 would be recommended in order to have sufficient complex formation for an efficient detection. For PCR-free protocols, very high stability constants of 10^{15}–10^{18} should be used for good hybridization.

However, it should be considered that, if the detection method is very sensitive and it is able to distinguish the target in the free and in the bound state, even very few copies of the complex can be detectable, thus allowing to considerably lower the detection limit even if the probe has dissociation constants largely above the concentration of the target.

Self-complementarity and probe folding are two of the characteristics of the probes to be strictly evaluated (and avoided) in the design of probes for sensing technologies, as illustrated by Minunni and coworkers in a recent paper using freely available software for secondary structure evaluation [18].

4.2.1.2 Sequence Selectivity

Sequence selectivity is also a very important issue in DNA detection, in fact, DNA probes do not display the ideal all-or-none behavior, since partial complexation is possible for sequences differing from the target for only few bases. The most difficult case is that of a single-base difference (single mismatch), for which the stability constant and melting temperature could be very close to that of a fully matched duplex; however since differences in a single base represent clinically interesting cases involving oncogenes (e.g. *K-ras*) [7] or genetic diseases (e.g. thalassemia [4] or cystic fibrosis [5]), and single nucleotide polymorphisms (SNPs) detection can be a very powerful tool for identification purposes, pushing the recognition properties of the probes to the maximum efficiency in mismatch recognition is one of the main goals in probe design.

Sequence selectivity can be expressed in sensory systems as the ratio between the signal obtained with a specific probe-sensor combination, using the full-matched DNA and that obtained using mismatched sequences. The most stringent conditions for this evaluation are those relative to single-base mismatch. It should be noted that thermodynamics of mismatch binding are dependent on the length of the T:P duplex and on the type of mismatch. For DNA probes a full set of mismatch data are available from databases reporting thermodynamic data for oligonucleotides [14].

Usually, both the increase in temperature and the dilution of samples have the effect of increasing sequence selectivity, since conditions for dissociation of mismatched targets are met before those of the full-matched sequence (Fig. 4.1a, b). A detailed study of temperature dependence of fluorescent signal has been carried out for beacon probes (see Sect. 4.3.2), and it was shown that proper choice of temperature was essential in order to have maximum selectivity for single mismatches. However, high temperatures are not always compatible with sensing devices. Dilution of both probe and targets have the effect of dissociating the less-stable mismatched sequence (Fig. 4.1a), but this causes also a decrease in the sensitivity of the method; this strategy is therefore much more efficient when

ultrasensitive techniques are used for detection; working at very low concentration is normally accompanied by very high sequence selectivity.

According to these concepts, at a fixed temperature and concentration, the increase of the T:P length induces an increase in stability (and hence in sensitivity if the target is at very low concentration), but a loss in sequence-selectivity. Thus the only way to further push the performances of an optimized DNA sensory systems is to change the chemical nature of the probes used.

4.2.2 Oligonucleotide Mimics

During the past decades, the limitations in the use of ON probes were partially overcome using newly designed DNA mimics, in which the essential features of DNA structure are conserved, in particular the presence of nucleobases able to perform Watson-Crick-like base pairing, whereas the other chemical groups are drastically changed. The quest for new, highly efficient, DNA binding molecules has been pursued for the last two decades very intensely and it is still a subject of many studies. Chemists have changed all the essential features of the DNA model: the phosphate group, the sugar moiety and nucleobases. The effect of these modifications have been extensively studied, [19] especially in connection with antisense technology [20]. The most relevant modifications which proved to be successful in sensory systems are those related to changes in the backbone, using locked nucleic acids (LNA), morpholino oligonucleotides (MO), peptide nucleic acids (PNA) and their analogs, (Fig. 4.2), and several other miscellaneous probes which will be discussed in the next paragraphs.

4.2.2.1 Locked Nucleic Acids (LNA)

LNA (Locked nucleic acid, Fig. 4.2a) was first described by Singh et al. in 1998 as a new class of conformational restricted oligonucleotide analogs [21]. LNAs are oligonucleotides containing at least one LNA monomer, i.e., one 2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl nucleotide (β-D-LNA), where a ribonucleoside is linked between the 2′-oxygen and the 4′-carbon atom with a methylene bridge [21, 22]. In LNA monomers, the sugar is locked in a C3′-endo conformation (N-type and RNA-like) by the methylene bridge [21, 23] and structural studies show that LNA is a RNA mimic, fitting seamlessly into an A-type duplex geometry [24]. α-L-LNA (α-L-ribo configuration), stereoisomeric analogs of LNA have also been extensively studied [25].

LNA oligonucleotides can be synthesized using conventional automated phosphoramidite chemistry and LNA monomers are compatible with other monomers, e.g. DNA, RNA, 2′-*O*-Me-RNA, and with phosphorothioate (PS) and phosphodiester linkages [26, 27]. The interest in LNA applications has steadily grown up in last years: these nucleic acids analogs in fact show a very efficient binding to

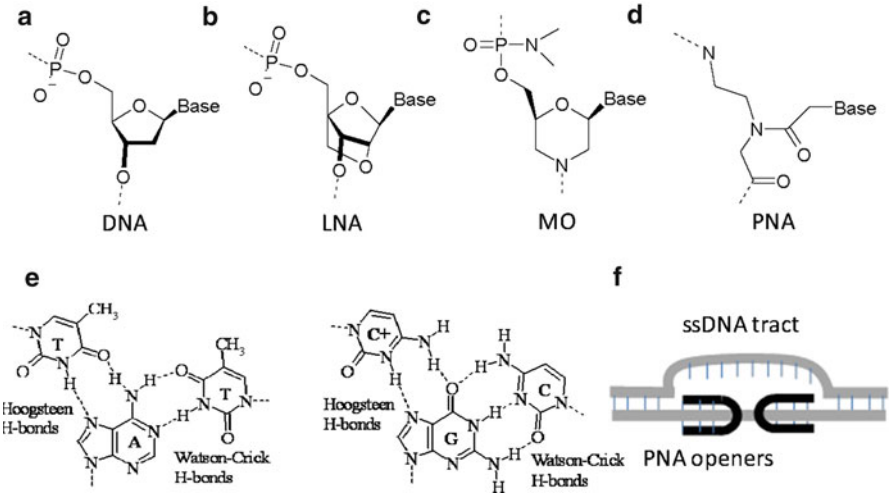

Fig. 4.2 Structures of (**a**) DNA (**b**) locked nucleic acids (*LNA*) (**c**) morpholino phosphoamidate oligonucleotides (*MO*) (**d**) peptide nucleic acids (*PNA*). (**e**) Hoogsteen base pairing occurring in triplex formation; (**f**) Mechanism of action of PNA openers via triplex formation

complementary nucleic acids and a high potency as antisense molecules *in vitro* and *in vivo*, together with a commercial availability as oligonucleotides (fully modified, mix-mers with DNA, RNA, other monomers and various modification) and as phosphoramidite building blocks [28].

LNAs act as natural nucleic acids with respect to Watson-Crick base pairing and fully modified LNA oligomers induce very high thermal stability of duplexes toward complementary RNA or DNA [26]. In particular, a large number of LNA:DNA hybrids have been structurally characterized by NMR spectroscopy and X-ray crystallography: these duplexes showed the common features for native nucleic acid duplexes, i.e. Watson–Crick base pairing, *anti* orientation of nucleobases, base stacking, and right-handed helical conformation [29].

The most conclusive feature of LNAs is the unprecedented hybridization to complementary nucleic acids, a property that made LNAs become general and versatile tools for specific high-affinity recognition of single-stranded DNA (ssDNA) and, due to their forced C3′-endo conformation, particularly suited for the binding of RNA.

Single-stranded LNA oligonucleotides can also interact with double-stranded DNA (dsDNA) by two possible types of hybridization, i.e. binding to the dsDNA by the formation of a triple-helical complex or binding to the dsDNA by single-strand targeting, thus displacing the other DNA strand by a so-called “strand invasion” process. In particular, gene expression could be up- or downregulated at the level of transcription via selective triple-helix formation, preferably at promoter sites, emphasizing new appealing approaches [30]. The properties of LNAs for targeting DNA thus make LNAs exceptional tools for nucleic acid probes and absolutely

crucial in this context is the freedom in design with excellent cooperativity between LNA monomers and other classes of monomers and labels. Then, a wide range of LNA-based approaches have been evaluated in the field of ultrasensitive detection of DNA, especially in developing PCR-free techniques.

LNA probes modified with pyrene units were used as "invaders" for dsDNA (vide infra) [31]. LNA capture probes have also been used for the development of electrochemical hybridization assays. For example LNA probes was employed in an electrochemical assay in which streptavidin-coated magnetic micro-beads were used as solid phase to immobilize the biotinylated capture probes. The target biotinylated DNA sequences were then recognized via hybridization with the LNA capture probe. After labeling the biotinylated hybrid with streptavidin–Horse Radish Peroxidase (HRP) conjugate, the electrochemical detection of the enzymatic product was performed onto the surface of a disposable screen-printed electrochemical cell (SPEC) [32].

In another approach, LNA oligonucleotides have been used in the development of sensitive and selective molecular beacons (MB) [33].

Thus, hybridization properties displayed by LNAs have made them very efficient tools for many applications in detecting DNA targets in various fields of research, especially diagnostics, genomics and biotechnology.

4.2.2.2 Morpholino Oligonucleotides

Morpholino oligonucleotides (MOs, Fig. 4.2c) are another class of very popular oligonucleotide analogs, first described by Summerton in 1989 [34]. MOs have standard nucleic acid bases bound to morpholine rings which are linked through phosphorodiamidate groups [35].

Replacement of anionic phosphates with the uncharged phosphorodiamidate groups eliminates ionization in the usual physiological pH range, so MOs in organisms or cells are present as uncharged molecules; in addition, the lack of charge makes them especially useful for electrostatic transduction. Furthermore, this particular structure makes MOs extremely resistant to nucleases and other degradative factors in biological systems [24]. Finally, it is worth to say that MOs excel in the properties of stability, efficacy, long-term activity, water solubility, low toxicity and specificity. The morpholino structure was devised in order to circumvent some cost-problems associated with other DNA mimics: in fact, MOs can be synthesized starting from cheap ribonucleosides, to which an amine group is introduced via a relatively simple ribose-to-morpholine transformation. Hence, the second step is represented by the oligo assembly of activated subunits into oligos of defined sequence, through coupling and deprotection reactions [24, 36].

MOs have been used as antisense to direct splicing of pre-mRNA [37], blocking of selected gene expression [38] and detecting RNA sequences [39]; in addition, in recent years, an increasing number of works have been published about the use of MOs systems for sensing DNA. In particular, MOs have been frequently employed in the field of surface hybridization technologies, especially in assays performed on solid supports on which immobilized MO probes are able to react

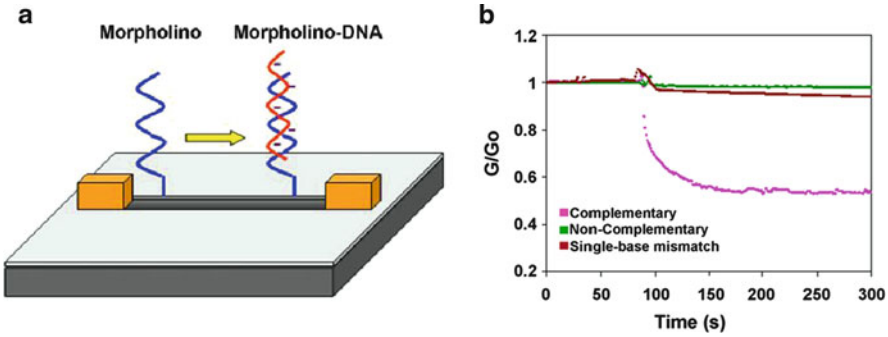

Fig. 4.3 (**a**) Schematic illustration of direct DNA detection using a morpholino capture probe and silicon nanowires (*SiNW*); (**b**) Real-time conductance response for a Morpholino-functionalized SiNW sensor during injection of 1 nM complementary, non-complementary and one-base mismatched DNAs (Reprinted from Ref. [44], Copyright 2010. With permission from Elsevier)

with analyte targets from solution. Kinetic and thermodynamic aspects of surface hybridization in MO-DNA systems have been studied [40, 41]. One example of an application in this field is represented by the work carried out by Tercero et al. [42], based on a label-free analysis of DNA by surface hybridization employing thiolated MO monolayers on a gold surface. Hybridization of uncharged morpholino monolayers with charged nucleic acid targets alters the dielectric and electrostatic characteristics of the surface environment, so that the signatures of hybridization can be monitored through the surface differential capacitance C_d. Based on a 10:1 signal-to-noise criterion, C_d measurements limits of quantification corresponding to several picograms of material per square millimeter were attained.

In another approach, an ultrasensitive electrochemical biosensor employing a MO oligomer as capture probe and a cationic redox polymer as signal generator for direct detection of DNA was proposed, [43] based on the immobilization of the morpholino oligomer on an indium-tin oxide (ITO) electrode and amperometric detection of target DNA by forming a DNA/cationic redox polymer bilayer on the electrode. The cationic redox polymer is introduced to the ITO electrode via electrostatic interaction with the hybridized DNA and it exhibits excellent electrocatalytic activity towards the oxidation of ascorbic acid, allowing direct voltammetric and amperometric detection of DNA.

MO-DNA hybridization was also exploited by Zhang et al. [44] in the achievement of morpholino-functionalized silicon nanowires as novel gene chip platforms for the sequence-specific, label free detection of DNA. In this case MO were covalently attached to functionalized silicon nanowires, and subsequent MO-DNA hybridization on silicon surface was monitored through time-dependent conductance measurements, thus obtaining limits of detection in the femtomolar range (Fig. 4.3).

All these properties make MO promising, though still not largely used, probes for sensing applications, especially in the field of surface optical and electrochemical sensors.

4.2.2.3 Peptide Nucleic Acids (PNA)

Peptide Nucleic Acids (PNAs) are DNA mimics in which the negatively charged sugar-phosphate backbone is replaced by a neutral polyamide backbone composed of N-(2-aminoethyl)glycine units (Fig. 4.2) [45]. They can be considered very promising tools due to their unique properties, since they can bind complementary DNA or RNA sequences following standard Watson-Crick rules [46], but, due to the lack of electrostatic repulsion, PNA/DNA and PNA/RNA complexes have improved thermal stability as compared to DNA/DNA and DNA/RNA duplexes [47]. The high affinity for the DNA target allows to decrease the limit of detection in many applications devoted to DNA recognition. Polypyrimidine PNA can form very stable triplexes with polypurine DNA of the type PNA/DNA/PNA, via both Watson-Crick and Hoogsteen base pairing (Fig. 4.2e). These structures are so stable that dsDNA undergoes displacement of the non-complementary strand, thus forming a D-loop structure. Though limited by sequence restriction, this process allows to directly targeting dsDNA, a goal which is intrinsically difficult with most of the other oligonucleotide probes. The formation of triplex structures is favoured by replacing cytosine with pseuisocytosine (J-base), which can form Hoogsteen hydrogen bonds at higher pHs. Thus, PNA-openers can be obtained (Fig. 4.2f), and their use allows to make one of the two strands available for recognition by a specific probe [49].

Another interesting feature of PNA probes is their selectivity: a single base-pairing mismatch in PNA/DNA duplexes is more destabilizing than in DNA/DNA complexes of the same length [46]. Moreover, since the increased affinity allows to obtain stable PNA-DNA and PNA-RNA complexes even with short PNA probes (10-mer or less), as compared to DNA probes, the possibility to use short sequences in post-PCR applications favours improved selectivity, which in some applications allows for an easy detection of single-point mutations and single nucleotide polymorphisms [50, 51].

PNA-DNA hybridization is less sensitive to the presence of salts, which are necessary to attenuate electrostatic repulsions in duplex DNA. Actually, PNA-DNA binding can be efficiently achieved even under very low salt concentrations, a condition that promotes the destabilization of RNA and DNA secondary structures resulting in an improved access to target sequences [52]. Low ionic strength conditions are essential when targeting a double-stranded DNA, in order to disfavour DNA-DNA duplex formation, and to allow PNA probes to invade the double helix, displacing the homologous DNA strand. The relative independence of their performances to the environment makes the analytical procedures more robust, especially in the case of the analysis of complex matrices such as biological fluids and foods.

Finally, an interesting property of PNAs, which is useful in many biological applications, is their stability to both nucleases and peptidases, since their “unnatural” skeleton prevents recognition by natural enzymes, making them highly persistent in biological fluids [53]. A major drawback is that enzymatic reactions, which are often used in combination with DNA probes, are not possible using PNA substitutes. Therefore, detection schemes involving e.g. DNA-ligase or DNA-polymerases

cannot be performed with PNAs. However, PNAs can easily be functionalized with recognition elements for proteins (such as biotin) and then coupled with enzymatic assays [54]. As a further consequence of their enzymatic and chemical stability, kits and sensory systems produced using PNA probes are superior to DNA-based ones for long-term storage.

For these reasons, PNAs have been used as analytical tools in PCR-base methods and in a wide variety of sensing techniques, including microarrays, electrochemical-, surface plasmon resonance (SPR)-biosensors, FISH, and single molecule detection (see the following paragraphs) [55, 56]. Ultrasensitive detection of DNA could be obtained using DNA capture by PNA and hybridization with Au nanoparticles in surface plasmon resonance imaging (SPRI) [57] with selectivity at the single base-mismatch level. The efficient capture of genomic DNA was accomplished and detection of as low as 11 DNA copies of unamplified genomic DNA from biological samples could be performed [58].

The excellent hybridization properties of PNA oligomers, combined with their unique chemistry, has been exploited in a variety of molecular biology tools, biomedical applications and diagnostic techniques [59]. Custom-made PNA probes are now commercially available, on account of the easily accomplished synthesis by the standard procedures usually adopted in peptide chemistry. The high chemical stability of PNAs allows the use of several different protecting groups for the terminal nitrogen (including Fmoc and Boc) and for the nucleobases (amides, Cbz or acid labile protecting groups), fully exploiting the diverse strategies developed in the last years in peptide synthesis [60].

4.2.2.4 Peptide Nucleic Acids Analogs

The PNAs scaffold has served as a model for the design of new compounds able to perform DNA recognition. Many modifications of the basic PNAs structure have been proposed, in order to improve their performances in term of affinity and specificity towards complementary oligonucleotides. A modification introduced in the PNAs structure can improve their performances as probe in three different ways: (i) improving DNA binding affinity; (ii) improving sequence specificity, in particular for directional preference (antiparallel vs parallel) and mismatch recognition; (iii) improving signal transduction.

Several reviews have covered the literature concerning new chemically modified PNAs [61, 62]. Base modification has been a very powerful tool for improving the DNA recognition process by PNA. One important variation of PNA which has proven to be very useful is the modification of adenine and thymine residues with pseudo-complementary bases 2,6-diaminopurine (Fig. 4.4a) and 2-thiouracil (Fig. 4.4b), which can form base pairs with thymine and adenine respectively, but are not able to bind to each other [63]. Using these bases, double-duplex invasion can be obtained (Fig. 4.4c), which enable to separate the two strands of a dsDNA; if properly designed, this process can be use to generate single-stranded tracts which can be recognized by specific probes or enzymes [48].

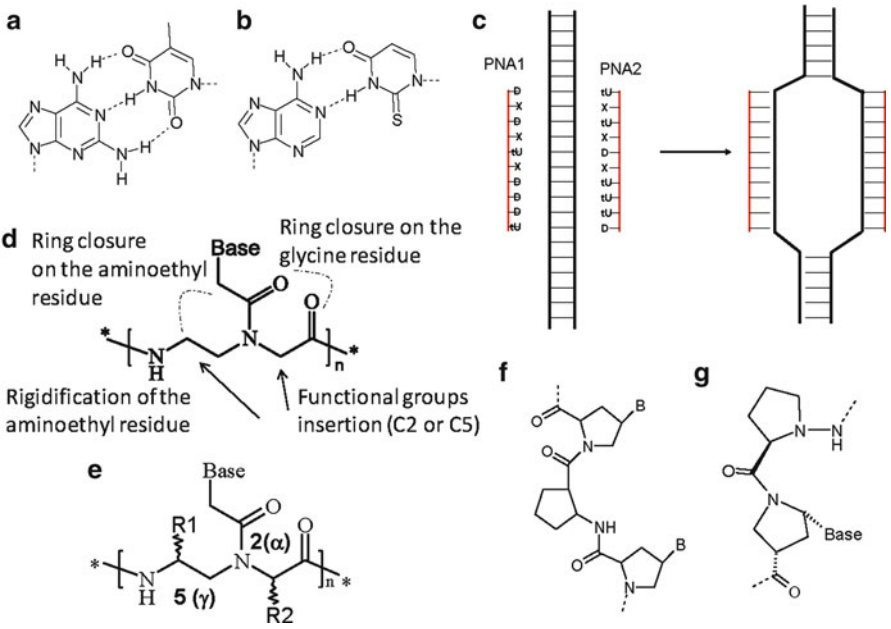

Fig. 4.4 Modified PNA structural variations. (**a**) 2,6-diaminopurine (D) paired with thymine; (**b**) 2-thiouracil (tU) paired with adenine. (**c**) Double duplex invasion process using D and tU containing PNA. (**d**) type of modifications reported in the literature for PNA backbone. (**e**) Chiral acyclic PNA bearing functional groups either on C2, C5, or both. (**f**) prolyl-(ACPC)-PNA. (**g**) pyrrodinyl-PNA

A very large amount of data is available for PNA backbone variation (Fig. 4.4d). Structure-activity relationships showed that the original design containing a 6-atom repeating unit and a 2-atom spacer between backbone and the nucleobase was optimal for DNA recognition [64]. Introduction of different functional groups with different charges/polarity/flexibility have been described and are extensively reviewed elsewhere [65]. PNAs have been constantly improved during the years, using the concept of "preorganization", i.e. the ability to adopt a conformation which is most suitable for DNA binding, thus minimizing the entropy loss of the binding process. The main strategies which have been used for achieving this goal are: (a) preorganization by cyclization of the PNA backbone (in the aminoethyl side or in the glycine side), (b) addition of substituents at C2 or C5 carbon of the monomers which has the effect of shifting the PNA preference towards a right-handed or left-handed helical conformation, according to the configuration of the new stereogenic centers, in turn affecting the stability of the PNA-DNA duplex through a control of the helix handedness. Many of these modifications included the presence of one or more stereogenic centers, and it was thoroughly demonstrated that there is a dramatic effect of chirality on DNA recognition [66, 67].

Using the linear *N*-(2-aminoethyl)glycine as a starting point, several PNA derivatives were obtained by insertion of side chains either at the C2 (α) or

C5 (γ) carbon atoms (Fig. 4.4e). These modifications have the effect of modulating the conformational properties of PNA. If the constraint is appropriate for the conformation required for DNA binding, this can actually result in improved DNA binding properties, whereas if not, a detrimental effect is obtained.

The insertion of stereogenic centers in one of the PNA strands induces a predominant helix handedness [68, 69]; lysine-based chiral PNAs, and in particular the "chiral box" PNA (with three chiral monomers inserted in the middle of the probe sequence) showed an increase of the sequence selectivity, both in terms of direction control and of recognition of a single base mismatch [70]. Therefore, this type of structure seems to be ideal for targeting point mutations in genes with therapeutic interest. For standard achiral decameric PNAs, the difference in melting temperature between antiparallel and parallel complexes is about 10 °C. The insertion of D-lysine side chains in the PNA strand had the effect of amplifying this difference in stability, an effect which is magnified by the above mentioned D-Lys "chiral box" PNA, which was shown to bind the complementary antiparallel-, but not the parallel-DNA, as evaluated by UV and CD melting and by ESI-MS spectra [70, 71]. Mismatch recognition was a property particularly pronounced in the "chiral box" containing PNAs and this was found to be true for different sequences. Using this model of PNA modification it was possible to detect very efficiently the single point mutations W1282X and R553X causing cystic fibrosis with surface plasmon resonance (SPR) biosensors [72] and capillary electrophoresis [73] respectively.

C5-substituted PNA could be used as very convenient scaffolds for linking functional groups to the PNA backbone [74]. In several different publications C5-modified PNA monomers have been used for the introduction of functional groups such as thiols [75] or carboxylates [76]. C5-substituted PNAs bearing small side chains derived from L-alanine and L-serine have strong preference for right-handed helicity, which favors DNA binding [77].

A systematic study performed on PNA containing one chiral monomer derived from D- or L- Lysine revealed that both the D-configuration at the C2 and the L-configuration at the C5-position induce right handedness, whereas the opposite stereochemistry induces a preference for left-handedness and therefore less DNA affinity [78]. Thus, the DNA binding affinity can be modulated from none to very strong by tuning the stereochemistry of a single monomer in a PNA decameric strand. On the base of these results, chiral PNA probes were designed for microarrays for recognizing ApoE genotypes related to the Alzheimer disease [79]. The "chiral box" model was tested using either only 2D or only 5L monomers derived from arginine [80], and an "extended chiral box" PNA containing both 2D and 5L arginine monomers [81].

Cyclic structures have been described with increasing binding affinity and sequence specificity [82]. In particular, excellent properties of DNA binding could be achieved using five-membered ring structures such as prolyl-(ACPC)-PNA (Fig. 4.4f) [83, 84] or pyrrolidinyl-PNA (Fig. 4.4g) [85], which were used in sensory applications. Selectivity for DNA over RNA [86] and for RNA over DNA [87] were also reported using cyclic PNA analogs.

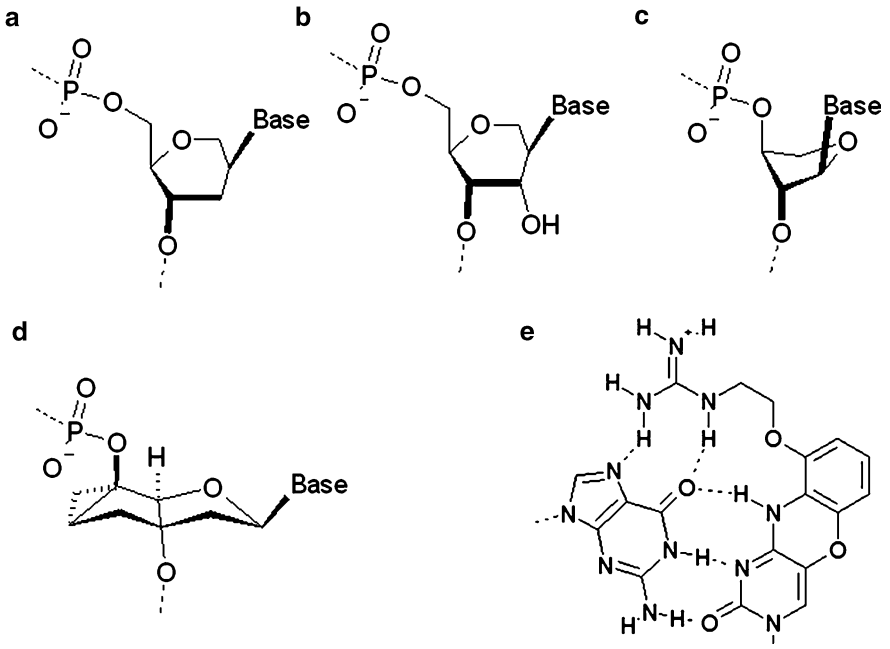

Fig. 4.5 Other DNA analogs and modified bases with remarkable properties. (**a**) Hexitol nucleic acids (*HNA*); (**b**) altritol nucleic acids (*ANA*); (**c**) threose nucleic acids (*TNA*); (**d**) tricyclo-DNA; (**e**) the guanidium G-clamp modified nucleobase, which can improve both binding and selectivity and has been used on ON and on PNA

4.2.2.5 Other Modified Oligonucleotides

Many other oligonucleotide analogs, though not as widely used as the four classes described above, have been described in the literature and tested in antisense technology, with binding affinities and selectivity which could be exploited for diagnostics.

A variety of sugar substitutions have been evaluated by Eschenmoser for understanding ON structural features [88]. Threose nucleic acids (TNA, Fig. 4.5c) are a simplified version of DNA in which the sugar moiety has been reduced to a simple four-atom unit. Despite having a repeat unit one atom shorter than natural nucleic acids, TNA is able to base pair with RNA, DNA and itself [89]; therefore TNA oligos have been proposed as ancestors of DNA in prebiotic chemistry. TNA are very interesting also because they can act as template for DNA synthesis and can be obtained from a DNA template by polymerases [90].

Hexitol nucleic acids (HNA) and altritol nucleic acids (ANA) are DNA mimics with six-membered rings (Fig. 4.5) developed as potential antisense drugs and recently tested as components of arrays for SNP detection [91].

Tricyclo-DNA are constrained oligonucleotide analogs which have been successfully used in antisense research to redirect pre-mRNA splicing

ONs and ON analogs containing modified nucleobases have also been a subject of intense research. Most notably, the introduction of "engineered" nucleobases led to an increase of both affinity and selectivity in antisense research. Nucleobases with extended aromatic cores have been shown to improve stacking interactions, thus stabilizing the double strand formed with complementary oligonucleotides. Combination of base stacking, properly designed hydrogen bonds, and electrostatic interactions led to very interesting structures, such as the G-clamp residue, able to bind strongly and selectively to guanine by simultaneous occurrence of all the above mentioned interactions (Fig. 4.5e) [92].

The research on nucleobase modification is centrally related to sequence selectivity and affinity. Benner and coworkers have developed a whole set of nucleobase-substitutes, expanding the natural genetic code, called artificially expanded genetic information system (AEGIS) that has as many as 12 nucleotides and as many as 6 base pairs. AEGIS nucleotides are already being applied as orthogonal binding elements in human diagnostic tools, including in FDA-approved assays for HIV, hepatitis B, and hepatitis C viruses. Kool and coworkers have introduced extended bases as tools for nucleic acid recognition [93].

Specific nucleobase substitutes are very powerful tools, and fine tuning of specificity can be obtained, as demonstrated by the design of special nucleobases allowing the detection of specific oxidative lesions such as 8-oxo-guanine [94].

Substitution of the nucleobase with aromatic rings has the effect of abolishing base specificity, thus allowing a particular position to act as an universal base, which can be useful in some applications. If the aromatic moiety is fluorescent, a change in fluorescence can be obtained upon forced intercalation, thus leading to a direct read-out of the recognition process [95].

Not all the above reported variations in the chemical structure of oligonucleotides have been so far used in diagnostics. However, the toolbox for development of new, more efficient and specific sensory systems is very rich and it is continuously improving.

4.3 Chemistry and Architectures of Probes in DNA Sensing

The design of probes for a specific sensing application cannot be done without considering the type of modifications and chemistry needed for the conjugation with reporting elements, sensor surfaces and nanostructures. Furthermore, ONs or ON analogs are often used in complex molecular architectures, where the design of the probe requires the presence of special recognition elements.

4.3.1 Chemistry for Bioconjugation and Probe Immobilization

The oligonucleotides and their analogs in most cases have to be linked either to one or more reporting units or to a surface. This is normally achieved using

an appropriate spacer and appropriate chemistry which is compatible with the oligonucleotide and with the groups to be linked.

Bioconjugation of oligonucleotides and their analogs is carried out with essentially three different strategies: (i) use of conjugated monomers bearing functional groups (fluorophores, electroactive moieties etc.) either on the backbone or on the nucleobase; (ii) modification during oligomer synthesis, (iii) post-synthetic conjugation using specific reaction schemes, in particular click chemistry [96]. Many types of fluorophores have been linked to oligonucleotide backbone, mainly through 2′-modification of a ribose unit. Variations of the LNA structure with the introduction of a nitrogen atom also allow to introduce substituents which can carry active reporting moieties. Peptide nucleic acids derivatives are very versatile structures for this goal, since the introduction of functional groups can be achieved through modification at C2 or C5 positions of the backbone.

Nucleobase modification is most easily obtained by modification of C5 or C6 of pyrimidines and C2 of adenine, though other substitutions have been reported.

Redox-active functional groups, e.g. pyrene, phenothiazine, porphyrin, Ru(II)$(bpy)_3$ or Os(II)$(bpy)_3$ complexes, dimethylaminonaphthalene, TAMRA, glycosides have been introduced using this strategy [97]

Several chemistries are available for linking probes to a solid surface, and many of them have been developed in the field of microarrays fabrication [98]. General strategies are for example: adsorption, covalent linking, and non-covalent interactions (mainly biotin-streptavidin). Adsorption by electrostatic interactions is one of the methods used, since DNA can bind to positively charged matrices, such as chitosan, or charged polymers such as poly(allylamine)-hydrochloride (PAAH)/sodium poly(styrenesulfonate) (PSS)/PAAH films. Adsorption on carbon and electroadsorption can also be performed using DNA or other probes. Covalent linking is best obtained using high-yield reactions forming stable bonds. Some of the most used reactions are reported in Fig. 4.6.

Thiol-modified probes can be linked to gold surfaces directly (Fig. 4.6a). On the contrary, maleimide-modified probes can react with thiols by conjugated addition (Fig. 4.6b).

Isothiocyanate and N-hydroxysuccinimidyl active ester groups can easily be coupled with nucleophilic groups, especially amines (Fig. 4.6c, d). Aldehyde moieties give rise to Schiff bases and oximes, the latter being more robust chemically. Reduction of Schiff bases to amines is a good way to increase the stability of biointerphases (Fig. 4.6e) [99]. Again click chemistry can efficiently be used in this context. Azido and alkyne groups can be used to form new bonds via copper catalyzed Huisgens 1,3-cycloaddition.

Linkage of LNA is essentially the same as oligonucleotides, due to similarity in their chemical structure. PNAs have been immobilized using Huisgen cycloaddition between azide surfaces and acetylene-carrying PNA [100], thiolated PNAs on gold or maleimide surfaces, [101–103] amino-capped PNAs on isothiocyanate, [104] succinimidyl ester, or aldehyde surfaces [99], and on surfaces partially prefunctionalized with cDNA [105].

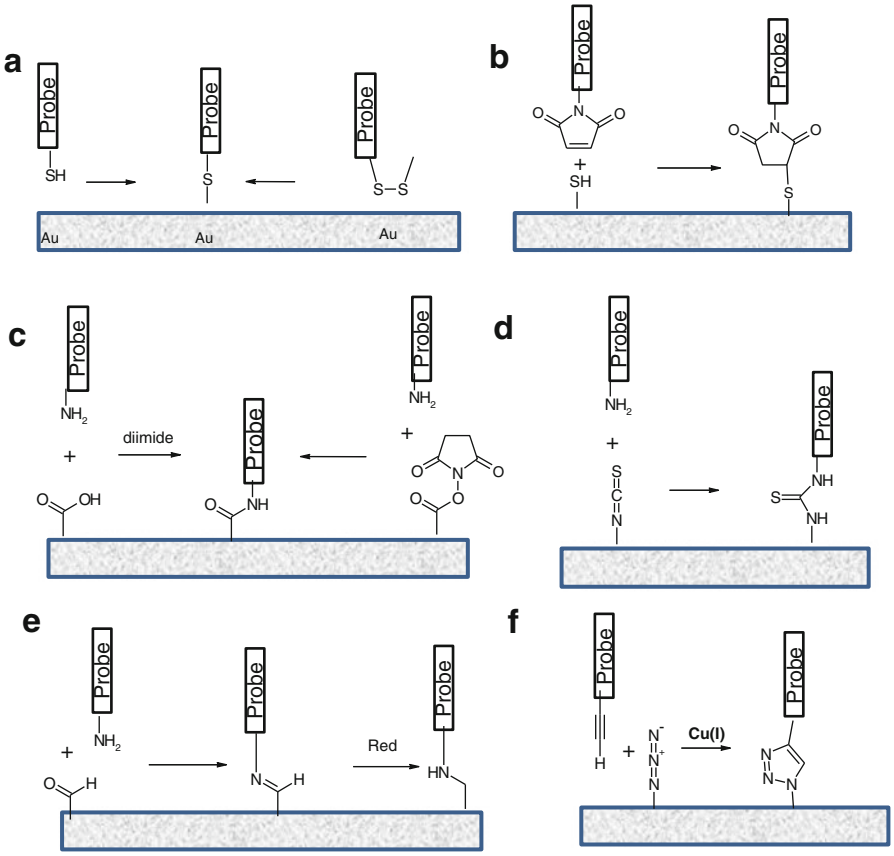

Fig. 4.6 Main chemistries used for probe deposition. (**a**) Thiols linked to gold surfaces; (**b**) thiolated residues on the surface reacted with maleimide-conjugated probe; (**c**) amide formation from carboxylic acid and a condensing agent (*left*) or from N-hydroxysuccinimidyl ester (*right*); (**d**) reaction of an amine-modified probe with a isothiocyanate group on the surface; (**e**) reaction of amine-modified probe with aldehyde residues forming a Schiff base (*center*) which can then be reduced using borohydride derivatives to a more stable amine group; (**f**) an example of click chemistry with alkyne-modified probe and azide-modified surface by a copper(I) catalyzed Huisgen cycloaddition

MOs modified with terminal amino groups have been coupled to sensor surfaces using reaction with aldehyde groups.

The same type of chemistries could be used for the covalent linking of ON analogs to nanostructures such as nanoparticles, nanorods, and quantum dots. However, in many cases, non covalent affinity binding can be used [106]. Adsorption of streptavidine on surfaces allows to use biotinylated probes, which can then be directly linked to the surface thanks to the very high stability constant of the biotin-streptavidin complex ($K = 10^{15}$).

4.3.2 *Fluorescent Probes for In Situ and Single Molecule Detection*

The most direct method for visualizing the presence of a specific sequence within a sample of interest is to hybridize its genomic DNA with fluorescently labeled probes and directly observing the signal obtained after removal of the excess of unbound probe. This is particularly interesting in the observation of tissues and single cells and is the basis of *in situ* hybridization techniques. Since targets can be very diluted in these analysis, it is not surprising that either long ON probes or ON analogs showing very high DNA affinity, such as PNA, have been used in these applications.

Fluorescence in situ hybridization (FISH) is a technique that uses fluorescent probes able to bind to only those parts of the sequence target with which they show a high degree of sequence complementarity. Fluorescence microscopy can subsequently be used for detection, after washing of unhybridized probes [107]; high-sensitivity detection, simultaneous assay of multiple species and automated data collection and analysis have advanced this field significantly [108]. Thus, FISH has become a key investigation procedure in the analysis of nucleic acids, with a significant impact on diagnostics, therapeutics and biomedical research in general. In particular, last years have seen important efforts in developing novel approaches in ultrasensitive detection of DNA sequence targets. For example, some research groups exploited the possibility of using PNA probes in order to realize PNA-FISH systems, taking advantage from PNA compatibility with a wide range of fluorochromes and its capacity to hybridize with high affinity and specificity to complementary sequences. In such a context, Agerholm et al. [108] proposed sequential FISH analyses of human blastomeres using PNA probes. After fixation of the blastomeres on poly-L-lysine-coated glass slides, FISH was carried out using labeled PNA probes and then monitoring the signal by fluorescence microscopy, pointing out the possibility to make up to five sequential FISH cycles without loss of signal. An analogue system based on PNA-FISH was also proposed for the analysis of human oocytes, polar bodies and preimplantation embryos [109].

PNA probes targeting specific rRNA sequences of yeast and bacteria with clinical, environmental, and industrial value have recently been developed and applied to a variety of rapid assay formats [110].

In a different approach, FISH can be combined with flow cytometry, in the so-called Flow-FISH technique. Flow cytometry consists in a procedure for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection apparatus: in Flow-FISH, this approach is combined with the use of labeled probes in order to collect data in fluorescence spectroscopy through the probe-target hybridization. In particular, many works have been published about the use of labeled PNA probes in such a procedure, as reported, for example, by Brind'Amour et al. [111] who exploited the use of a PNA-based Flow-FISH in the analysis of repetitive DNA in chromosome and by Baerloche et al. [112] who reported a similar protocol to measure the average length of telomere repeats in cells.

Recently, Robertson et al. [113] suggested the use of LNA-modified oligonucleotide probes, instead of the typical PNA probes, describing a method based on flow–FISH for the specific, high-sensitivity, low-background detection of mRNA, emphasizing the applicability of LNA-FISH techniques in detecting target sequences.

Alternatively to Flow-FISH and traditional procedures performed after fixation of the sample on a slide, FISH can also be carried out in suspension (S-FISH), with the sample placed on a polished concave slide just as the final step of the procedure [114]. For example, Vandewoestyne et al. [115] made use of S-FISH combined with automatic detection and laser capture microdissection (LCM) for profiling of male cells in male/female mixtures. In another approach, FISH can be performed exploiting the properties of quantum dots (QDs) [116]. In particular, various method for direct QD labeling of oligonucleotide probes have been developed, in order to make possible FISH approaches, for example, in detection of breast-cancer cells, in which nanocrystal fluorophores were significantly brighter and more photostable than some organic fluorophores [117]. QDs have also been used as FISH labels for a Y-chromosome-specific sequence in human-sperm cells [118] and for the construction of QD-labeled DNA probes for detecting specific sequences in *E.coli* plasmids [119].

Finally, it is worth to say that FISH can also be performed through another protocol, the so-called Fiber-FISH, which allows high resolution mapping chromatine or DNA genes and chromosomal regions on fibers. In this way it is possible to obtain high resolution mappings from some hundreds to the limit of 1 kb [120, 121].

Single molecule fluorescence spectroscopy (SMFS) can be performed using highly localised light excitation of fluorophores, which can be obtained by confocal microscopy with very high numerical aperture (NA), by total internal reflection microscopy (TIRM) or by zero-mode waveguides (ZMW). SMFS allows to measure not only fluorescence intensity but also other parameters connected with fluorescence. These techniques have been reviewed recently and an excellent recent paper by Ranasinghe and Brown describes apparatus, materials and strategies for DNA analysis by single molecule detection [122].

One problem of these techniques is that using very small volumes for detection, relatively high concentrations are needed in order to have even a single molecule in the measuring volume, and high measuring times are required in order to sample a sufficient number of molecules for statistically significant results. The combination of these measurement with microfluidics and recycling of the solutions can greatly reduce the time required and lower the detection limits; recirculation of the sample through a nL chamber and micro-evaporation led to detection of ssDNA at initial concentrations as low as 50 aM in an acquisition time of 100 s after a preconcentration step [123].

In order to avoid read-out of fluorescent probes, single molecule two-colour coincidence detection (TCCD), in which two fluorophores are simultaneously detected as a function of time and only coincident burst are counted [124]. Ultrasensitive detection of oligonucleotides with 50 fM limit of detection even in the presence of 1,000 fold excess of unrelated DNA was achieved using two color (Rhodamine green and Alexa Fluor 647) single molecule fluorescence coincidence detection

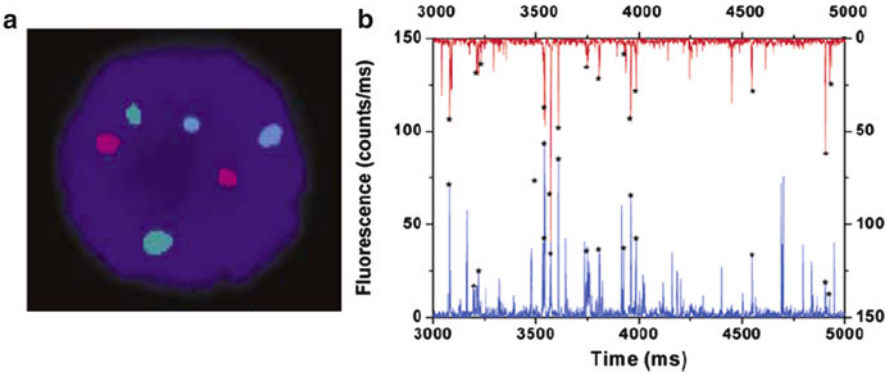

Fig. 4.7 (**a**) Result of the PNA-FISH technique applied to a male blastomere. Three different types of chromosomes are colored in *red, green* and *blue* by using specific PNA probes labeled with different fluorophores (Ref. [108], by permission of Oxford University Press) (**b**) Fluorescence bursts (expansion between 3,000 and 5,000 ms) on both the *blue*- and *red*-excited channels for a 50 pM solution of dual-labeled 40-base duplex DNA. Coincident bursts on both channels are marked with an *asterisk* (Reprinted with permission from Ref. [125]. Copyright 2001 American Chemical Society)

[125]. Hybridization detection was achieved by the simultaneous presence of signal burst in both channels (Fig. 4.7b), which is unlikely to occur by coincidence in very dilute solution (single molecule regime) for unpaired ON. In this case the target DNA was labeled, but the most convenient way for detection of non-fluorescent nucleic acids targets is to use of two fluorescent probes which co-localize upon hybridization to the same unlabelled target molecule, or give rise to FRET effect. Castro and Williams first described this approach for amplification-free detection of genomic DNA, using two 15mer peptide nucleic acid (PNA) probes [126, 127]. This procedure was then extended to the detection of cDNA produced by reverse transcription [128], or to mRNA [129].

In order to remove background fluorescence, an excess of quencher-labelled oligonucleotides can be used, as shown in the detection of microRNA (miR) using LNA/DNA mixmers [130].

Other properties can be used to distinguish between free and bound probes, as recently demonstrated by Hook and coworkers in the detection of unlabelled DNA using residence time of oligonucleotide-modified unilammelar vesicles in a sandwich-type assay with TIR fluorescence microscopy (TIRFM) [131].

These advanced fluorescent-based techniques become more efficient when using self-reporting probes described in the next paragraph [132, 133].

4.3.3 Self-Reporting Probes

Molecular beacons (MBs) are single-stranded oligonucleotide probes that contain a loop-and-stem structure [134]. The loop portion of the beacon contains the

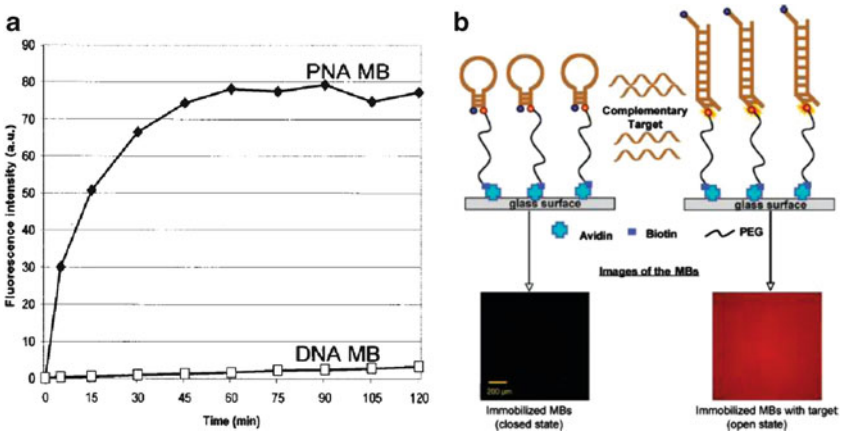

Fig. 4.8 (**a**) Example of a PNA molecular beacon (*PNA MB*) used in flow cytometry for the detection of E. Coli, in comparison with a DNA beacon (*DNA MB*) of the same size (Reprinted with permission from Ref. [136]. Copyright 2003 American Society for Microbiology). (**b**) Schematic illustration of the LNA-MBs-based technique described by Martinez et al. When the complementary target binds to the beacon, the fluorophore is separated from the quencher and its fluorescence is restored (Reprinted with permission from Ref. [33]. Copyright 2009 American Chemical Society)

sequence complementary to a target (usually 15–30 bases), whereas the two stems are complementary to each other (5–7 bases). A quencher is covalently attached at the end of one stem, while a fluorophore is covalently attached to the end of the other. DABCYL and the more effective black hole quencher (BHQ) can be used, but also gold nano-particles and gold surfaces have been successfully applied. Because the fluorophore and quencher are in close proximity, MBs do not fluoresce until hybridization with the target nucleic acid sequence. In the presence of a target, the hairpin structure of MBs undergoes conformational changes that force the fluorophore and quencher moieties to separate, thus restoring fluorescence intensity. The use and design of MB probes in optical detection of DNA are described in details in another chapter of this book [135], and only few relevant examples involving modified DNA structures will be discussed here.

PNA beacons were described by several groups using a stemless design, in order to avoid excessive stability of the stem tract, since PNA:PNA duplexes are far more stable than DNA:DNA ones. Frank-Kamenetskii and co-workers described the performances of these beacons and compared them with ON beacons, showing improved performances. PNA beacons were used in FISH, flow cytometry [136] (Fig. 4.8a) and HPLC, [137] to generate specific signals.

Martinez et al. [33] recently experimented LNA-based beacons attached on a glass surface, obtaining detection limits for the DNA sequence targets in the low nanomolar range.

Smart probes use guanine as quencher and therefore incorporation of a single fluorophoric unit is necessary in the synthetic process [138]. Other switching mechanism can be used, in particular, fluorescence resonance energy transfer

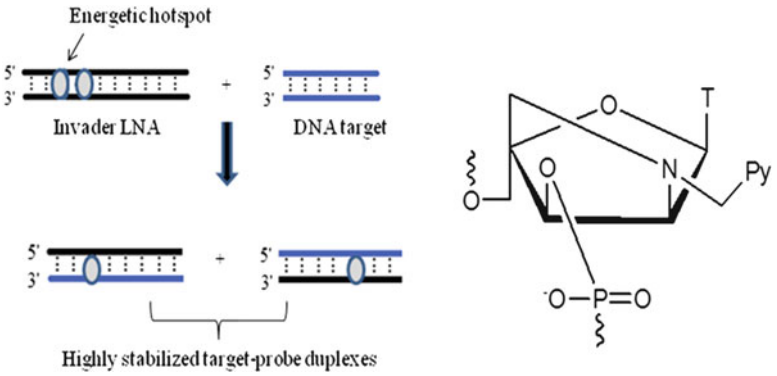

Fig. 4.9 On the *left*, a schematic illustration of the formation of the probe-target duplexes; the term "energetic hotspot" refers to the particular zipper arrangement of the probe. On the *right*, 2′-N-(pyren-1-yl)methyl-2′-amino-α-L-LNA used as intercalator-modified-LNA monomer

(FRET), excimer formation, and forced intercalation [139], and DNA-templated reactions can generate a fluorescent signal [140].

Pyrene units are well known for their tendency to form excimers, i.e. excited state dimers, which emit light to longer wavelength than the isolated fluorophore, and the pyrene unit has been extensively used in the design of ON derivatives [141].

In this context, Sau et al. [31] recently proposed *invader* LNA probes, i.e. DNA duplexes with *"interstrand zipper arrangements"* of intercalator-functionalized 2′-amino-α-L-LNA monomers, demonstrating their ability to recognize short sequence dsDNA targets, precisely directing the appendend intercalators into the core of DNA duplexes. These invader LNA probes take advantage of the "nearest neighbour exclusion principle", i.e. intercalating units of invader LNA monomers are poorly accomodated in probe duplexes but extremely well tolerated in probe-target duplexes, whose formation is thus favored by thermodynamic reasons. The detection of probe-target duplexes was eventually carried out by steady-state fluorescence spectroscopy, thanks to the pyrene substituents used as intercalators. Infact, during the recognition of the dsDNA targets, the pyrene moieties of the probes are forced apart, causing a decay of the intensity of the excimer signals at their typical emission wavelength (Fig. 4.9).

Intercalating probes in which a fluorophore is either switched on or off by interaction of the oligonucleotide with the DNA target can also be used. Thiazole orange is particularly suited, since its fluorescent properties change dramatically upon interaction with nucleic acids [142]. PNA probes bearing thiazole orange tethered at their end have been described by Kubista et al. and proven to be very efficient: these probes are commercialized as Light-up probes [143].

Reacting probes have been described, using DNA template reactions to generate a fluorescent signal [140]. These probes have the advantage to accumulate signal during time, if the reaction is designed in order to have catalytic activity by DNA or RNA, but of course is not reversible and is better suited for in vivo studies or FISH than for sensor technologies [144].

4.3.4 Surface and Nanostructure Architectures Using Advanced Probes

Probes must be designed also according to the devices used in sensory systems, and appropriate linking group and linking strategies has to be considered. Thus a brief description of most common sensor architecture is provided in this paragraph.

The principal types of formats used for sensor development rely on surface chemistry and architectures, and are depicted in Fig. 4.10. A sensing surface can act as simple support (as in the case of microarrays) or as an active sensing element (which is the case in most sensory systems). Probes can be adsorbed on the surface, and this approach has been sometimes used on glass surfaces, especially in the field of optical fibers. Adsorbtion of streptavidine allows to obtain activated surfaces which can be linked to biotinylated probes with non-covalent, yet very stable, bonds. If the probes are chemically attached on the surface, the latter must be suitable for linking of specific functional groups or must be activated by introduction of appropriate functional groups. Probes can be directly linked to the surface, but normally a 15–25 atom spacer should be added to the tail of the probe in order to allow sufficient space for hybridization, as tested for PNA microarrays [145]. Direct linking is necessary when the direct readout of the hybridization is necessary, such as in carbon nanotube electrochemical devices allowing the detection of single molecule target DNA [146]. In order to avoid probe overcrowding, dilute solutions are used for deposition, and normally the empty space on the surface is filled with a diluter molecule, thus forming a membrane-like monolayer (Fig 4.10a). 2D architectures can be obtained by covering a self assembled monolayer (SAM) with a second layer bearing the probes, thus obtaining a spatially controlled disposition in which the spacing between the surface and the probes can be controlled at the nanometer scale, thus allowing complete prediction of the physical properties of the surface assembly (Fig. 4.10b). A typical example of this approach is described by Knoll and coworkers in another chapter of this book using biotinylated thiols and a second layer of streptavidin molecules, which in turn are linked to biotinylated probes [147]. In alternative, a binding matrix bearing suitable functional groups can be linked to the surface, and probes can be attached to this matrix. This approach allows to have 3D instead of 2D architectures, in which the number of captured targets is enhanced without overcrowding (Fig. 4.10c). Polymers and polysaccharides (e.g. dextran, chitosan) can be used as surface layers. These matrices should be highly hydrophilic in order to be permeable to aqueous phase in which the target DNA molecules are dissolved. Hydrogels are especially suited as 3D matrices, since they are able to swell and shrink by change of the solvent, with a concentration effect which can enhance sensitivity considerably [148].

Gold surfaces are particularly suitable as sensor surface not only for their optical or electrochemical properties, but also for their ability to bind thiolated molecules. Thiolated probes can be deposited, followed by deposition of a short alkanethiol such as mercaptohexanol (MCH) which can prevent non-specific Au–DNA interaction. The introduction of a third “backfiller” bearing a carboxylic acid group has been claimed to greatly enhance the performances of sensors [149].

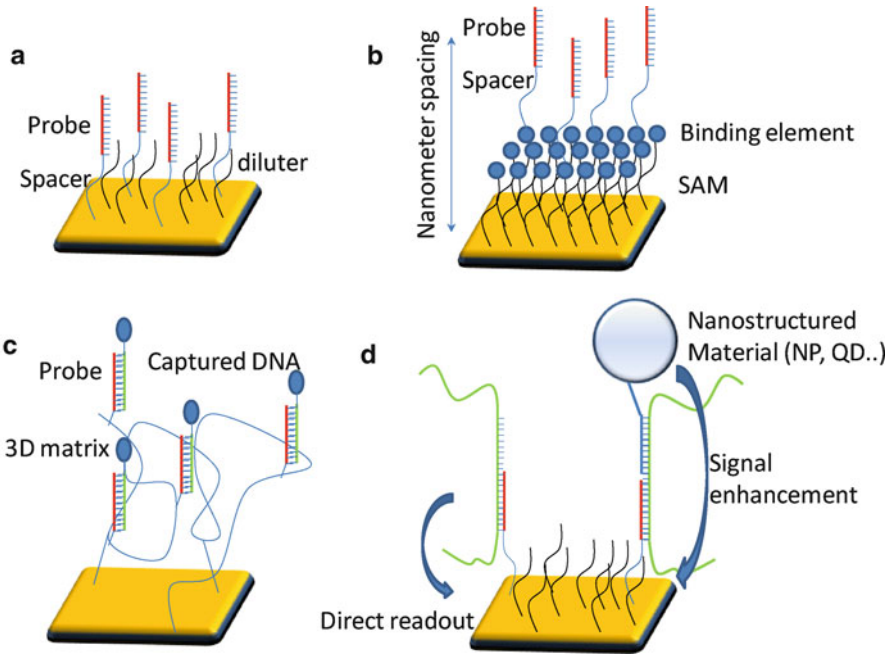

Fig. 4.10 Schematic representation of surface architectures used in sensor technology. (**a**) Spacer-bearing probes directly linked to the surface with a diluter molecule; (**b**) Probes attached to a functional element at the end of a self-assembled monolayer (*SAM*); (**c**) probes linked to a 3D matrix enabling higher loading; (**d**) two examples of read-out: on the *left* the recognition element is directly read, on the *right* a sandwich-type assay in which the captured DNA is bound to a second oligonucleotide linked to a nanostructure for signal amplification

Glass surfaces can be modified using trialkoxysilanes with suitable amino or thiol groups, and the activated surface is then reacted with bifunctional molecules to which the probes can be attached. Silicon surfaces are first oxidized to produce silanolic groups and then modified as silica surfaces. Carbon-based layers, such as carbon nanotubes and graphene can be obtained with appropriate (i.e. carboxylic) groups by oxidation of the material itself or by transformation of precursor materials (e.g. chemically reduced graphene oxide or electrochemically reduced graphene oxide) [150].

Paper and polymeric materials are also interesting surfaces, since the development of low cost sensing devices can be envisaged, [151] and many chemical methods allow proper derivatization of these materials.

Finally, the readout process can be direct or the target molecule can bind polymeric structures or probe-labeled nanostructures (nanoparticles, quantum dots, dendrimers, unilamellar vesicles, SWCN, etc.), in a sandwich-type assay, which also can employ the specific probes above reported as a second recognition element (Fig. 4.10d). These decoration strategies will be described in other chapters of this book [10, 152].

4.4 Currently Used Probes in Ultrasensitive Techniques and PCR-Free Detection of Genomic DNA

Although the above reported examples of probes and probe-surface architectures have been described for oligonucleotide mimicking the DNA target even at very low copy number, or single molecule, only a few works have been published showing actual genomic DNA to demonstrate the use of the techniques for direct detection without amplification. One of the reason for this is the difficulty to target double stranded DNA by displacing one of the two strands, though strategies for overcome this problem have been developed, several examples reported in the text together with further examples are reported in Table 4.1 [153–161] for DNA probes and in Table 4.2 [162–164] for other probes.

DNA molecules have been the working horse for the development of most genosensor technologies and thus the majority of methods rely on these type of probes. The possibility to have enzymatic degradation of DNA:RNA hybrids have been the bases of a very efficient sensory system based on depletion of surface RNA probes, as described by Corn and coworkers [162]. Artificial probes cannot be easily used in combination with enzymatic reactions.

Table 4.1 Selected examples of unamplified genomic DNA/RNA detection using DNA probes

Probe	Method	Limit of detection	Ref.
DNA	FISH	See references	[107] and references therein
DNA	Single molecule	See references	[122–125, 131–133]
DNA	Piezoelectric sensing	40,000 copies	[153]
DNA	Fluorescence	20 copies, 3 zM	[154]
DNA	QCM	50 copies 0.17 aM	[155]
DNA	Total internal reflection ellipsometry	10-1,000 μg/mL for genomic 1 fg/mL for single gene	[156]
DNA	Gold nanoparticle-based detection By evanescent wave light	7.5×10^7 target copies	[157]
DNA	Colorimetric scatter of gold nanoparticle probes	66 pg/μL, 20,000 copies (33 zmoles)	[158]
DNA	Colorimetric scatter of gold nanoparticles	150,000 copies (500 ng of total genomic DNA)	[159]
DNA	Colorimetric scatter of gold nanoparticle probe	2.9 amoles 0.5 μg of unamplified RNA	[160]
DNA	Nanoparticle-amplified SPR imaging-polyadenylation reactions	10 fM (5 amoles)	[161]

Table 4.2 Selected examples of unamplified genomic DNA/RNA detection using other probes

Probe	Method	Limit of detection	Ref.
RNA	SPR imaging with ablation of the probes by RNase H	1 fM (oligonucleotides) 7 fM (genomic) or 5.8 μg/mL	[162]
LNA	Flow-FISH	n.r.	[113]
LNA	Single-molecule detection	500 fM	[130]
PNA	QCM with RecA enhancement	8.6 pg/L	[163]
PNA	Single-molecule detection	Minimum 1,200 molecules See references	[126–129]
PNA	Surface acoustic wave	1.21 pg/L	[164]
PNA	SPR imaging with nanoparticles	41 zM unamplified DNA	[57, 58]
PNA	FISH	See references	[108–111]
PNA	Enzymatic with colorimetric detection	10 zmole	[54]

n.r not reported

However, in sensory systems, the use of modified oligonucleotides has been increasingly important and some of these has been proved to be suitable for unamplified DNA detection. PNAs and LNAs are good candidates for this type of strategy, and this justifies the appearance of more and more examples of methods using these probes instead of DNA. MOs have also been demonstrated to be efficient probes, though their use has not be reported so far for real genomic DNA detection.

4.5 Conclusion

Among the factors which allowed ever improving performances of sensing techniques, the use of chemically modified and properly modified probes is of major importance. Though most of the works reported in early studies took advantage of the availability of oligonucleotide probes, recent works more and more rely on improved probe structures. The combination of advanced techniques and highly selective molecular recognition events, such as those obtained by PNA, LNA and MO, will probably allow DNA sensing techniques to surpass the required threshold for becoming widespread large-scale items, thus enabling DNA detection to be used in simple, affordable, and delocalized analysis for personalized and point-of-care diagnostics, even in limited resources areas.

The quest for even higher affinities and specificity by chemically modification of probes is still open and the so far obtained structures have still not be fully explored in diagnostics. Thus, research in this field is one of the most interesting challenges for application of our ability to synthesize bio-inspired biomaterials.

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Chapter 5
Charged Conjugated Polymers

Fengting Lv and Shu Wang

Abstract Signal amplification property and versatility in molecular design of conjugated polymers make them attractive as signal transduction materials. Cationic conjugated polymers (CCP) have demonstrated great potential as DNA-sensing materials since electrostatic interactions between CCP and negatively charged DNA have been well developed to modify optical properties and thereby detect hybridization states. In this chapter, we focus on the state-of-art of CCP-based non-amplified genomic DNA detection in homogenous solution as well as on microarray format. Representative applications of poly(thiophene)-based DNA sensors which display colorimetric output due to conformational changes, and poly(fluorene)-based DNA sensors which utilize CCP as energy donors are highlighted. The developments of amplified single nucleotide polymorphisms and DNA methylation detection employing CCP as sensory signal are briefly discussed. Integrated signal amplification effect of CCP with high-throughput DNA microarray format will yield superior platform for genomic DNA detection.

5.1 Introduction

Simple, sensitive, and specific chemical and biological sensing is becoming ever more dependent upon the development of novel materials. Polymers, quantum dots, crystals, and nanostructures have made a profound impact and have endowed modern sensory systems with superior performance. Conjugated polymers (CPs), characterized by a backbone with a delocalized electronic structure, have emerged as one of the most important classes of transduction materials. For the past three decades, conjugated polymers have attracted much attention as an active component

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for their potential applications such as light-emitting diodes (LEDs), photovoltaic cells, field effect transistors (FETs), and chemical and biological sensors [1]. The chemical structures of conjugated polymers offer several advantages as the responsive basis for chemical and biological detection schemes based on optical methods. The conjugated units in close proximity of the polymer backbone allows for efficient electronic coupling and therefore fast intra- and interchain energy transfer, which indicated that an environmental change at a single site can affect the properties of the collective system, producing large signal amplification [2–11]. Trace detection of analytes including ions, biomacromolecules, and explosives have been successfully accomplished by utilizing this amplification mechanism [12–22]. A wide range of conjugated polymers, for example, poly(thiophene)s (PT), poly-(*p*-phenylenevinylene)s (PPV), poly(*p*-phenyleneethynylene)s (PPE), and poly(fluorene)s (PF) have been reported in the literature as promising materials for these applications.

Because the interchain electron transfer interactions of conjugated polymers are relatively strong compared with the van der Waals and hydrogen bonding interchain interactions, conjugated polymers tend to be insoluble. Water solubility, a prerequisite for interrogating biological substrates, was typically achieved by incorporating charged pendant groups or polar groups onto the CP backbone. The introduction of various substitutions along the conjugated backbone can not only improve the processability of these aromatic polymers but can also modify their physical properties, which can lead to physical phenomena that are not found in the parent unsubstituted polymers. Conjugated polymers bearing ionic pendant groups are also called conjugated polyelectrolytes (CPEs) and these water-soluble materials combine the properties of polyelectrolytes, which coordinate electrostatic forces with oppositely charged analytes, with the attractive optical and electronic properties of conjugated polymers [6]. Conjugated polyelectrolytes have demonstrated great potential as biosensory materials, especially in DNA-sensing applications, which are driven by the formation of stable aggregates with cationic conjugated polymer through the strong electrostatic interactions. The primary advantage of this strategy is that it relies upon noncovalent interactions and therefore does not require covalent attaching of single-stranded DNA (ss-DNA) probe onto conjugated polymer backbone.

Reliable and sensitive DNA detection is of vast scientific and economic importance, and novel techniques and materials that determine DNA hybridization and sequence characterization are under intense investigation [23–26] for applications, such as medical diagnostics, identification of genetic mutations, gene delivery monitoring, and specific genomic techniques. In recent years, much effort has been devoted to improve the selectivity, sensitivity, and miniaturization to allow detection in small sample volumes. Gene chips and DNA microarrays which perform high-throughput screening of hundreds to thousands of targets in a single experiment are dominating the market as one of the most powerful fluorescent DNA detection techniques [27–29]. However, most available methods involve polymerase chain reaction (PCR) protocols to increase the concentration of specific nucleic acid sequences, which tend to complicate the procedures and therefore prolong the

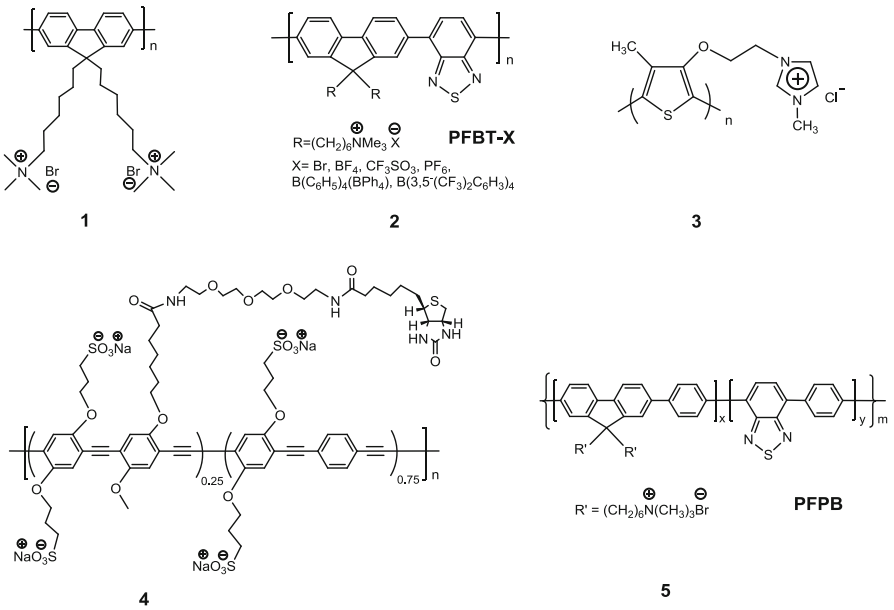

Fig. 5.1 The molecular structures of typical water-soluble CPEs

overall detection time. In response to this limitation, signal-amplification systems constructed by water-soluble cationic conjugated polyelectrolytes have been developed by taking advantage of their unique optical properties. The molecular structures of typical water-soluble CPEs are shown in Fig. 5.1.

The purpose of this review is to highlight some of the fundamental properties of cationic conjugated polymers and their remarkable applications in optical non amplified DNA sensory processes. Since there has been great progress made in the detection of single nucleotide polymorphisms (SNP) and DNA methylation with PCR-associated target preparation utilizing cationic conjugated polymers as sensory signal, all these developments are also addressed.

5.2 Synthesis, Optical Properties, and Sensing Mechanisms

5.2.1 Synthesis

The opportunity to synthesize new conducting polymers with improved and desired properties began to attract the attention of synthetic chemists in the 1980s. In this review, typical synthetic approaches of some classical water soluble conjugated polymers are provided. The reader interested in the exhaustive list of published procedures should refer to the literature [5, 14, 30].

Poly(thiophene)s with ionic side chains have been widely used for the specific detection of DNA sequences which relies upon conformational changes of the conjugated polymer backbone upon ssDNA hybridization with complementary ssDNA. The synthesis of water soluble poly(thiophene) was initiated by Wudl group in 1987, where the corresponding methyl sulfonate monomers were electropolymerized first and then converted into the sodium salts [31]. Recently, the Leclerc [32] and Inganäs [33] groups have also developed new water soluble poly(thiophene) derivatives, which were prepared from an oxidation polymerization of monomers in chloroform with $FeCl_3$ as the oxidizing agent. Figure 5.2a illustrates the synthesis of poly[3-(3′-N,N,N-triethylamino-1′-propyloxy)-4-methyl-2,5-thiophene hydrochloride] (PMNT) [32].

By virtue of high fluorescence quantum yields in water, PPE and their derivatives become one of the most studied classes of conjugated polymers in fluorescent biosensor. The Sonagashira method is widely employed to prepare high molecular weight PPEs by coupling a dihaloarene and a diethynyl-arene in a DMF/water mixture with $Pd(PPh_3)_4$/CuI as catalyst [34]. The overall synthetic route of PPEs is illustrated in Fig. 5.2b. The groups of Schanze, Swager and Bunz have synthesized a series of water soluble PPEs employing the similar method [4, 35, 36].

Wudl [37] and coworkers reported the synthesis of water soluble PPV by using Wessling polymerization procedure. As depicted in Fig. 5.2c, the monomer was first polymerized in the presence of base, and further hydrolyzed in DMF/water mixture under reflux to get the non-conjugated polymer. Upon heating or acid treatment, the fully conjugated poly(2,5-methoxy-propyloxysulfonate phenylene vinylene) (MPS-PPV) was obtained.

Cationic PFs materials serve as donors in fluorescence resonance energy transfer (FRET) DNA assays which display signal amplification. The palladium-catalyzed Suzuki coupling reaction has gained considerable popularity for synthesis of water-soluble PFs due to the mild reaction conditions. Bazan and co-workers [38] initially prepared poly[9,9-bis(6′-[N,N,N-trimethylammonium]hexyl)fluorene-co-phenylene dibromide] (PFP) by quaternization with methyl iodide of pendant amine groups on a neutral precursor. Since it is difficult to purify the monomer containing amine groups, they developed an improved synthetic approach by using monomers containing bromo groups, as shown in Fig. 5.2d. Subsequent quaternization proceeds with excellent yield via the reaction of the precursor polymer with trimethylamine in THF/water, followed by precipitation from acetone.

5.2.2 Optical Properties

When a CPE exists in solution in a non-aggregated state, the photophysical properties (fluorescence quantum yields, absorption and emission maximum, and lifetimes) of this material are remarkably similar to those of the neutral analogue dissolved in non-polar organic solvent [39]. In fact, photophysical studies provide

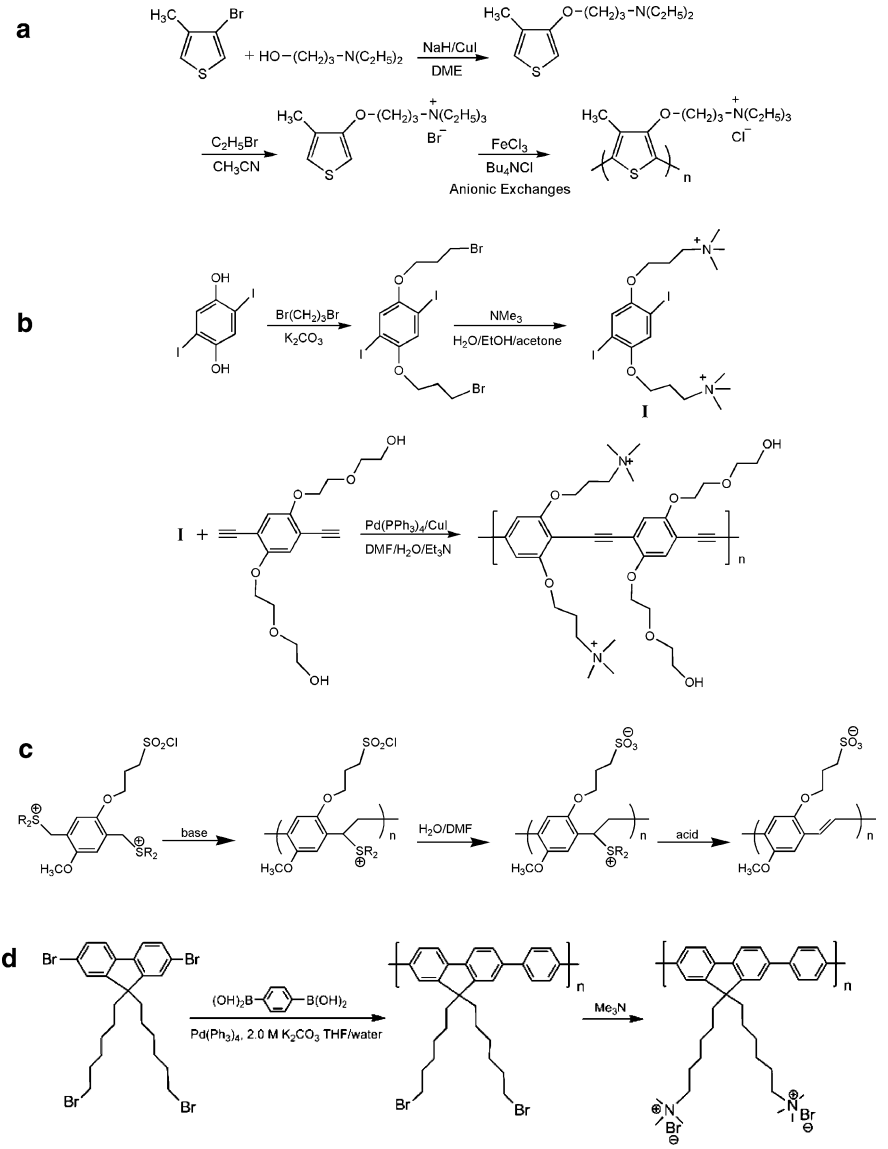

Fig. 5.2 The synthetic scheme of PMNT (**a**) [32], PPEs (**b**) [34], MPS-PPV (**c**) [37], PFP (**d**) [38]

very clear evidence that in aqueous solution CPEs have a very strong tendency to aggregate, and this aggregation phenomenon has a substantial influence on the photophysical properties of the polymers. The number of charges per repeat unit and the nature of the linkers between the backbone and the charged groups influence solubility and thus aggregation tendency. Understanding the correlation between

structure and solution behavior of CPEs will provide unique opportunities for fine-tuning of CPEs structures and therefore improving detection sensitivity and selectivity.

Bazan and co-workers studied the solvent-dependent aggregation properties of cationic poly(fluorene) (P1, chemical structure shown in Fig. 5.1) [40]. The backbone and alkyl side chain are hydrophobic moieties, while the cationic charged quaternary amines control electrostatic interactions. Dissociation of the charged ionogenic groups requires polar solvents, while the hydrophobic segments are better accommodated in non-polar solvents. There exist two different types of aggregates depending on the solvent medium. Single chain behavior or minor aggregation occurs when the THF content is in the range from 30 to 80%. In pure water, the polymer likely forms tight aggregates with chains coming together and forming the inner core, which lead to lower emission intensities due to π-π stacking and self-quenching. Upon addition of THF to aqueous solutions, the aggregates are broken up and therefore self-quenching is reduced. With further increasing the THF content higher than 80%, the ionic interactions of charged groups with the nonpolar medium lead to the groups becoming buried within a new aggregate structure. The overall aggregation driving force is the amphiphilic nature of the cationic PF. The obtained solvent-dependent aggregated information of cationic PFs is beneficial to gain insight into their signal transduction mechanism.

Compared with their neutral analog PPV, the red-shift of fluorescence emission and significantly decreased fluorescence quantum yield for MPS-PPV were reported by Whitten and co-workers [41]. Similarly, Schanze et al. [42] reported the solvent-dependence of the absorption and fluorescence of PPE type CPE, which also revealed that aggregation has a strong effect upon the photophysics of CPEs.

Another work carried out in Bazan group demonstrated that increasing the counter anion size can decrease the interchain contacts of PFBT-X and lead to a substantial increase of quantum yield in the bulk [43]. The main backbone of PFBT-X (P2, chemical structure shown in Fig. 5.1) is composed of alternating fluorene and benzothiadiazole (BT) units, and dynamic light scattering analysis indicated that the suppression of aggregation of PFBT-X in water by the large and hydrophobic anion, $B(3,5\text{-}(CF_3)_2C_6H_3)_4$. Simple ion exchange methods can be employed to generate cationic conjugated polymers with different charge compensating ions, which make regulating their optoelectronic properties for specific applications easier.

Water-soluble dendritic poly(fluorene) with positively charged amine groups on the exterior were synthesized by Wang and co-workers [44]. By virtue of the higher cationic charge density, this novel cationic conjugated polymer is less aggregated in aqueous solution and exhibits an improvement of fluorescent DNA assays signal, whereas higher generation is not easily available due to the steric hindrance of the side chain and the lower yield.

Besides tuning polymer properties by rational design and synthesis at the level of the conjugated repeat unit, the groups of Whitten [45] and Bazan [38] demonstrate that by complexing with appropriate surfactants, one may control the geometric conformation of the polymer, and thereby tune its optical and chemical properties.

5.2.3 Signal Transduction Mechanism

CPE-based DNA sensing systems in homogeneous media and heterogeneous state have been successfully accomplished by utilizing two main signal transduction mechanisms. One is DNA-induced conformational or aggregation change of CPEs, and another mechanism is quenching the fluorescence of CPEs through electron transfer or energy transfer.

Cationic polythiophenes transduce DNA hybridization into a colorimetric output based on conformational changes of the polymer upon interaction with single-stranded DNA or double-stranded DNA. Polythiophenes are known to exhibit interesting chromic features (change of color induced by a conformational change of the conjugated backbone) in the presence of different stimuli [10]. In many cases, such chromic effects also have a strong influence on the fluorescence properties of these conjugated polymers. The optical features afforded by polythiophenes provide the basis for specific detection of non-amplified DNA sequences. Specifically, an aqueous solution of cationic polythiophenes is yellow with an absorption wavelength near 400 nm which is related to a random-coil (non-planar) conformation of the polythiophene derivative. After complex with single-stranded DNA, the highly conjugated and planar conformation, which exhibit relatively red-shifted absorption wavelength, is predominant. A fluorometric detection of oligonucleotide hybridization is accessible since the fluorescence of polythiophenes is quenched in the planar, aggregated form. This change in fluorescence intensity is mainly due to a modification in the delocalization of π electrons along the carbon chain backbone that occurs when switching between the two conformations.

Water-soluble CPEs exhibit strong photoluminescence and rapid transport of the singlet excitation throughout the conjugated backbone. An important characteristic of these materials is the efficient quenching of the CPE fluorescence that can be achieved by quencher-labeled target strand DNA through either electron transfer or energy transfer. This amplified quenching ("superquenching") can be attributed to a combination of Coulombic and hydrophobic interactions in aqueous media and to rapid migration of excitons through the polymer following photoexcitation. PPE derivatives as one of the representative CPEs were employed to develop DNA sensors by this superquenching mechanism [46–48].

The quencher can be designed as an energy acceptor to allow for efficient fluorescence resonance energy transfer from the CPEs to the acceptor. The unique characteristic of CPEs is their ability to amplify signals of dyes labeled on DNA; therefore lower concentration of analytes can be easily detected, in comparison to methods using direct excitation of small molecular dyes. In comparison to intensity-based methods, these techniques provide large changes in emission profiles and open the opportunity for ratiometric fluorescence measurements [49]. Furthermore, assays of this type are less prone to false positives since non-specific interactions between the CPEs and DNA could be effectively eliminated. In FRET-based DNA assays, the light harvesting CPE and a fluorophore capable of introduction into a probe structure are generally designed to function as the donor and the acceptor,

respectively. According to Förster theory [49], FRET is a long-range energy transfer which arises from dipole-dipole interactions. FRET rate (k_t) is influenced by three factors which are the donor-acceptor distance (r), the orientation factor (k), and the overlap integral (J) between the emission of the CPEs and the absorption of the acceptor. The overlap integral ($J(\lambda)$) expresses the degree of spectral overlap between the donor emission (described by emission intensity, $F_D(\lambda)$) and the acceptor absorption (described by molar absorbance, ε_A (λ)), as described in Eq. 5.1. The components of the DNA sensor can be carefully chosen so that their optical properties meet this requirement and then the FRET process is optimized. Cationic poly(fluorene-co-phenylene)s based DNA assays involving FRET have been extensively developed and reviewed [5, 7].

$$k_{t(r)} \propto \frac{1}{r^6} \cdot \kappa^2 \cdot J(\lambda)$$
$$J(\lambda) = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \tag{5.1}$$

5.3 Signal-Amplified DNA Detection

5.3.1 PT-Based DNA Detection

The Leclerc group pioneered DNA detection with conjugated polymers in homogenous solution. In 2002, they reported new water-soluble cationic polythiophene derivatives, which can easily transduce oligonucleotide hybridization with a specific 20-mer capture probe into a clear optical (colorimetric or fluorometric) output [32]. Three types of negatively charged oligonucleotides were used: a capture-probe sequence (X1: 5′-CATGATTGAACCATCCACCA-3′), a perfect complementary target (Y1: 3′-GTACTAACTTGGTAGGTGGT-5′), X1 and Y1 are a specific oligonucleotide pair for Candida albicans. A two-mismatch complementary target (Y2: 3′-GTACTAACTTCGAAGGTGGT-5′), and a one-mismatch complementary target (Y3: 3′-GTACTAACTTCGTAGGTGGT-5′). Y2 is a specific oligonucleotide for Candida dubliniensis, and Y3 is a variant designed to have single mismatches with either C. albicans or C. dubliniensis sequences. An aqueous solution of cationic poly(3-alkoxy-4-methylthiophene)s (P3, chemical structure shown in Fig. 5.1) is yellow with an absorption wavelength near 400 nm (Fig. 5.3 left panel curve a) which is related to a random-coil conformation. Upon addition of capture oligonucleotide X1, the mixture becomes red ($\lambda_{max} = 527$ nm) (Fig. 5.3 left panel curve b), the highly conjugated and planar conformation dominated due to the formation of a so-called duplex between the polythiophene and the oligonucleotide probe (see Fig. 5.4). After mixing with the complementary oligonucleotide Y1, the solution becomes yellow ($\lambda_{max} = 421$ nm) (Fig. 5.3 left panel curve c); presumably caused by the formation of a new complex termed a triplex (*c.f.* Fig. 5.4), formed

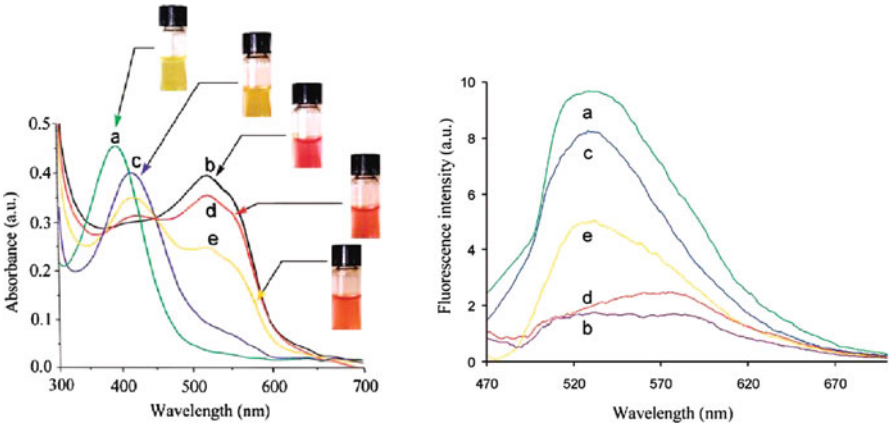

Fig. 5.3 *Left panel*: UV-vis absorption spectra of a solution of (**a**) poly(3-alkoxy-4-methylthiophene)s, (**b**) poly(3-alkoxy-4-methylthiophene)s/X1 duplex, (**c**) poly(3-alkoxy-4-methylthiophene)s/X1/Y1 perfect match triplex, (**d**) poly(3-alkoxy-4-methylthiophene)s/X1/Y2 mixture with two mismatches, and (**e**) poly(3-alkoxy-4-methylthiophene)s/X1/Y3 mixture with one mismatch. *Right panel*: Fluorescence spectra corresponding to the different assays in *left panel* (Reprinted with permission from Ref. [8]. Copyright (2004) American Chemical Society)

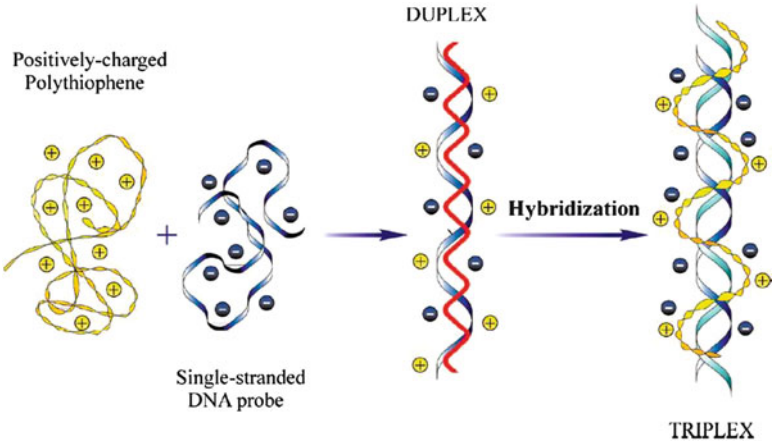

Fig. 5.4 Schematic description of the formation polythiophene/ss-DNA duplex and polythiophene/ds-DNA triplex complex (Reprinted with permission from Ref. [8]. Copyright (2008) American Chemical Society)

by complexation of the polymer with the hybridized nucleic acids. A schematic description of duplex and triplex formation is shown in Fig. 5.4. They also investigated the specificity of this polymeric optical transducer in the presence of imperfect or incomplete hybridizations. As shown in Fig. 5.3 left panel curve d, a distinct absorption spectrum is observed in the case of polymer/X1 complexed with

oligonucleotide targets with two mismatches (Y2). It is also possible to distinguish only one mismatch (Fig. 5.3 left panel curve e). It is interesting to note that the spectra of duplex and triplex forms show an isosbestic point, which indicates the presence of only two distinct conformational structures for the polymeric transducer. The detection limit of this colorimetric method is about 1×10^{13} molecules of 20-mers oligonucleotide (a concentration of 2×10^{-7} M) in a total volume of 100 μL.

A fluorometric detection of oligonucleotide hybridization is possible since the fluorescence of polythiophenes is quenched in the planar, aggregated form. The yellow form of poly(3-alkoxy-4-methylthiophene)s is fluorescent (quantum yield of 0.03 with a maximum of emission at 530 nm, *c.f.* Fig. 5.3 right panel curve a). Upon addition of capture oligonucleotide probe X1, the fluorescence intensity decreases and the maximum of emission is slightly red-shifted (Fig. 5.3 right panel curve b). When hybridized with the complementary strand Y1, the formation of a polymeric triplex leads to a five-fold rise in fluorescence intensity (Fig. 5.3 right panel curve c). Upon addition of the oligonucleotide target having two mismatches (Y2), the fluorescence intensity is weak and not significantly modified (Fig. 5.3 right panel curve d). Moreover, it is possible to distinguish oligonucleotides with one mismatch (Y3) (Fig. 5.3 right panel curve e). By utilizing the fluorometric output of poly(3-alkoxy-4-methylthiophene)s, the detection limit of the perfect complementary oligonucleotide (20-mers) is as few as 3×10^6 molecules in a volume of 200 μL, which is a concentration of 2×10^{-14} M. Similar results have been obtained with other polythiophene derivatives [50].

Following the aforementioned work, the same group developed a DNA detection platform by using a custom-built fluorimeter based on a high-intensity blue diode (as the excitation source) and a nondispersive interference filter [51]. A few hundred copies of either DNA or RNA can be specifically detected. The possibility to follow hybridization reactions in real time at such low concentrations could be used to study kinetics and thermodynamics of nucleic acids in various biological systems. In addition, similar selectivity and sensitivity was obtained when the fluorescent polymeric hybridization transducer was grafted on magnetic microbeads, which allowed the possibility of performing both preconcentration and detection steps simultaneously [52].

This simple, rapid, and versatile methodology based on different electrostatic interactions and conformational structures between cationic polythiophenes, and single-stranded oligonucleotides or double-stranded (hybridized) nucleic acids is greatly advantageous because this assay does not require any chemical reaction of the probes or the analytes.

Building on this initial concept, in 2005 they reported the detection of DNA targets in aqueous solutions at the zeptomolar range using a physical amplification method described as fluorescence chain reaction (FCR), which is a combination of electrostatic interactions, chromism, and a Förster resonance energy transfer mechanism, leading to novel fluorescence signal amplification detection [53]. Neutral duplexes made from a cationic polythiophene, serves as a potential donor, and oligonucleotide capture probes labeled with Alexa Fluor 546 (acceptor). The choice of Alexa Fluor 546 depends on the need to get a good overlap between

its excitation spectrum and the emission spectrum of the polymer donor, and an excellent fluorescence quantum yield of the acceptor. In the starting conditions, the stoichiometric duplex gives a red color and fluorescence quenching. After hybridization with the perfectly complementary DNA target, a new absorption feature appears at 420 nm, which is related to the formation of a triplex. Upon irradiation at 420 nm, the fluorescence band at 530 nm of the resulting triplex (the donor in this FRET scheme) overlaps neatly with the absorption spectrum of Alexa Fluor 546, and efficient energy transfer to the neighboring acceptor (emission maximum at 572 nm) is observed. Upon addition of noncomplementary or mismatched DNA to the duplex, the complex remains in the red non-fluorescent form, preventing the FRET mechanism from occurring. Consequently, the fluorescence intensity measured under the same experimental conditions with the perfect complementary ss-DNA strand is always higher than that obtained with targets having mismatch. The detection limit can be further enhanced by increasing the number of duplex probes, in the case of 10^{10} copies of duplex probes as starting materials, 30 copies of 20-mer target oligonucleotides are easily detected from a volume of 3 mL, and perfectly complementary targets can still be distinguished at such low concentrations from sequences having two or even one mismatch. The detection limit is five copies in 3 mL, or 3 zM, with a custom blue-LED fluorimeter, whereas a somewhat higher detection limit of 30 copies in 3 mL, or 18 zM, was obtained using a commercial spectrofluorometer. Moreover, this novel signal amplification detection strategy can also be used to direct detect ds-DNA at ultralow concentration levels. Because the probe-to-target hybridization reaction is in competition with rehybridization of the ds-DNA, most previously reported direct DNA detection techniques rely on the availability of the target sequence as ss-DNA. Since the polythiophene has a higher affinity toward ds-DNA compared to that of the ss-DNA probes, the amplified quenching of the signal in previous studies [32, 51] may lead to false positive signals, whereas this novel approach, involving "turn-on" signal amplification, is promising. The massive intrinsic signal amplification provided by this novel integrated molecular system allows the specific detection of as little as five dsDNA copies in a 3 mL sample volume in only 5 min, without the need for prior PCR amplification or chemical tagging of the genetic target. All these features provided by this novel sensing platform will lead to major advances in genomics.

For the purpose of detecting multitarget simultaneously, they extended the FCR detection scheme to microarray-based multiplex detection by hybrid self assembled polythiophene/DNA probe complexes for the direct and specific detection of DNA [54].

Two capture probes were used for DNA detection: one single-stranded DNA probe labeled with Cy3 chromophore, 5′-NH_2-C6-CATGATTGAACCATCCACCA-Cy3-3′ (NH_2-P1), and one unlabeled DNA probe, 5′-NH_2-C6-CATGATTGAACCA TCCACCA-3′ (NH_2-P2). Two DNA targets, one perfectly matched to the DNA probes, 3′-GTACTAACTTGGTAGGTGGT-5′ (T1), which corresponds to a conserved region of the Candida albicans yeast genome, and one target having one mismatched base, 3′-GTACTAACTTCGTAGGTGGT-5′ (T2), were investigated. The average polythiophene/DNA aggregate diameter is around

200–250 nm, while the height is around 20–30 nm. The diameter of the spots is about 1.5–1.7 mm, each including about 1×10^{12} probes. Figure 5.5b exhibits fluorescence images from a spotted slide after hybridization with the perfect complementary target oligonucleotide and a target with one mismatch for concentrations ranging from 1×10^{-15} to 1×10^{-6} M. The image measured at the fluorescence wavelength specific to the Cy3 chromophore shows a clear contrast between perfect complementary targets and those having one mismatch at concentrations greater than 10^{-14} M. As shown in Fig. 5.5b, the fluorescence intensity at 530 nm (fluorescence from the polythiophene itself) is very weak, even for the perfect complementary target. This observation indicates that the FRET mechanism is highly efficient. For a perfect complementary target oligonucleotide, a LOD of 5×10^{-16} M from a sample volume of 0.4 μL, corresponding to 300 DNA molecules. The detection sensitivity obtained with the system was improved by a factor of ca. 1,500 over that obtained using an unlabeled duplex (NH_2-P2/polythiophene complexes).

5.3.2 PPE-Based DNA Detection

Whitten and co-workers developed DNA sensors employing PPE derivatives with advantages of high fluorescence yields in both solution and absorbed states coupled with lower nonspecific effects in solid phase. In 2002, they reported an assay for a target single strand 20-base sequence of DNA coding for the anthrax lethal factor (ALF), which based on fluorescent polymer superquenching [46]. The sensor was constructed using a carboxylic acid functionalized polystyrene microsphere covalently functionalized with streptavidin. Biotinylated PPE (PPE-B, P4 chemical structure shown in Fig. 5.1) and DNA capture strands were simultaneous coated on polystyrene microsphere. Addition of the target strand would not attenuate the fluorescence of the microsphere, whereas addition of a quencher-labeled target strand (DNA-QTL) would reduce the fluorescence of the sensor. Target strand nucleic acids compete with a DNA-QTL for a capture strand associated closely with a fluorescent polyelectrolyte. The direct competition assay did not provide a sensitive assay, so they developed a two-step assay, in which the sensor was first incubated with target DNA and DNA-QTL was added in a second step, providing a sensitive quenching inhibition (turn-on) assay. The level of quenching decreases with increasing concentration of target DNA. An assay with similar sensitivity was obtained by pre-incubating the biotinylated capture strand with a mixture containing a constant amount of DNA-QTL and variable amounts of target DNA and then adding the mixture to microspheres pre-treated with only PPE-B.

Another improved assay for target DNA was developed using a modified sensor and replacing the biotinylated capture DNA with a biotinylated peptide nucleic acid (PNA) capture strand [47]. In this case, the polystyrene microspheres were functionalized with quaternary ammonium groups, PPE-B was precomplexed with Neutravidin at an empirically ratio that provided the strongest superquenching

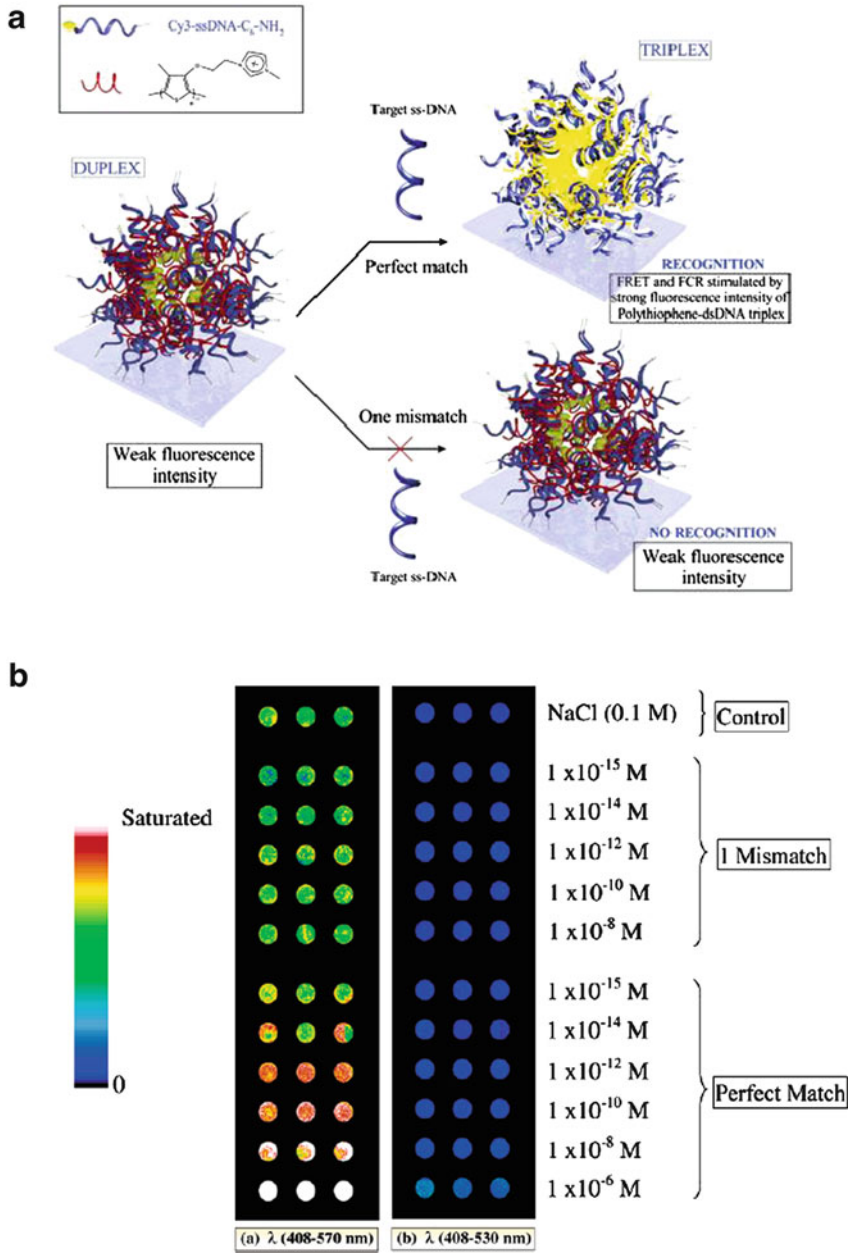

Fig. 5.5 (**a**) Schematic description of the specific recognition of target ssDNA by duplex aggregates onto glass slides with visualization of signal amplification detection mechanism based on the conformational change of cationic polythiophene and fast energy transfers within the micelle. (**b**) Fluorometric detection of hybridization of 20-mer DNA oligomers on arrays. *Left*: Excitation at 408 nm, emission at 570 nm, and *right*: excitation at 408 nm, emission at 530 nm (Reprinted with permission from Ref. [54]. Copyright (2006) American Chemical Society)

effect, and the complex was added to the ammonium functionalized microspheres. It was found that a 15 base capture PNA strand gave excellent results in sensing compared to a 20-base capture DNA. This assay was carried out by first incubating the biotinylated capture strand with the microsphere sensor described above, subsequently adding variable amounts of target DNA and then adding the DNA-QTL and measuring the intensity of the fluorescence. Thus the assay functions in a fluorescence "turn-on" mode. Additionally, the authors evaluated the sensor to discriminate single nucleotide mismatches, and the experimental data demonstrated that the sensor was capable of detecting single nucleotide mismatches within 20 min for subpicomolar amounts of the DNA target at 40 °C. These assays offer comparable or improved sensitivity to other conventional fluorescence microarray-based assays for DNA [55, 56], which do not involve amplification by a polymerase chain reaction.

Schanze and co-workers constructed a novel molecular beacon by linking oligonucleotide to PPE [48]. The oligonucleotide took part in the polymerization process and was incorporated into the PPE chain as an end capping unit. Molecular-beacon sequence was 5′-PPE-CCTAGCTCTAAATCACTATGGTCGCGCTAGG-DABCYL-3′, a universal quencher DABCYL (4-(4-(dimethylamino)phenylazo) benzoic acid) was selected as the quencher, PPE chain as the fluorophore to amplify the fluorescence signal. When the molecular beacon is in its closed state, the polymer chain will be brought close to the quencher, and the fluorescence of PPE will be strongly suppressed because of the superquenching property of the conjugated polymer. After target-DNA binding, the fluorescence of the PPE would be restored as a result of the increased separation distance between the conjugated polymer and the quencher. Selective label-free detection of target oligonucleotides with an amplified fluorescence signal was successfully achieved by using this novel molecular beacon.

5.3.3 PF-Based DNA Detection

Bazan and co-workers have shown in a series of publications that effective FRET transduction mechanism can be employed for the detection of DNA. Much of the FRET work was stimulated by the demonstration of specific DNA sequence detection using a dye-labeled PNA probe and cationic poly-(fluorene-*alt*-phenylene) (PFP) [57]. As shown in Fig. 5.6 left panel, a PNA probe (5′-CAGTCCAGTGATACG-3′) with fluorescein at the 5′ position was used as PNA-C*, and electrostatic attraction brings the donor (PFP) and anionic DNA into close proximity. If the ssDNA target hybridizes to a complementary PNA-Fl probe, PFP and acceptor (Fl) are close enough that excitation of PFP results in FRET and emission from fluorescein. If the target is not complementary to the PNA probe, the polymer to fluorescein distance is large, and no fluorescein emission is observed (Fig. 5.6 right panel). Signal amplification by the PFP provides a fluorescein emission that is over 25 times higher than that obtained by the direct excitation of

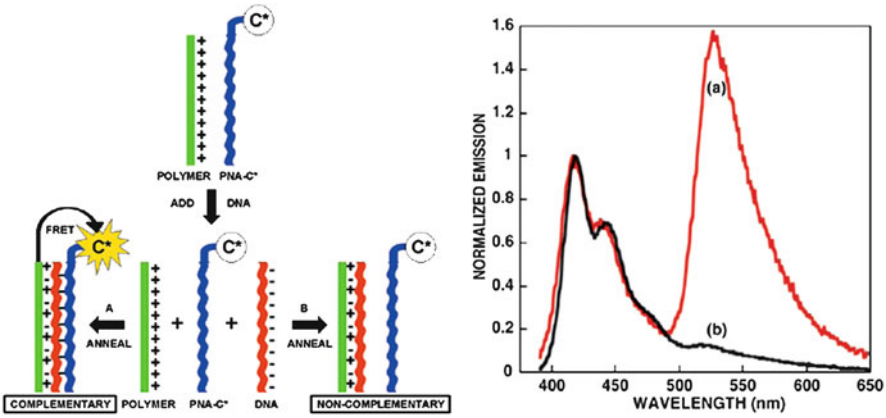

Fig. 5.6 *Left panel*: Schematic representation for the use of a water-soluble CP with a specific PNA-C* optical reporter probe to detect a complementary ssDNA sequence. *Right panel*: Emission spectra of PNA-Fl in the presence of complementary. (**a**) and noncomplementary, (**b**) DNA by excitation of PFP. The spectra are normalized with respect to the emission of PFP (Reprinted with permission from Ref. [57]. Copyright (2002) National Academy of Sciences, USA)

the dye at its absorption maximum. This amplification reflects the higher optical density of the polymer, and the very efficient FRET step.

By adding an S1 nuclease enzyme, they developed a new strategy that is sensitive to single nucleotide polymorphisms [58]. The recognition is accomplished by sequence-specific hybridization between the uncharged PNA-Fl probe and the ssDNA sequence of interest. Treatment with S1 nuclease leads to digestion of DNA, except for those regions "protected" by a perfectly matching hybridized PNA. PFP only works as excitation donor to Fl for PNA/ssDNA duplex. The overall method was tested by measuring the ability of the sensor system to detect wild type human DNA sequences, as opposed to sequences containing a single base mutation.Specifically, the PNA probe was complementary to a region of the gene encoding the microtubule associated protein Tau. The probe sequence covers a known point mutation implicated in a dominant neurodegenerative dementia known as FTDP-17, which has many clinical and molecular similarities to Alzheimer's disease. Using an appropriate PNA probe, unambiguous FRET signaling is achieved only for the wild type DNA and not the mutant sequence harboring the single nucleotide polymorphism.

The sensing strategy in Fig. 5.6 was modified to replace the PNA-Fl probes with more readily available ssDNA-Fl as the signaling reporter because DNA/DNA interactions are more common in biology and are therefore better catalogued and understood [59]. The higher local charge density of the double strand results in a stronger electrostatic attraction between the dsDNA-Fl and the conjugated polyelectrolyte relative to the situation with ssDNA, upon adding a complementary strand ssDNA, the hybridized strand will result in an efficient FRET from PFP to Fl.

In the case of a noncomplementary strand, the nonhybridized strand will interfere with the ssDNA-Fl-PFP interactions and FRET from PFP to Fl does not occur.

Liu et al. [60] reported a strategy of using a probe-immobilized silica nanoparticle (NP) surface as a platform for DNA detection which took advantage of the optical amplification of cationic conjugated polymers and easy separation of NPs. The approach begins with probe immobilized NPs in solution to capture the target labeled with fluorescein and bring fluorophores close to the surface, after separation, PFP was added to the system. Only when the complementary sequence was present in the initial solution would PFP and fluorescein be present in the final mixture, allowing intense acceptor emission upon polymer excitation. Excess DNA probes on the NP surface complexed with PFP to increase the local concentration of donor units and fluorescein self-quenching was minimized, leading to over 110 fold signal amplification. The nearly homogeneous solution of silica NPs facilitates DNA hybridization and meanwhile provides the advantage of solid state sensors that allow separation and base mismatch detection.

Ethidium bromide (EB) intercalate within the internally stacked bases of dsDNA, resulting in an increase in its fluorescence quantum yield. The scheme for DNA detection by utilizing the FRET between PFP and EB was designed by Bazan group [61]. Actually, it turns out that the FRET efficiency between PFP and EB is not high when complementary ssDNA was introduced to the sensing system, especially compared to previously reported studies with CCPs and fluorescein-labeled DNA or PNA, which may be attributed to nonoptimized transition dipole alignment and electrostatic repulsion between PFP and EB. In order to enhance the FRET efficiency to gain higher signal, fluorescein was attached to ssDNA terminus, which function as a fluorescence resonance gate for transferring conjugated polymer excitations to dyes intercalated within dsDNA. FRET from PFP to Fl, followed by a second FRET to EB, becomes energetically feasible. The overall process provides a substantial improvement of selectivity over previous homogeneous conjugated polymer based DNA sensors. DNA conformational transition can be detected by utilizing this two-step FRET principle [62].

Our group has developed a DNA detection method which combines optical amplification properties of cationic conjugated polyelectrolytes with highly selective target-induced DNA strand displacement [63]. Efficient strand displacement allowed for formation of a hairpin shape DNA which could be recognized and cleaved by Hae III endonuclease, or by formation of an active DNAzyme which was inhibited before strand displacement. The occurrence of cleavage after strand displacement afforded a weaker FRET between PFP and the cleavage product. Since the existence of a mismatch in the target DNA could lower the efficiency of the displacement reaction, the assay was able to detect even a single mismatch in the target DNA. The detection limits of the target DNA are 2.14 pmol for the Hae III cleavage system and 0.75 pmol for the DNAzyme system.

Very recently, our group developed a dual-amplified fluorescence DNA detection scheme taking advantages of signal amplification property of conjugated polymers and recyclable autocatalytic hybridization amplification of DNA [64]. The detection principle is illustrated in Fig. 5.7. The hairpin DNA_A (probe A) and DNA_B labeled

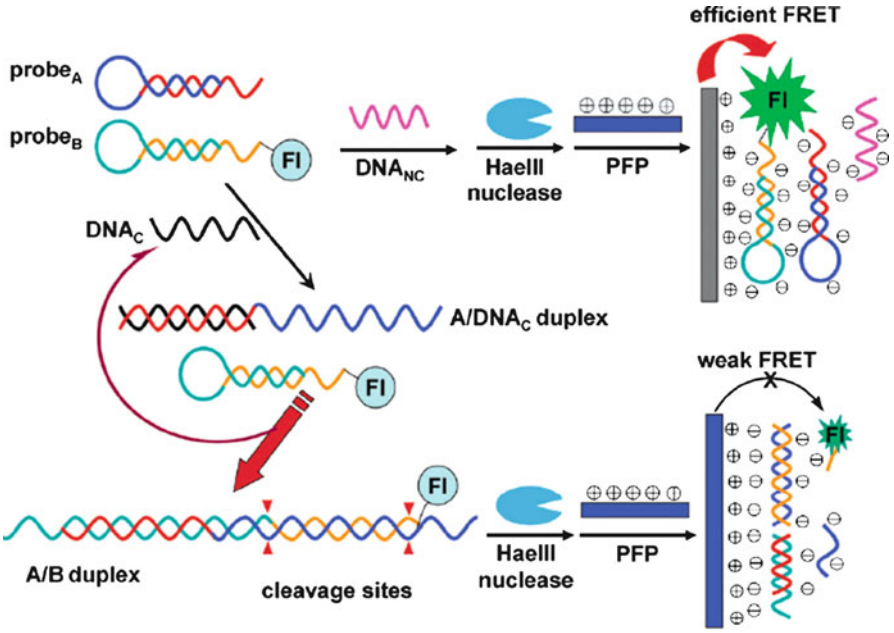

Fig. 5.7 Schematic representation of the dual-amplification DNA detection. DNA_C is complementary to probe A, DNA_{NC} is non-complementary to probe A. HaeIII is the restriction endonuclease for probe A/probe B duplex cleavage and the cleavage sites are indicated by *arrows* (Reprinted from Ref. [64]. With permission by the Royal Society of Chemistry)

with a fluorescein dye at 50-terminus (probe B) act as the signaling probes. Hairpin probe A and probe B co-exist metastably in the absence of target DNA. The autocatalytic hybridization process of probe A and probe B is initiated by adding the target DNA (DNA_C) which is complementary to probe A. The formed probe A/DNA_C duplex with a 3′ single strand can then hybridize with the 5′ single strand of probe B to simultaneously release the target DNA_C. The released DNA_C can then hybridize with another hairpin probe A and the cycle starts anew. Thus, the autocatalytic hybridization of DNA induced by the target DNA_C can produce large amounts of probe A/probe B duplex labeled with fluorescein at its 5′-terminus. The probe A/probe B duplex can then be cleaved away by the endonuclease HaeIII. Owing to the two amplification processes, the new detection approach achieves high detection sensitivity with a 10 fmol detection limit, thus greatly improving the sensitivity of DNA detection using cationic conjugated polyelectrolytes.

Aggregation-induced interchain energy transfer in polymers of PFPB, 5% of the phenylene units in the typical poly(fluorine-*co*-phenylene) structure substituted with BT units chain (P5, chemical structure shown in Fig. 5.1), can be used to design DNA sensor [65, 66]. The emission of PFPB is predominantly blue in dilute conditions, and emit green light for concentrated solutions. A similar blue to green color change occurs upon addition of either ssDNA or dsDNA, and these color

changes can be used to generate calibration curves that can be used to determine the concentration of DNA in solution [66]. When combined with a Cy5-labeled PNA probe (PNA-Cy5), the polymer can be used to design a three-color DNA detection assay. PFPB/PNA-Cy5 emits blue color in the absence of ssDNA, and it emits green light due to the interchain FRET characteristic of PFPB when non-complementary DNA is present ($ssDNAN_C$). When bound to the complementary $ssDNA_C$, PFBT/PNA-Cy5 emits a red color due to the intramolecular FRET of PFBT followed by a subsequent FRET step to Cy5.

Bazan and co-workers extended the well developed homogenous cationic conjugated polymers (CCPs)-PNA/DNA sensory method to solid platforms [67]. They utilized surface-bound PNA probe labeled with Cy5 to hybridize complementary ssDNA (ssDNAc), after addition of synthesized poly[9,9′-bis-(6″-(N,N,N-trimethylammonium) hexyl) fluorene-*co-alt*-4,7-(2,1,3,-benzothiadiazole) dibromide] (PFBT) to ssDNAc/PNA-Cy5, an intense red emission from Cy5 was observed. There was no energy transfer for noncomplementary ssDNA/PNA-Cy5 under the same experimental conditions. In order to demonstrate that the light-harvesting properties of PFBT could be incorporated into platforms suitable for microarray technologies, they used unlabeled PNA chemically binding to surface-modified glass surface, and the target ssDNA was labeled with Cy5, when PNA hybridized with complementary ssDNA, excitation of PFBT resulted in FRET to Cy5, while no Cy5 emission was observed when the surface was treated with non-complementary ssDNA-Cy5. Moreover, they designed a solid-state assay that did not require labeled target ssDNA. The design strategy was that ssDNA hybridization to immobilized PNA resulted in a change in surface charge from neutral to negative. Exposure of the surface to the cationic PFBT resulted in electrostatic binding only on those surfaces that bear the duplex structures. By measuring the PFBT emission, it is possible to detect the target DNA. The selectivity of this method relies on the successful removal of CPE from nonhybridized PNA surfaces.

Building on the above work, in 2007 Bazan et al. [68] created a PNA microarray to detect unlabeled target DNA utilizing PFBT as optical transducer where the basic sensing strategy was depicted in Fig. 5.8a. Since PFBT has two separated absorption bands, at 330 and 455 nm, and that it also has absorption at 488 nm both in solution and in solid state. By exciting the conjugated polymer at 488 nm on a commercial microarray scanner, the presence of the target is directly indicated by the fluorescence emission of the polymer. The long target complementary DNA derived from the pathogenic bacteria was also detected with this protocol. A representative image and related information are shown in Fig. 5.8b. Each spot has a diameter of ca. 200 mm. The probe sequences in Fig. 5.8b correspond to segments found in Staphylococcus aureus and Staphylococcus epidermidis. As expected, higher signals were obtained using completely matched DNA sequence. Furthermore, the sensitivity was also improved in comparison to previous reported label-free DNA microarrays based on cationic polythiophene [69]. All these features including high sensitivity, eliminating the necessity of target labeling, and detection on a commercial microarray scanner directly made this simple and rapid methodology highly promising for the future of genomics research.

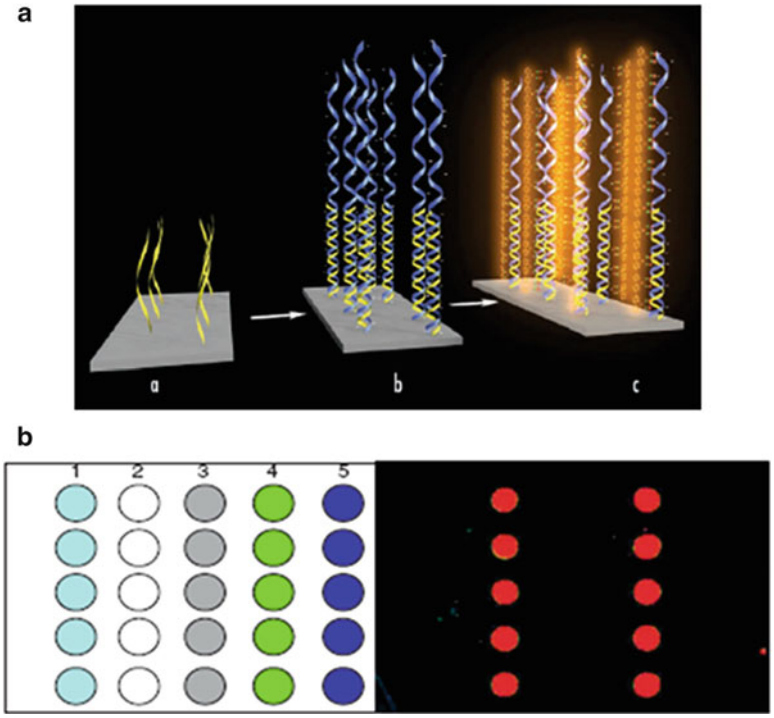

Fig. 5.8 (**a**) Label-free ssDNA detection using immobilized PNA and PFBT. *a*: Surface bound PNA (shown in *yellow*), *b*: hybridization with ssDNA (shown in *blue*), *c*: electrostatic adsorption of PFBT onto the PNA/ssDNA surface (Reprinted with permission from Ref. [5]. Copyright 2008 Springer). (**b**) Array results obtained from hybridization with a mixture of two unlabeled oligomeric targets, 5′-TGCGAAAAAA GTCTGAATTGCAGGGAGCAG-3′ (probe 2 match) and 5′-TGCAACTCATCT GGTTTAGGATCTGGTTGA-3′ (probe 4 match). *Left*: Layout of PNA probes. *1*: negative control, 5′-ACTTGTAAATGGTAG-3′; *2*: positive control, 5′-CTGCTCCCTGCAATT-3′; *3*: negative control, 5′-GATATTCGCACTGA-3′; *4*: positive control, 5′-TCAACCAGATCCTA-3′; and *5*: negative control, 5′-TCAAGCAAATCCCAT-3′. *Right*: Scanned array image of polymer emission (Reprinted with permission from Ref. [68]. Copyright 2007 Nature Publishing Group)

5.3.4 PCR-Associated PF-Based DNA SNP and Methylation Detection

SNP is the most common sequence variation in the human genome. SNPs can be used as genetic markers for mapping genes, defining population structure, and performing gene association studies, as well as a fundamental tool in drug discovery and identification of genetic and inherited diseases [70–72]. Our recent studies showed that conjugated polyelectrolytes can be used for detecting SNP, and the detection model for SNPs is indicated in Fig. 5.9 based on FRET between PFP and fluorescein using a single-base extension (SBE) reaction [73]. The target DNA

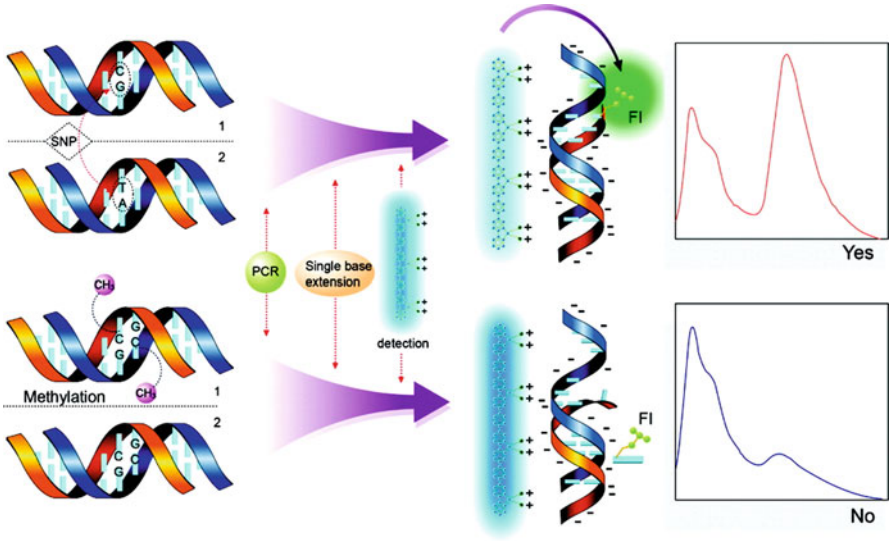

Fig. 5.9 Schematic representation of SNP genotyping based on CCP and allelic-specific primer extension, and DNA methylation detection (Reprinted with permission from Ref. [16]. Copyright (2010) American Chemical Society)

fragment is part of p53 exon8 containing a polymorphic site, where the nucleotide G in a wild-type target is replaced by A in a mutant. The 3′-terminal base of the probe is T that is complementary to mutant type target sequence and is not complementary to the wild type target at the 3′-terminus. For the mutant target, the probe is fully complementary, and the fluorescein-labeled base G is incorporated into the probe by an extension reaction in the presence of Taq DNA polymerase. Upon adding PFP, strong electrostatic interactions between DNA and PFP bring the fluorescein close to PFP, and efficient FRET from PFP to fluorescein occurs. For the wild target, the 3′-terminal base of the probe is not complementary, thus the base extension reaction is blocked and the dGTP-Fl remains in the solution. In this case, upon adding the PFP, FRET from PFP to fluorescein is inefficient. The method has high sensitivity and can detect as low as 2% allele frequency. The sensing platform was employed to discriminate the SNP genotypes of 76 individuals of Chinese ancestry [74].

Replacing the single base extension reaction by incorporating dGTP-Fl and dUTP-Fl during the PCR extension, one step SNP detection was achieved which can be used to detect genomic DNA. In this approach, when PCR amplification occurs, dGTP-Fl and dUTP-Fl are incorporated into formed PCR amplicons. By detecting the existence of PCR product with the FRET signaling from PFP to fluorescein-labeled PCR amplicons, the SNP genotype can be identified. The genotyping of 50 ng of genomic DNA from a human lung cancer cell is easily detected using this method [75].

Along these lines, we designed an improved method for multi-color and one-tube SNP genotyping assays based on PFP/DNA assemblies using fluorescein-modified

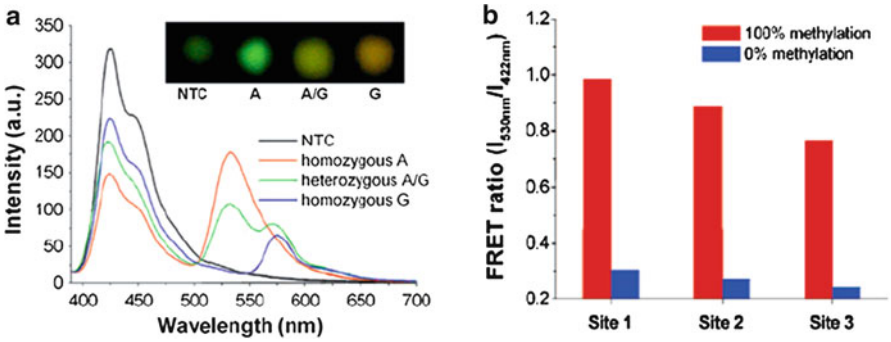

Fig. 5.10 (**a**) Emission spectra from solutions containing PFP and extension products of homozygous A, heterozygous G/A, and homozygous G. No-template control (NTC) was used as the blank. The excitation wavelength is 380 nm. Inset in Figure is the images of extension products under a 300 nm UV light with a 550–650 nm band-pass optical filter (Reprinted from Ref. [76]. With permission by the Royal Society of Chemistry). (**b**) FRET ratio (I_{530nm}/I_{422nm}) from solutions containing PFP and single nucleotide base extension products of methylated plasmid and nonmethylated plasmid using methylation specific probes in HEPES buffer solution. The probes used in extension reactions were p16-1m for site 1, p16-2m for site 2, and p16-3m for site 3 (Reprinted with permission from Ref. [81]. Copyright (2008) American Chemical Society)

dUTP and Cy3-modified dCTP in single base extension reactions [76]. A sequence on exon 8 of human p53 containing a polymorphic site is used as a DNA target in which the nucleotide G in the wild type is replaced by A in the mutant target. This G-A point mutant alters the coding of an amino acid (Arg-Trp) [77]. Thus three kinds of SNP genotypes are possible: homozygous G, heterozygous G/A, and homozygous A. The dye incorporated to probe DNA by polymerase is dependent on the template DNA and whether it is homozygous or heterozygous. Multi-step FRET is present if both dyes are incorporated to the probe DNA in one tube, the corresponding emission spectra and images of extension products with PFP dropped onto a glass slide under 300 nm UV light with transmission mode are shown in Fig. 5.10a. As a high throughput system (HTS)-compatible and label-free technique, the method is promising for the sensitive detection of practical SNP samples.

DNA methylation is an important component of epigenetic regulation that can be used as a biomarker for early cancer diagnosis and determination of specific cancer types [78–80]. Our recent studies showed that conjugated polyelectrolytes can be involved to detect DNA methylation [81]. The system relies on a highly selective single base extension reaction and significant optical amplification of conjugated polymers, which is illustrated in Fig. 5.9. Bisulfite treatment changes nonmethylated C into U but does not influence the methylated C, and the resulting U is substituted by T after PCR amplification [82]. Thus, the DNA methylation status can convert into a C/T polymorphism. After single nucleotide base extension with Taq polymerase in the presence of dGTP-Fl and methylation specific probe, the dGTP-Fl is incorporated into the probe for the methylated DNA, but not for nonmethylated DNA. Upon addition of PFP, strong electrostatic interactions

between DNA and PFP bring the fluorescein close to PFP, and efficient FRET from PFP to fluorescein occurs for the methylated DNA but not for nonmethylated DNA.

The methylation status of a specific CpG site can be monitored by fluorescence spectra of the PFP or fluorescein emission changes in aqueous solutions. We established this platform by using a pUC57 plasmid containing a 283-bp DNA fragment from the promoter region of a tumor-suppressor gene (p16) as a model. Plasmid pUC57 that carried the sequence was fully methylated by methylase (M.SssI). The FRET ratios (I_{530nm}/I_{422nm}) for specific extension are three to five times higher than that of the nonspecific extension (*c.f.* Fig. 5.10b), which demonstrates the good selectivity of this assay method for the detection of the methylation status of specific CpG sites. The improved fluorescein emission by FRET shows the optical amplification by PFP, and analyte DNA at the picomolar level can be easily detected. Based on the detection model, methylation status of three CpG sites in the p16 promoter region of the human colon cancer cell line HT29 has been detected. As expected, significant FRET signaling for the methylation-specific probe implies that hypermethylation occurs in the p16 promoter region of HT29 cells.

Utilizing the novel detection strategy, the methylated statuses of the p16, HPP1 and GALR2 promoters of five cancer cell lines (HT29, HepG2, A498, HL60 and M17) were assayed. Genomic DNA from cancer cells is pretreated with a methylation-sensitive restriction endonuclease, followed by PCR amplification in the presence of fluorescein-labeled dNTP and Taq polymerase. The PCR only occurs for methylated DNA, and DNA methylation of the gene sequence of interest is detected as a result of FRET between CCP and fluorescein that is incorporated into DNA. The obtained assay results provide an association study between the cancers and susceptibility genes, which shows great potential for early cancer diagnosis [83].

5.4 Summary and Outlook

In summary, the recent reports presented in this chapter show that substantial efforts have been placed in developing homogeneous and heterogeneous non-amplified genomic DNA assays that take advantage of the optical amplification afforded by CPEs. Cationic conjugated polymers have great potential to be incorporated into the widely used microarray technology for simplified process with improved detection sensitivity, and the combination will open unique possibilities in efficient DNA detection. Although we have described a number of possible applications in the present chapter, there is considerable room for improvement in the intrinsic emissive polymer properties to enhance the detection sensitivity. Therefore, rationally design and synthesize functionalized conjugated polymers is still one of the challenges in polymer science, and optimizing CP structure with a better understanding of the forces that control the association between conjugated polyelectrolytes and DNA will yield superior detection platforms. Moreover, widely use of an optimized fluorescence detection procedure based on a high-intensity blue diode as the excitation source should display more sensitive and specific detection capability. Furthermore,

convenient methods that are suitable for rapid assessment of the identity of SNP, and DNA methylation without the need of DNA amplification are highly desirable. We believe that there is great promise for the use of conjugated polymers-based fluorescence sensors to accomplish this objective in future investigations, which will broaden the application field of conjugated polymers and offer new assay strategies for detecting genomic disorders.

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Part III
Detection Strategies – Sensors

Chapter 6
Optical Detection of Non-amplified Genomic DNA

Di Li and Chunhai Fan

Abstract Nucleic acid sequences are unique to every living organisms including animals, plants and even bacteria and virus, which provide a practical molecular target for the identification and diagnosis of various diseases. DNA contains heterocyclic rings that has inherent optical absorbance at 260 nm, which is widely used to quantify single and double stranded DNA in biology. However, this simple quantification method could not differentiate sequences; therefore it is not suitable for sequence-specific analyte detection. In addition to a few exceptions such as chiral-related circular dichroism spectra, DNA hybridization does not produce significant changes in optical signals, thus an optical label is generally needed for sequence-specific DNA detection with optical means. During the last two decades, we have witnessed explosive progress in the area of optical DNA detection, especially with the help of simultaneously rapidly developed nanomaterials. In this chapter, we will summarize recent advances in optical DNA detection including colorimetric, fluorescent, luminescent, surface plasmon resonance (SPR) and Raman scattering assays. Challenges and problems remained to be addressed are also discussed.

6.1 Introduction

Optical detection has long been a powerful tool for biomedical applications. Optical sensors based on bio-recognition events possess some unique advantages over other analytical methods. To name a few, light beam produces much less interference to biological events compared with electronic, electrochemical or magnetic signal sources. Optical signals are immune to electromagnetic interference, capable of

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performing remote sensing and providing multiplexed detection within a single device. Optical labels are safe and ease of manipulation compared with classical radioactive labels.

The basic principle of DNA optical sensor is to generate a recordable optical signal upon sequence-specific hybridization. While a few excellent reviews have given comprehensive summarize of the history and updated process of DNA sensors and DNA microarrays [1–7], we will focus on some recent advances on optical detection of non-amplified genomic DNA in this chapter. In the end, we will present a brief outlook for questions remained to be addressed and future potential for these optical DNA sensors.

6.2 Optical Methods for DNA Analysis

6.2.1 Colorimetric Assays for Nucleic Acid Detection Using Au Nanoparticles

Colorimetric biosensors, which mean a detection method based on the color change induced by a bio-recognition event, are particularly impressive since they minimize or eliminate the necessity of using expensive and complicated instruments, sometimes the detection can even be realized by unaided naked eyes.

The milestone work was the development of colorimetric DNA sensor using Au nanoparticles (AuNPs) from Mirkin's group in 1996, which in a sense opens the era of bionanotechnology [8, 9]. Herein we first provide a short description of the interesting optical propertiesy of AuNPs in order to better understand the principle of AuNPs-based colorimetric DNA sensor. AuNPs, or Au colloid has a long history that could date back to at least to the fourth century AD when it was used to stain the Lycurgus cup owing to its brilliant wine color [7, 10]. But correlation of the color of Au colloidal with its small size was first was heralded by Michael Faraday in 1857 in a lecture entitled "Experimental Relations of Gold (and other Metals) to Light" [11]. We must pay a tribute to his insight, even 150 years ago, he noted, "The state of division of these particles must be extreme; they have not as yet been seen by any power of the microscope". The explosion of nanotechnology in the last two decades has confirmed that Au colloids prepared by Faraday were actually spherical AuNPs with a diameter of 10–100 nm. The intense red color of AuNPs is due to the interaction of incident light with a collective oscillation of free electrons in the particles known as localized surface plasmon resonance. When AuNPs aggregate, color of AuNPs changed from red to blue, which was attributed to the interaction of interacting particle surface plasmons and aggregate scattering properties. This simple red-to-blue color change has motivated scientists to design colorimetric sensing strategies by utilizing the analyte-induced AuNPs aggregation. For example, AuNPs loaded with antibodies or antigens has been used in commercialized test strips for HIV or pregnancy testing.

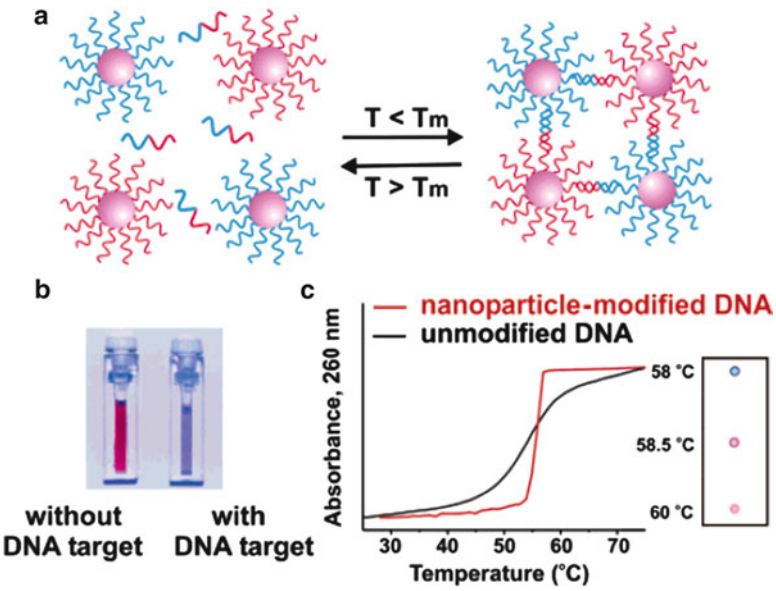

Fig. 6.1 Upon hybridization with the complementary target DNA, probe DNA -functionalized AuNPs will aggregate (**a**), resulting in a *red*-to-*blue* color change (**b**). The aggregation process is accompanied by a sharp increase of the absorbance in the melting profile as compared with regularly DNA hybridization in solution phase (**c**) (Reproduced with permission from Ref. [7]. Copyright (2007) American Chemical Society)

Mirkin's group pioneered the use of aggregation-induced color change of AuNPs in nucleic acid detection. Briefly, AuNPs of 13 nm in diameter was modified with thiolated-probe DNA via Au-S chemistry. Thus upon the addition of target DNA that cross-linked the probe DNA-modified AuNPs through sequence-specific hybridization, a red-to-purple color change is therefore observed. More interestingly, given the nature of DNA hybridization is reversible depending temperature, the aggregation process is reversible; denaturation of the hybridized DNA duplex at elevated temperature (above the melting temperature, Tm) causes the dissociation of aggregates into dispersed AuNPs (Fig. 6.1). Further studies indicated that the melting profiles of the nanoparticle-labeled DNA aggregates were extraordinarily sharp, occurring over a temperature range much narrower than the transition for unlabeled or conventional fluorophore-labeled DNA [12, 13]. By elaborate optimization of influence factors such as AuNPs size, densities of probe DNA on AuNPs and salt concentrations, this colorimetric assay enables the detection of target DNA with a limit of detection (LOD) down to nM.

This simple colorimetric technique offered several advantages for quantitative analysis of nucleic acids: (1) AuNPs is ease of conjugation with thiolated probe DNA. The large surface area of nanomaterials ensures a spherical AuNPs of 13 nm load hundreds of capture probe DNA, meanwhile the three dimensional

(3D) assembly of probe with AuNPs lowers the steric hindrance and favors target-probe hybridization; (2) AuNPs possesses extremely high extinction coefficients (e.g., 2.7×10^8 M^{-1} cm^{-1} at ~520 nm for 13 nm spherical AuNPs, ca. 1,000 times higher than those of organic dyes) [14], thus slight aggregation may result in intense color, suggesting relatively high sensitivity; (3) The simple red-to-blue color change readout does not require expensive, sophisticated instrumentation; (4) last but not least, the sharp absorbance changes in Tm profile could be utilized to discriminate between perfectly matched target oligonucleotides and targets with single base-pair mismatches, holding huge promise in single nucleotide polymorphisms (SNPs) analysis. Therefore, ever since first developed by Mirkin and co-workers, this platform has been increasingly applied for the detection of a wide spectrum of analytes ranging from nucleic acids to proteins, saccharides, small molecules, metal ions, and even cells. It is quickly becoming an important alternative detection tool, holding great potential in clinical diagnostics, drug discovery and environmental contaminant analysis [15, 16].

In addition to this target nucleic acid-induced cross-linking aggregations, Rothberg and Li have proposed an alternative colorimetric method using unmodified citrate-reduced AuNPs in 2003 [17–19]. They found that single-stranded (ss) DNA can be attached on citrate-reduced AuNPs through DNA base-gold interactions and can stabilize AuNPs electrostatically from salt-induced aggregation. In contrast, double stranded (ds-) DNA shows little binding affinity to AuNPs, and, therefore it provides little stabilization against salt. In other words, at an appropriate high salt concentration (e.g., 200 mM of NaCl), citrate-capped AuNPs are stabilized in the presence of ss-DNA, but aggregate in the presence of ds-DNA (Fig. 6.2). This simple phenomenon realized a rapid, label-free sequence-specific detection of target DNA with a LOD also of nM range. Later on, our group further investigated this system and provided a slightly modified mechanism [20–22]. The different affinity of ss- and ds-DNA to AuNPs is attributed to at least three effects. First, DNA bases attach to AuNPs with high affinity via Au-N coordination. Importantly, DNA bases are encapsulated within the negatively charged phosphate backbone of ds-DNA, while they are exposed in ss-DNA. Second, the surface of AuNPs is negatively charged because they are stabilized by citrate ions. Since the charge density of ds-DNA is higher than that of ss-DNA, the electrostatic repulsion between AuNPs and DNA is larger for ds-DNA. Third, ds-DNA is much more rigid than ss-DNA; thus ss-DNA possesses higher freedom to wrap on AuNPs than ds-DNA.

6.2.2 Fluorescent Nucleic Acid Assays

6.2.2.1 Fluorescent Assays Based on Molecular Beacons

Molecular beacons (MB), which were first described in 1996 by Tyagi and Kramer [23], are a class of newly developed probes that are widely used as fluorescent probes. Tyagi and Kramer named this fluorescent DNA probe “molecular beacon”,

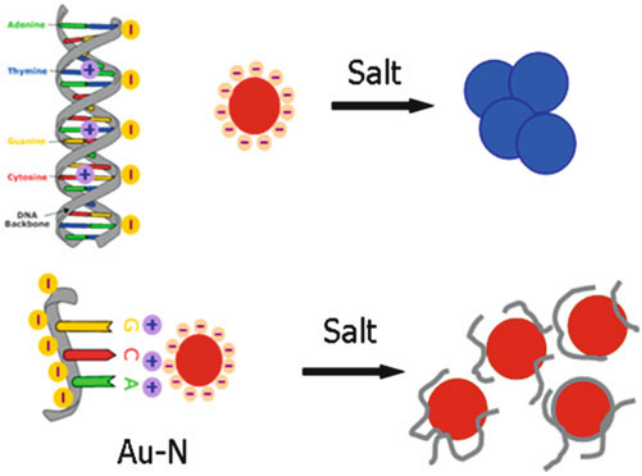

Fig. 6.2 ds- and ss-DNA reveal different affinity of unmodified citrate-reduced negatively-charged AuNPs. ss-DNA possesses higher affinity to AuNPs, it could be attached on AuNPs surface that stabilize AuNPs against salt-induced aggregation, while ds-DNA doesn't

because it emits a light signal upon hybridization with target sequence. Generally, a typical MB is a hairpin structured single stranded oligonucleotide that is modified with a fluorophore and a quencher group at each end of its stem end. The sequence of a MB is composed with two segments including a recognition region of about 15–30 bases flanked by two short complementary stem sequences, which force the entire sequence to form a stem–loop structure at room temperature. In the absence of target DNA, the fluorophore and quencher is forced to be close proximity, where fluorescence is quenched effectively. While in the presence of target DNA that is hybridized with the loop segment of MB, and the resulting duplex structure between target DNA and loop is thermodynamically stable compared with the weaker stem helix, thus the stem-loop structure is forced to open. As a result, the spatial separation of fluorophore and quencher blocks the energy transfer and restores the fluorescence signal (Fig. 6.3a). The application of MBs in DNA sensing was covered by a number of reviews [24–26].

The distinct advantage of MB lies in its ability to real-time monitoring the quantity of target DNA, therefore MB is widely used in RT-PCR to provide a real time measurement of PCR progress [27–29]. In addition to DNA, MB was also employed for real-time quantification of mRNA both *in vitro* and *in vivo* [30, 31]. For example, with the help of confocal fluorescence microscope, MBs could provide a real-time visualization and localization the detection of mRNA in living cells [32].

Despite the variety of applications of MBs in biosensors, the basic principle of detection remains unchanged: the generation of a fluorescent signal is based on hybridization between target oligonucleotides and MBs in a 1:1 stoichiometric ratio. That is, one target oligonucleotide could open only one MB to give fluorescent

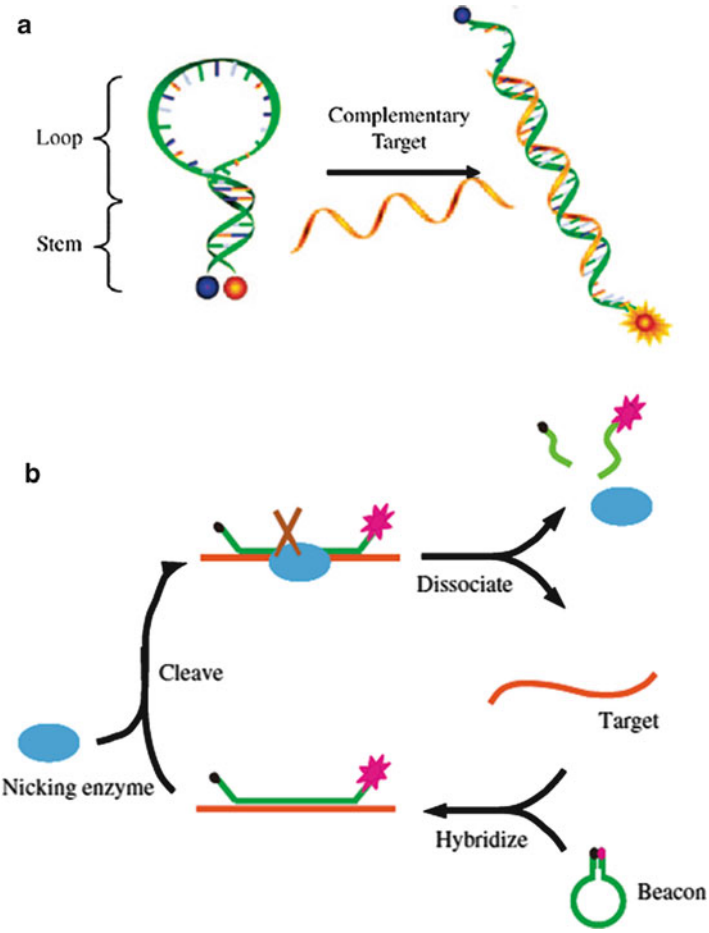

Fig. 6.3 (**a**) Mechanism of a molecular beacon. The MB adopts a stem–loop structure and thus holds the fluorophore (*orange*) and quencher (*blue*) in close proximity. As a result, the fluorescence emission of the fluorophore is strongly suppressed (in the absence of a target). Hybridization with target DNA opens the hairpin structure and recovers the fluorescent signal to allow readout the hybridization event (Reproduced with permission from Ref. [24]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA) (**b**) Working principle of a basic NESA strategy. A nicking enzyme is introduced to cleaves the MB that already be opened by target DNA, resulting dissociation of target from the cleavaged beacon and in turn be released to solution. The as-released target nucleic aid could further hybridize to another beacon and initiate the second cycle of cleavage (Reproduced with permission from Ref. [34]. Copyright (2008) Oxford University Press)

signal. Given the instrument limit of commercial fluorophotometers, MBs could only detect nucleic acids down to nanomolar range.

Recently, a strategy called nicking enzyme signal amplification (NESA) was designed to improve the sensitivity of MB-based fluorescence assay [33–35]. Nicking enzymes are a kind of restriction endonucleases that can recognize a

specific sequence along a double-strand DNA; however, they cleave only one specific strand instead of both strands, leaving a nick in the DNA (Fig. 6.3b). In brief, this strategy introduces a nicking enzyme recognition site in the loop of MB. Upon hybridization with target nuclei acid that yields the duplex recognition domain for nicking enzyme, the nicking enzyme specifically cleaves the MB and this process induces a decreased stability of the two duplexes obtained. Thus target nucleic acid is dissociated from the cleaved beacon and released into solution. The released target nucleic acid could further hybridize to another beacon and initiate the second cycle of cleavage. Eventually, one target nucleic acid could go through many cycles, resulting in cleavage of many beacons. Therefore the limit of detection (LOD) could be significantly pushed down to picomolar range [34].

Despite the three orders of magnitude improvement in sensitivity, the NESA still suffers from one major drawback. It requires the target sequence contains a nicking enzyme recognition site, thus is not appropriate for detection of target with random sequences. Several extended visions of NESA strategy were designed for random sequence detection. For example, Xie and co-workers coupled rolling circle amplification (RCA) with NESA that further improves the LOD down to 85 fM [34]. More recently, Zhou and co-workers [36] presented another modified version of NESA named cascade enzymatic signal amplification (CESA), which they integrated a invasive signal amplification with NESA. Unlike RCA that requires a e padlock probe DNA to capture target nucleic acid, invasive signal amplification does not rely on specific recognition sequences but on a specific structure formed by the specific binding of an upstream probe and a downstream probe to a target DNA; so any target sequence can be detected.

A further improved LOD of 1 fM was achieved in this strategy. In addition to nicking enzyme, sequence-independent exo-exonuclease III and DNA polymerase were also used to digest MBs that “recycles” target nucleic acid to improve the sensitivity [37]. The coupling of MBs with enzymatic digestion cycling possesses great potential for ultrasensitive detection, real-time imaging and location of nucleic acid in-vivo.

6.2.2.2 Fluorescent Assays Based on Nanomaterials

Organic Dye Fluorophores Incorporated in Nano-Scale Matrix

While organic dyes have to be powerful fluorescent labels in bio-diagnostics, they also suffer from intrinsic drawbacks, for instance, limited sensitivity and photo-stability. Moreover, most organic dyes present a certain level of toxicity that hinders their application in vivo cellular studies and imaging. To overcome these disadvantages, researchers attempted to incorporate dyes inside silica NPs [38], which provide an effective barrier keeping the dye from the surrounding environment, both photobleaching and photodegradation phenomena that often affect conventional dyes can be minimized. Using appropriate synthetic conditions, a large number of dye molecules can be incorporated inside a single silica particle

(there can be tens of thousands of dye molecules). Even though there remain some fluorescence quenching phenomena within an NP with a large amount of dye incorporated in a small volume, the goal of obtaining a particle with brighter luminescence is largely successful. Dye-doped NPs produce a highly amplified optical signal compared with a single dye molecule.

Tan's group has pioneered the application of this dye-doped Silica NPs in nucleic acid detection and imagining [39]. They first attached a capture probe DNA on a dye-doped Silica NPs via biotin-avidin interactions. The dye-doped Silica NPs modified capture probe was further used in a sandwich-type fluorescent DNA assay [40]. Hybridization of target DNA with the capture probe brings the dye-doped silica NP to the surface. This provides a large number of dye molecules on the surface for signaling. By monitoring the luminescent intensity from the surface-bound NPs, DNA target molecules can be detected with increased sensitivity (as low as 0.8 pM). The protocol could be also used in array-based multiple DNA analysis and imaging by using different dye-doped Silica NPs. Zhou et al. [41] prepared a class of dye-doped core-shell NPs by attaching thiolated DNA probes to the surface of AuNPs. The AuNPs were then coated with a 10–15 nm silica layer through the (3-mercaptopropyl)trimethoxysilane (MPTS). Two-color DNA microarray-based detection and imaging was demonstrated using Cy3- and Cy5-doped NPs in sandwich hybridization.

Fluorescent Nanomaterials

Quantum dots (QDs) are a class of II-IV or III-V semiconductor nanoparticles with a size scale approximately less than 10 nm [42, 43]. The distinct optical property of QDs is the size-dependent fluorescence properties. QDs possess narrow size-tunable fluorescence spectra with narrow full width-at-half-maxima. The absorption spectra of QDs are broad. The combination of broad absorption spectra and narrow symmetric emission spectra enables QDs attractive for multiplex targets sensing [44]. Meanwhile, QDs have higher quantum yields and are more robust against photobleaching compared with organic dyes, thus also great attention was given to its application in imaging [45].

The size-tunable fluorescence emission and simultaneous excitation feature of QDs render them as ideal fluorophores for wavelength-and-intensity multiple labeling. About a decade ago, Nie and co-workers pioneered the use of QDs embedded beads for multiple DNA detection [46]. The principle of multiplexed optical coding is based on multicolor QDs embedded in polymer beads. The polymer beads were modified with DNA probes. Target DNA was directly labeled with a fluorescent dye. Fluorescence spectra of the single-bead level yield both the coding and the target signals. The coding signals identify the DNA sequence, whereas the target signal indicates the presence and the abundance of that sequence (Fig. 6.4).

In addition to direct labeling, QDs is mainly used as electron donor in fluorescence DNA assays. FRET (or Förster resonance energy transfer) is a through space

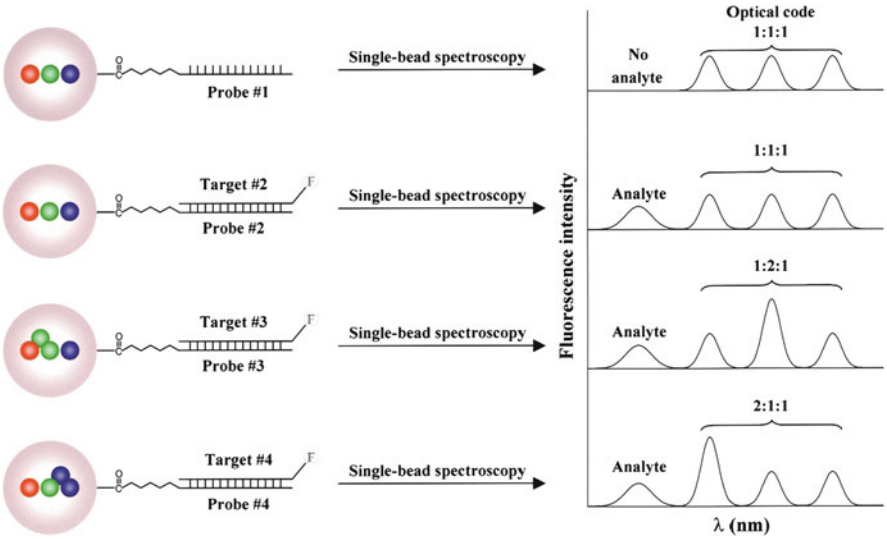

Fig. 6.4 Schematic illustration of DNA hybridization assays using QD-tagged beads. Multicolor optical coding for DNA could be achieved by embedding different-sized QDs into polymeric microbeads at precisely controlled ratios (Reproduced with permission from Ref. [46]. Copyright (2001) Nature Publishing Group)

dipolar coupling interaction that allows electronic energy to be transferred from a donor to an acceptor [47, 48]. The rate of energy transfer depends on the distance between the donor and the acceptor, their relative orientations, and the spectral overlap. The fluorescence emission of QDs has a spectra overlap with the excitation of regular organic dyes. Therefore by careful arrangement of their distance from DNA and of their orientation, FRET may be observed between QDs and dyes.

A typical FRET sensor for nucleic acid, involves a probe DNA attached on a QDs surface. Then a dye labeled target DNA is hybridized with the immobilized probe DNA. As a result, the target DNA brings the fluorophore in close proximity of QDs enough to raise FRET. Several FRET-based sensing strategies for nucleic acids were proposed in the last decades (Fig. 6.5). For example, Ozkan and co-workers modified a CdSe/ZnS QDs with a stem-loop structured probe DNA with an organic quencher 4-(4′-dimethylaminophenylazo) benzoic acid (DABCYL) conjugated at one end of the stem (Fig. 6.5a). In the closed state, the fluorescence of QDs was quenched by DABCYL. In the presence of target that complementary to the loop segment of the probe DNA, DABCYL was brought away from QDs, resulting recovery of fluorescence from QDs [49]. Krull's group designed a strategy to simultaneously detect two target nucleic acid sequences using FRET (Fig. 6.5b) [50]. These two targets were labeled with Cy3 and Alexa Fluor 647, respectively. Probe sequences complementary to these labeled targets were paired with a green QDs and a red QDs. Upon probe–target hybridization, the induced donor-acceptor proximity resulted in FRET-sensitized acceptor fluorescence.

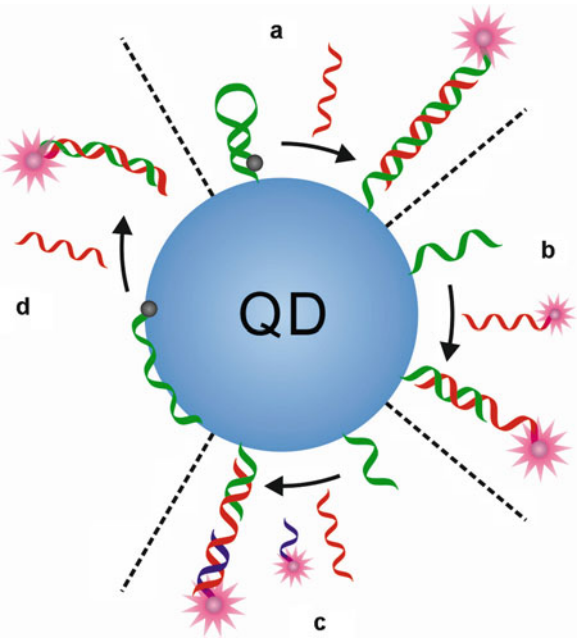

Fig. 6.5 FRET-based sensing strategy for nucleic acids using QDs as donors: (**a**) a QD-based molecular beacon (Ref. [49]); (**b**) hybridization assay for DNA (Ref. [50]); (**c**) a sandwich assay for DNA (Ref. [51]); (**d**) cationic polymer linked QDs and DNA (Ref. [52])

Wang and co-workers have raised a sandwich-typed FRET assay for DNA (Fig. 6.5c) [51]. A streptavidin-coated CdSe/ZnS QDs with emission at 605 nm was paired with Cy5 as an acceptor. The sandwich was constructed with a biotinylated probe DNA complementary to half of target sequence and a Cy5-labeled probe complementary to the other half of target. The strong biotin–streptavidin binding interaction brought the sandwich to the surface of the QD. FRET between QD donor and Cy5 acceptor was observed. Travas-Sejdic employed a cationic polymer as a linker to electrostatically link a dye-labeled probe DNA and QDs (Fig. 6.5d) [52]. The differential interaction of ss- and ds-DNA with $CdTe^{+}$ results in differential changes of FRET efficiency, which was used to recognize the hybridization event.

Besides II-IV semiconductor nanoparticles QDs, Silicon nanoparticlesor nanowires are another class of fluorescence QDs. While bulk silicon is not fluorescent, silicon at nanoscale could generate intense room temperature photoluminescence, which is generally believed to result from a combination of quantum confinement effects. Although of quantum yield (QY) of silicon-based QDs is relatively low (<10 %), they still possesses some unique superiority over II-IV semiconductor nanoparticles QDs in bio-labeling and imaging. For example, the potential toxicity problem of the II-VI QDs associated with release of heavy metal ions (e.g. Cd ions) has not yet been fully addressed, which limits their widespread biological and medical applications [50, 53]. Nontoxic silicon-based QDs reveal the potential as promising candidates. Intense exploration has been done to improve the QY of silicon QDs to facilitate their applications in bio-labeling.

Kortshagen and co-workers recently reported successful preparation of SiQDs with remarkably high ensemble quantum yields exceeding 60 % by using plasma-assisted synthesis with strict removal of oxygen and elaborate surface passivation [54]. Especially, we and our collaborators recently developed a new class of fluorescent silicon nanospheres (SiNSs), each containing several hundreds of SiQDs [55, 56]. The as-prepared nanospheres possess excellent aqueous dispersibility, strong fluorescence (quantum yield: >15 %), robust photo-stability and favorable biocompatibility. Nevertheless the process of synthesis SiQDs with higher QYs, their application in DNA detection assay is still far behind that of QDs, and we believe that this is a promising field for future research.

In addition to QDs, up-converting rare-earth nanophosphors (UCNPs) are another class of fluorescence nanoparticles [57]. In contrast to QDs that adsorb UV light and emit visible light, UCNPs adsorb low energy light, usually near-infrared (NIR) or infrared (IR), and emit high energy light (UV or visible) through multi photon absorptions or energy transfers. UCNPs consist of rare earth atoms that are embedded in a crystalline host matrix. Among the varies of up converting materials, NaYF4 nanocrystals are reported as the most effective host for infrared-to-visible up conversion when doped with Er or Yb ions [58]. Although the quantum yield of UCNPs is not even comparable with QDs, is still possesses some unparalleled advantages in bio-labeling applications. For example, the noninvasive and deep penetration of NIR radiation could realize deep tissue imaging. Meanwhile, since the background fluorescence of living organisms could not be excited by NIR light, UCNPs labeling provides unmatched sharp contrast in imaging.

The bottle-neck of UCNPs in nucleic acid assay comes from the tough conjugation of DNA with UCNPs. Despite recent advances in synthesis of UCNPs with controllable size and shape, the as-synthesized UCNPs are usually coated with hydrophobic organic ligands and suffer from poor water-solubility. Currently, several surface modification strategies have been proposed to enable UCNPs water-soluble and conjugate with oligonucleotides. One general strategy to convert hydrophobic nanoparticles water soluble is to encapsulate with silica or amphiphilic copolymers [59]. For example, Tanke's group has coated Y2O2S:Er UCNPs with silica, which was then conjugated with biotin [60]. This biotinylated-UCNPS were successfully employed as fluorescence reported in nucleic acid arrays. Compared with Cy5 label, the assays with UCNPs reveals four times improved sensitivity. Another versatile strategy for synthesizing water-soluble and carboxylic acid-functionalized UCNPs was performed by directly oxidizing oleic acid ligand to azelaic acid ($HOOC(CH_2)_7COOH$) with the Lemieux-von Rudloff reagent. The free carboxylic acid groups on their surface enable further conjugation with DNA probes [61]. By using this protocol, Huang and co-workers modified UCNPs with a DNA capture probe and constructed a sandwich-type DNA assay including a reporter dye-labeled DNA on UCNPs. Upon illumination of the UCNPs with NIR light, the visible emission light of UCNPs could be adsorbed by the dye, producing FRET from UCNPs to dye; the fluorescence of the dye could be observed as the readout of this UCNPs-based DNA assay.

Nanomaterials as Fluorescence Quenchers

Fluorescent detection methods are still the most employed in nucleic acid assay due to the commercial availability of a wide spectrum of fluorophores, the ease of fluorescent labeling, and the inherent capability for real-time detection. While fluorescent detection is usually highly sensitive, the detection sensitivity is often limited by the presence of background emission. While organic quenchers have proven their utility, they suffer from relatively low quenching efficiency, and, more importantly, quenching efficiencies often vary significantly from one dye to another.

Interestingly, some metallic or carbon nanomaterials have shown ultrahigh fluorescence quenching ability. For example, the quenching mechanism of metal clusters has been well theoretically studied and proven to be a resonant energy transfer process [62–64]. Meanwhile, AuNPs have a Stern–Volmer quenching constant (KSV) that is greater with several (>5) orders of magnitude than that of typical small molecule dye quencher pairs [65]. This "super-quenching" ability of AuNPs has found great implications in fluorescence-based nucleic acid assays that great suppress the background. Dubertret et al. designed an AuNPs-based nanobeacon that replaced organic quenchers with 1.4-nm AuNPs, and found that AuNPs served as a universal quencher that could quench a range of fluorophores with 100-fold higher quenching efficiency than organic quenchers [66]. This so called "nanobeacon" not only led to improved sensitivity but also remarkably high selectivity for single-base mismatch discrimination. Nie and coworkers later reported a stem-less probe on 2.5-nm AuNPs that still demonstrated target-induced conformational change and fluorescence variation in response to the binding of target DNA [67]. Although these small-sized AuNPs revealed decent performance as effective quenchers in nanobeacon, their crowded surface could only accommodate one or several oligonucleotides probes. Recently, we employed larger-sized AuNPs of 15 nm in diameter to construct multicolor nanobeacons [68]. Importantly, it is possible to anchor many DNA probes at one single particle due to the increased surface area. As a proof-of-concept experiment, three DNA probes designed for three tumor-suppressor genes were immobilized at the AuNPs surface, each carrying a unique fluorophore. This nanobeacon exhibited rapid hybridization kinetics (minutes), and could respond specifically to different gene targets (Fig. 6.6). The organic dye in the nanobeacon could also be replaced by fluorescent QDs [15, 69], providing new opportunities for nanobeacons incorporating multiple functional nanoparticles.

In addition to metallic nanoparticles, some carbon nanomaterials including one dimensional (1D) single-walled carbon nanotubes (SWNTs) and two dimensional (2D) graphene have been observed to interact differentially with ss- and ds-DNA, which also enables them to be used as effective quenchers in fluorescence DNA detection. To take SWNTs as an example, ss-DNA has been demonstrated to interact noncovalently with SWNTs [70, 71]. In detail, ss-DNA molecules could wrap around SWNTs by means of π-stacking interactions between the nucleotide bases and the sidewalls of individual SWNT, resulting stable complexes, while for dsDNA, the affinity is significantly weaker. This difference in the binding

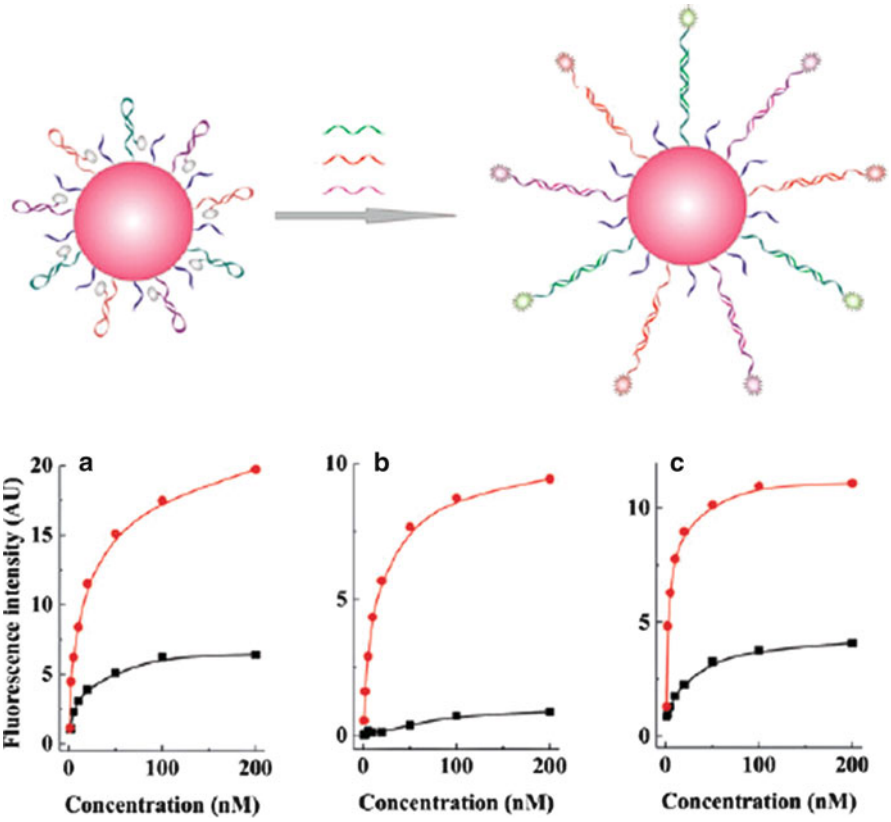

Fig. 6.6 Multicolor nanobeacons for multiplexing detection of three different cancer DNA markers. The multicolor nanobeacons are hybridized with three perfectly matched targets and three single mismatched targets, showing high sequence specificity that arises from the conformational constraint (Reproduced with permission from Ref. [68]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA)

interactions of SWNTs with ssDNA and dsDNA has provided the basis for their use to report DNA hybridization. Plus, SWNTs was found as an effective quencher to quench fluorophores nearby. This quenching was found to be quite universal to a variety of fluorophores, as well as QDs, and it is thought that either energy transfer or electron transfer might contribute to such high quenching efficiency [72, 73].

Tan and Yang combined the super quenching feature of SWNTs together with its' ability to differentiate ss- and ds-DNA, and designed a SWNTs-based strategy for DNA sensing [74]. They demonstrated that fluorophore-labeled DNA probes were efficiently quenched in the presence of SWNTs while they were released from SWNTs upon hybridization, leading to fluorescence recovery [74, 75]. This strategy, however, needs a fluorophore-tagged ss-DNA probe, which increases the detection cost. They also moved one-step further to introduce a DNA intercalating

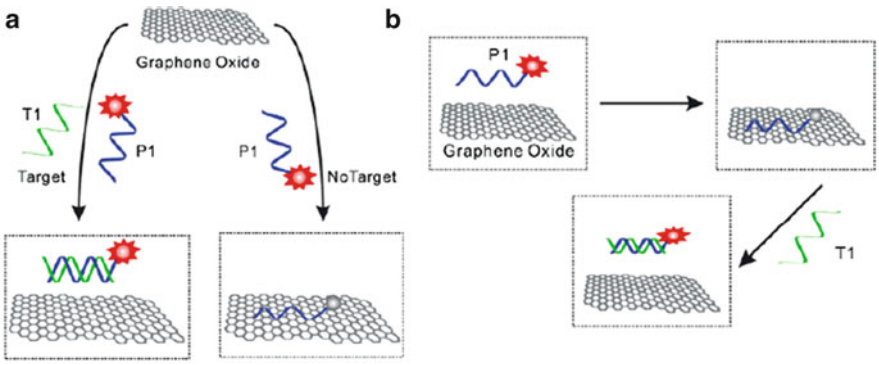

Fig. 6.7 (**a**) Scheme for the fluorescent DNA detection based on the ss-DNA/ds-DNA discrimination ability of GO. (**b**) Scheme for the target hybridization-induced probe liberation from GO (Reproduced with permission from Ref. [80]. Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim)

dye ethidium bromide (EB) to replace fluorophore and developed a cost-effective label free method for fluorescence DNA detection [76].

As a planar molecule, ethidium bromide can absorb on the sidewall of SWNTs. A mixture of the dye, the capturing ssDNA, and SWNTs formed a self-assembly of three components, generating complete quenching of fluorescence. After the introduction of target DNA, ds-DNA formed between the capturing DNA and the target. The adsorbed ethidium bromide preferred to intercalate into the hybridized bases, which disturbed the interaction of the dye and SWNTs, resulting in fluorescence recovery.

Graphene is a single layer of carbon atoms in a closely packed honeycomb two-dimensional lattice [77, 78]. Recently, graphene was also found to be an excellent super fluorescence quencher, however, different from SWNTs, the quenching mechanism of graphene was theoretically and experimentally attributed to be only long-range resonance energy transfer [64, 79]. We carried out molecular dynamics (MD) simulation to describe the interaction of graphene oxide (GO) with ss- and ds-DNA, which indicated that ss-DNA adsorbed strongly to graphene oxide (GO) sheets while ds-DNA did not [80]. Thus, similar to SWNTs, Lu et al. employed GO to adsorb and quench dye-labeled DNA probes, which could be liberated upon hybridization, leading to fluorescence increment [81]. He et al. designed a new approach that relied on different binding kinetic of ss-DNA and ds-DNA to GO, which led to sequence-specific detection with high sensitivity and rapid response time (<5 min) [50]. More significantly, due to the availability of large planar surface of GO, multicolor DNA detection was realized by using differentially labeled DNA probes (Fig. 6.7). Another unparalleled advantage of carbon nanomaterials, is their ability to cross cell membrane [82, 83]. Thus, in vivo analysis or imaging nucleic acid is highly anticipated [64, 84].

6.2.3 *Chemiluminescent and Electro-Generated Chemiluminescent DNA Assay*

6.2.3.1 Chemiluminescent DNA Assay

Luminescence is the emission of light without heat, and more specially the generation of light from chemical reactions (chemiluminescence, CL). This long history of CL has been summarized in a few review articles [85–87]. Since excitation light is not required for sample radiation, problems frequently encountered in fluorescence, such as light scattering, source instability and high backgrounds due to unselective photoexcitation are absent in CL assay. Consequently, CL has found great advantages in biodiagnostics [88].

In a typical CL reaction, an organic dye, which is not luminescent in the ground state, was oxidized by a strong oxidizing agent in the presence of a catalyst (chemical or biological). The most commonly used dye is luminol, while oxidation of luminol with hydrogen peroxide (oxidizing agent) in the presence of a catalyst results in the conversion of the ground state of luminol into an activated state (chemically induced electronic excited states). A strong blue emission (at 450 nm wavelength) can be observed as a result of the decay of the excited states back to the ground state [89]. The most intensively used catalysts that enhance the CL of luminol is horseradish peroxidase (HRP). The mechanism of HRP enhanced CL has been extensively studied [86], suggesting HRP reacts with H_2O_2 to form an oxidized HRP (HRP I) that reacts with the anion of luminol to form a half reduced enzyme (HRP II) and a radical of luminol. The enzyme returns to the reduced form (HRP) by reaction with a second molecule of luminol. The catalytic phenols form preferentially phenoxy radicals in contact with horseradish peroxidase and act as electron-transfer mediators to increase the efficiency of luminol radical formation. However, the luminol-based CL assay is mainly applied to immunoassays instead of DNA detection, which was attributed to the poor sensitivity. In the early phase of luminol-based DNA assay, luminol was directly conjugated with DNA as reporter probe in a sandwich-typed CL assay. A luminol derivative, N-(4-aminobutyl)-N-etylisoluminol (ABEI) is the only tracer used in immunoassay. However, luminol is more efficient in its free state. Upon conjugation with DNA, it lost most of CL properties; thereby this ABEI-tagged CL assay did not reveal satisfied performance. An alternative protocol is to use HRP as an enzyme label in sandwich-typed DNA assay and luminol-H_2O_2 mixture was added to generate CL signal and report the hybridization event, however, which is also found limited success.

In addition to HRP, some other catalysts, including Co^{2+} [90], Cu^{2+} [91, 92], or AuNPs [93, 94] could also enhance the CL of luminol-H_2O_2 system, although the detailed mechanism is not completely clear yet. Among these enhancers, AuNPs has found widely implications in CL DNA assay owing to the ease conjugation of DNA with AuNPs. For example, Willner's group reported that horseradish peroxidase-like DNAzyme-functionalized AuNPs could act as catalytic labels for the amplified detection of DNA with a LOD of 0.1 nM [93]. Li and co-workers demonstrated a

sandwich-typed CL assay for DNA detection using tadpole-shaped AuNPs [94]. Zhang and co-workers found that Au nanoaggregates induced by salt possesses higher enhancement towards the CL of luminol-H_2O_2 than AuNPs [95]. The Au nanoaggregates were used as catalyst in a CL DNA assay and a LOD of 1.1 fM was achieved.

Historically, the CL of luminol system has been preceded by a more sensitive dye of acridinium. Interaction of an acridinium ester (AE) with an alkaline hydrogen peroxide solution would trigger a short flash of light at 430 nm within 5 s or less. Nelson's group have synthesized a number of derivatives of this AE and characterized their chemiluminescent properties [96]. These derivatives display significant differences in the kinetics of the chemiluminescence reaction as well as optimal pH for light production. The first acridinium derivative of practical use was 4-(2-succinimidyl-oxycarbonylethyl)-phenyl-10-methyl-acridinium-9- carboxylate (AE-NHS). AE-NHS does not show the best quantum yield and is not very stable especially at room temperature although its stability is increased after coupling. More efficient compounds are found in thiol, sulphonamide, hydroxamic, oxime and chloroxime series [86]. After covalent conjugation to DNA helix, acridinium labels show increased stability toward hydrolysis. These property enables AE-derivatives to be used in important applications in Chemiluminescence DNA assay, including simultaneous detection of *gag* and *pol* regions of HIV [97] and discrimination of the single-base mismatch between wild-type and mutant sequences corresponding to the reverse transcriptase coding region of HIV-1 [98].

6.2.3.2 Electro-Generated Chemiluminescent Assay

Electro-generated chemiluminescence, or electrochemiluminescence (ECL) is an analogue of CL. ECL is chemiluminescence triggered by electrochemical techniques that undergoes a high-energy electron transfer reaction to generate an excited state. The combination of electrochemistry with chemiluminescence, has proven to be a powerful analytical tool [99, 100]. For example, in the classical ECL of tris(2,2′-bipyridyl) ruthenium $(Ru(bpy)_3)^{2+}$ that was developed by Bard [101], its ECL mechanism has been well-illustrated. $(Ru(bpy)_3)^{2+}$ ECL reaction occurs by oxidizing $(Ru(bpy)_3)^{2+}$ to produce$(Ru(bpy)_3)^{2+*}$ in the presence of a strong reducing agent. The most commonly used reducing agent for this type of ECL reaction is tripropylamine (TPrA). This mechanism is termed the oxidative-reduction mode [102, 103]. It has been proposed that its oxidative-reduction ECL mechanism is as follows:

$$(Ru(bpy)_3)^{2+} - e^- \rightarrow (Ru(bpy)_3)^{3+} \tag{6.1}$$

$$TprA - e^- \rightarrow TprA^{\bullet +} \tag{6.2}$$

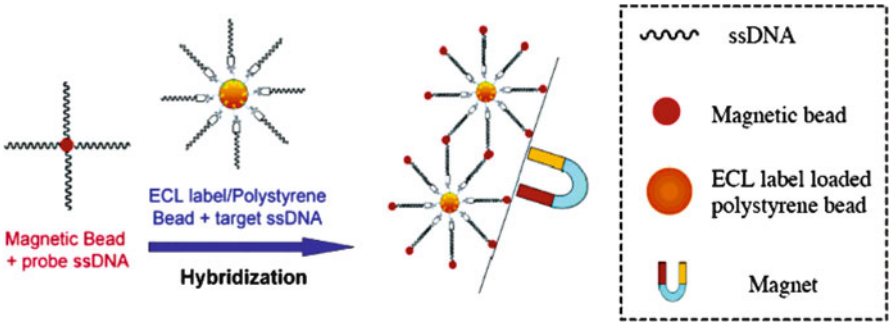

Fig. 6.8 Schematic diagram of DNA hybridization on a polystyrene bead as the ECL label carrier and a magnetic bead for the separation of analyte-contained ECL label/polystyrene beads (Reproduced with permission from Ref. [106]. Copyright (2004) American Chemical Society)

$$\mathrm{TprA}^{\bullet+} \rightarrow \mathrm{TprA}^{\bullet} + \mathrm{H}^{+} \tag{6.3}$$

$$(\mathrm{Ru(bpy)_3})^{3+} + \mathrm{TprA}^{\bullet} \rightarrow (\mathrm{Ru(bpy)_3})^{2+*} + \text{products} \tag{6.4}$$

$$(\mathrm{Ru(bpy)_3})^{2+*} \rightarrow (\mathrm{Ru(bpy)_3})^{2+} + \mathrm{h}\nu \tag{6.5}$$

The distinct advantage of ECL is its high sensitivity. $Ru(bpy)_3^{2+}$ can be highly sensitively detected at subpicomolar concentrations with an extremely wide dynamic range of greater than six orders of magnitude. Consequently, this super high sensitivity of ECL has found great implications in DNA sensors. The application of ECL in DNA assay was summarized in an excellent review [104].

This field was pioneered by Bard's group in 1995 [105], lately they integrated ECL with magnetic concentration to further improve the sensitivity [106]. In their protocol (Fig. 6.8), $Ru(bpy)_3^{2+}$ label was encapsulated in polystyrene (PS) beads. The advantage of PS beads is that one PS bead could carry up to 7.5×10^9 molecules of $Ru(bpy)_3^{2+}$. Then biotinylated target DNA was modified on the surface of these $Ru(bpy)_3^{2+}$ loaded PS beads via biotin-avidin interaction. The target DNA was complementary to a probe DNA that was modified on a magnetic beads surface. Upon addition of a magnetic field, the target DNA-PS beads containing $Ru(bpy)_3^{2+}$ were magnetically separated and transferred into an acetonitrile solution where the polystyrene beads dissolve the ECL label is released. This is followed by ECL detection and a LOD of 1 fM was achieved.

In addition to these solution phase ECL assays, solid state ECL DNA assays were also developed. The first model assay was also proposed by Bard's group [107]. In brief, they first modified a Au(111) electrode with a capture probe DNA. Upon hybridization with target DNA carrying a $Ru(bpy)_3^{2+}$ ECL label, $Ru(bpy)_3^{2+}$ was brought to the surface of electrodes and generated ECL signal. This simple method, however, requires the covalent modification of target DNA

with $Ru(bpy)_3^{2+}$, which is actually inappropriate for clinical diagnostics. Thereby, some sandwich-typed hybridization assays were further developed [108, 109]. An alternative type of hybridization assay was presented by Zhang and co-workers by using the conformational change of probe DNA [110]. The modified Au electrode with thiolated hairpin probe DNA was tagged with $Ru(bpy)_3^{2+}$. In the absence of target ss-DNA, the ECL probe immobilized on the surface of the electrode was in its folded configuration, in which its termini were held in close proximity to the electrode, and thus a strong ECL signal could be generated. In the presence of target ss-DNA, a stem-loop of the ECL probe on the electrode was converted into a linear double-helix configuration due to hybridization, resulting in the tag moving away from the electrode surface, which in turn decreased the ECL signal. This method avoids the introduce of a $Ru(bpy)_3^{2+}$-tagged reporter DNA probe; however the "signal-off" detection mode is not a favorite option in sensor design.

In these works, the employed solid state ECL reporter DNA probe carries only one $Ru(bpy)_3^{2+}$ label, thus it produces only one ECL photon for one hybridization event. Motivated by the work of Bard's group in solution phase [107], researchers also employed polymer beads and nanoparticles that load multiple number of $Ru(bpy)_3^{2+}$ as ECL reporters, thereby one hybridization event could correspond to multiple ECL photons, which may improve the sensitivity. For example, AuNPs prepared by the classical citrate-reduced method is negatively charged, thereby AuNPs are "sticked" to aggregates upon interaction of positively charged $Ru(bpy)_3^{2+}$ [111]. The resulted Au nanoaggregates carry a large number of $Ru(bpy)_3^{2+}$, which might be a powerful label for nucleic acid detection. Another example was reported by Zhang and co-workers by using AuNPs [112]. The large surface area of AuNPs enables them to load multiple number, or multiple kind, of biomolecules. For example, one AuNPs of 12 nm in diameter could carry up to a few hundreds of ss-DNA [113]. Zhang et al. modified a thiolated ss-DNA with a $Ru(bpy)_3^{2+}$ at the other terminal, and the $Ru(bpy)_3^{2+}$-functionalized ss-DNA was further covalently attached to AuNPs, thereby one as-prepared AuNP was loaded with hundreds of $Ru(bpy)_3^{2+}$. The resulted DNA-$Ru(bpy)_3^{2+}$-AuNPs was then used as reporter probe in a sandwich-typed ECL DNA assay and a LOD of 5 pM was achieved.

Some DNA intercalators were recently reported as co-reactants for ECL DNA hybridization assays. Park and co-workers found some duplex DNA intercalators including doxorubicin, daunorubicin or 2-phenylindole shows favorable ECL with $Ru(bpy)_3^{2+}$ at +1.19 V (versus Ag/AgCl), while the non-intercalated ssDNA does not [114]. Several pathogens were detected using this ECL approach, and a good specificity of single point mutations for hepatitis disease was obtained by the 2-phenylindole-intercalated $Ru(bpy)_3^{2+}$ ECL.

It should also be noted that a brand-new ECL system using semiconductor QDs was proposed by Ju's group [115–117]. They found that aqueous-soluble CdTe or CdSe QDs show intensive anodic ECL with a peak value at +1.17 V (vs Ag/AgCl) in basic buffer at an indium tin oxide (ITO) electrode. The ECL emission was demonstrated to involve the participation of superoxide ion produced at the ITO surface, which could inject an electron into the 1Se quantum-confined orbital of CdTe to form QDs anions. The collision between these anions and the oxidation

products of QDs led to the formation of the excited state of QDs and ECL emission. Therefore, CdTe QDs could also be employed as ECL labels in DNA assay and a LOD at fM scale was achieved [118].

6.2.4 Surface Plasmon Resonance (SPR) Nucleic Acid Assays

6.2.4.1 SPR Spectroscopy

Surface plasmon resonance (SPR) spectroscopy is a surface analysis method based on changes in the optical reflectivity of a thin metal film (typically gold) when analytes adsorb or bind to its surface or to any material coated onto its surface (see Chap. 9). Surface plasmon (SP) is defined as the plasma oscillations that can propagate at the interface of a metal and a dielectric medium. The electromagnetic field of a SP is confined at the metal–dielectric boundary and decreases exponentially into both media, thereby SP is extremely sensitive to changes in the refractive index of the dielectric. In a typical SPR spectroscopy, a polarized monochromatic light beam is passed through a prism and its attached gold-coated glass slide, and reflected off the thin gold coating, which is in contact with the liquid solution of interest. Excitation of SP at the gold/solution interface results in nearly complete attenuation of the specular reflected light intensity for incident angles very near the SPR angle. The specular reflected light intensity versus angle at fixed wavelength or versus wavelength at fixed angle reflects the refractive index change at the interface.

Ever since the first SPR instrument (BIAcore) was commercialized in 1990 [119, 120], SPR has been a powerful label-free method for real-time monitoring the biomolecular recognition events at surfaces including immunological analysis, studies of protein-protein interaction, molecular-biological studies on the mechanisms of gene expression, signal transduction and cell-cell interactions, the screening of new ligands, the quantification of protein adsorption and immobilization, the evaluation of surfaces for biocompatibility, epitope mapping, the determination of affinity constants, and the examination of the kinetics of binding [121–123]. In addition to quantitatively detection of analytes, one distinct feature of SPR sensor is that it could also monitor the conformational changes of surface-immobilized biomolecules.

The application of SPR in nucleic acid detection was started in the middle of 1990s [124, 125]. The early stage SPR sensors include one capture on the SPR substrate and the complementary target strand in solution. The hybridization of target strand with capture probe resulted changes of SPR angles. Unfortunately, the inability of conventional SPR to measure extremely small changes in refractive index hinders its application in ultrasensitive detection. To address this drawback, several approaches have been developed. Among them, substantial interest has been focused on utilizing external labels to enhance the sensitivity of the current technique. Keating and co-workers introduced sandwich-typed assay and an AuNPs labeled reporter probe [126]. Since the bulk refractive index of AuNPs is significantly higher than that of DNA and the electromagnetic interaction between metallic nanoparticles and metal surfaces may also help influence the plasmon mode

propagation, the introduced AuNPs label significantly amplifies the SPR response. With the help of AuNPs, LOD for a target was improved from 10 nM of regular assay to 10 pM.

6.2.4.2 SPR Microscopy

Recently, another SPR-related technique, SPR microscopy (SPRM), or "SPR imaging" (SPRi), has been developed to provide high-throughput real time information about the amount and distribution of adsorbed molecules at the interfaces with high spatial resolution [121]. The invention of SPR microscopy [127, 128], led the application of SPR to the imaging of biomolecular surface interactions. In principle of taking SPR images is by introducing a parallel light beam for uniform illumination of a surface at a fixed angle and recorded the reflected beam forming the image at a CCD camera. This configuration is sufficient to determine thickness with Angstrom-level resolution.

The thickness-sensitive feature of SPR microscopy enables it to be a powerful tool to study protein-protein, protein-DNA interactions that induce significant surface topography changes [129]. Corn's group has been very active in the development of SPRi-based detection of oligonucleotides [129–131]. In their early work, a single stranded capture probe DNA was first immobilized on SPR substrate. Upon hybridization with target DNA, the SPR image evolved rapidly, resulting in brighter spots, showing the potential of SPRi in DNA detection [130]. However, DNA hybridization does not induce significant changes in the layer height, thus this proof-of-concept label-free method is actually found limited practical success. Later on, the same group further amplified the SPRi response by introducing a sandwich-typed hybridization and a streptavidin-tagged reporter probe to improve the thickness of the DNA layer resulted by target-induced hybridization [132]. The combination of DNA hybridization and streptavidin-biotin binding improved the LOD for DNA hybridization using SPR imaging by a factor of ~4. Interestingly, the SPRi signal could be further improved by multilayers of streptavidin/DNA formed by successively assembly of biotin-labeled target DNA and streptavidin. This in situ monitoring of DNA hybridization, could be easily integrated to 1D arrays, or 2D arrays created by microfluidic techniques, to realize high-throughput nucleic acid analysis [133]. This hybridization event occurred at the SPR substrate, could be cycled by digestion with ExoIII. The enzymatically-assisted SPR imaging process realized a 10^2–10^3 improvement of sensitivity for a 16-mer target ssDNA [134].

6.2.5 Surface-Enhanced Raman Scattering (SERS) in Nucleic Acid Detection

Raman is a vibrational spectroscopic technique to study vibrational, rotational, and other low-frequency modes of molecules. Raman spectrum is obtained from the

inelastic scattering, or Raman scattering, of monochromatic light in the visible, near infrared, or near ultraviolet range. The limit of conventional Raman spectroscopy is its weak intensity, often requiring the use of powerful and costly laser sources for excitation. Interestingly, Raman scattering could be enhanced by factors of up to 10^8 when a compound is adsorbed on or near special metal surface, or 10^{14}–10^{15} when adsorbed on rough coinage metallic (Cu, Ag, Au) nanoparticles surface [135–137]. This phenomenon is called surface-enhanced Raman scarring (SERS). After decades of debate, the mechanism of SERS could be sorted into two classes which were called electromagnetic and chemical, respectively [137]. Briefly, the former focus on the enhanced electromagnetic fields which can be supported on metal surfaces with appropriate morphologies, while the latter on changes in the electronic structure of molecules which occur upon adsorption and which can lead to resonance.

In addition to ultra-high enhancement factor, SERS also possesses other attractive advantages, such as narrow Raman band, and high resistance to interferences of environmental factors (e.g., humidity, oxygen, and foreign species), thereby has been considered as an attractive method for DNA detection [138, 139]. Mirkin and coworkers pioneered the use of SERS for DNA targets detection [140]. They designed a Raman nanoprobe that consists of 13-nm-diameter AuNPs functionalized with Raman dye-labeled oligonucleotides. After Ag staining, the Ag particles can grow around the Cy3-labeled nanoprobes, leading to large Raman scattering enhancements. Thus, they demonstrated that six different oligonucleotide targets could be simultaneously detected with AuNPs labeled with different Raman probes. Significantly, the detection limit was down to 20 fM due to the strong SERS signal intensity. Moskovits and co-workers proposed an alternative SERS-based strategy for DNA detection (Fig. 6.9) [141]. By self-assembling probe-tethered Ag nanoparticles to a smooth Ag film using the complementary target species, they created electromagnetic "hot spots" on smooth Ag surface, which strongly enhance the Raman signal of the species present in the hot spot. Kim and Lee [142] replaced the smooth metal surfaces with rough metal nanowires to further improve the interface roughness and in turn enhancement factor, and demonstrated a nanoparticle-on-nanowire configuration SERS sensor for multiplex pathogen DNA. Very recently, our group proposed an alternative Ag nanoparticle-on-Silicon nanowire SERS platform and found that the in situ grew AgNPs on Silicon nanowires (SiNWs@AgNPs) revealed a SERS enhancement factor of $\sim 10^{10}$ [143]. Then a sandwich-typed DNA assay with A Raman tag was further constructed on the SiNWs@AgNPs substrate (Fig. 6.10). This Ag nanoparticle-on-Silicon nanowire platform revealized a remarkably low LOD of $\sim$1 fM for DNA target. Faulds and Graham also intensively devoted to the design of novel SERS-based DNA sensor [144–146]. One of their representative work is that they prepared a kind of dye-coded, DNA-functionalized silver nanoparticles [147]. Target DNA-induced sequence-specific hybridization produced the assembly of individual nanoparticles to nanoaggregates, which strongly enhanced the SERS signal of surfaces-coated dyes.

In addition to these efforts of bringing SERS tag-loaded nanoparticles to the close proximity of metal (bulk or nano) surfaces with DNA, another type of SERS-based

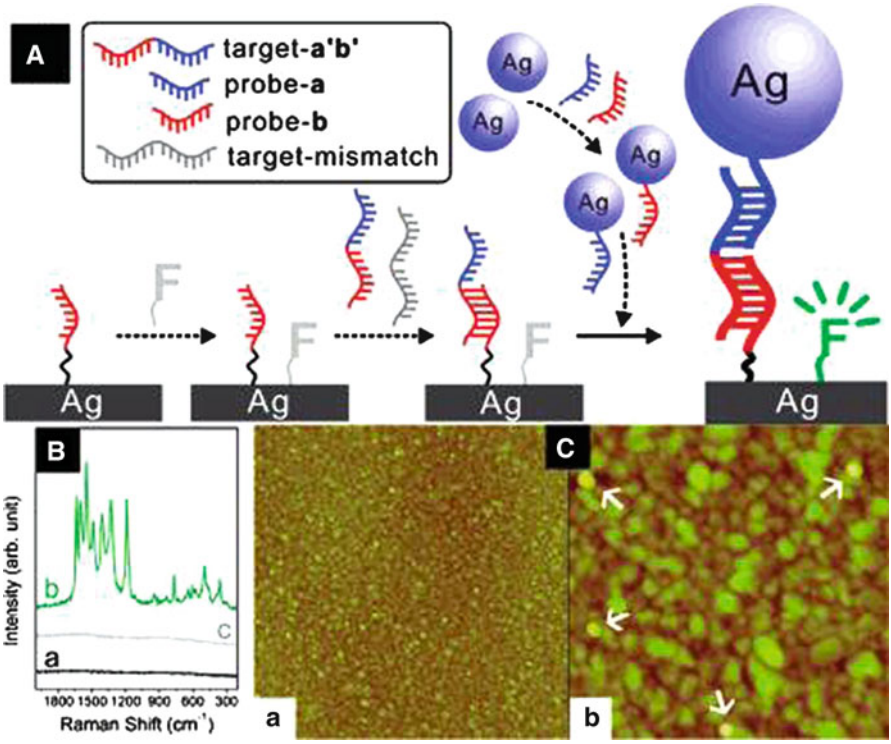

Fig. 6.9 (**A**) Schematic illustration for the detection of ss- DNA by SERS. (**B**) SERS spectra (vertically offset for clarity) indicating the target DNA hybridization-induced enhanced Raman signal. (**C**) Representative AFM images confirming the attachment of AgNPs to Au surfaces by target DNA (Reproduced with permission from Ref. [141]. Copyright (2007) American Chemical Society)

sensor involves the attachment of DNA-labeled SERS tag to the close surface of metal nanoparticles. For example, Vo-Dinh and co-workers [148] represented a plasmonics-based nanoprobe, referred to as a "molecular sentinel" (MS), consisting of a DNA hairpin loop having a Raman label molecule at one end and a metal nanoparticle at the other end. The nanoprobes combines the modulation of the plasmonics effect to change the SERS intensity of the label and the specificity of a DNA hairpin loop sequence to recognize and discriminate a variety of molecular target sequences. Upon hybridization with complementary target sequence that opens the hairpin capture probe, the Raman label was physically removed away from the metal nanoparticle, thus quenching the SERS signal. This "molecular sentinel" strategy was successfully employed for SERS detection o HIV-I virus segment. Johnson and co-workers [149] presented indirect nucleic acid capture strategy to draw SERS tag to the surface of AuNPs with the help of hybridization with target DNA. In their design, a 53-mer West Nile Virus (WNV) genome target DNA was

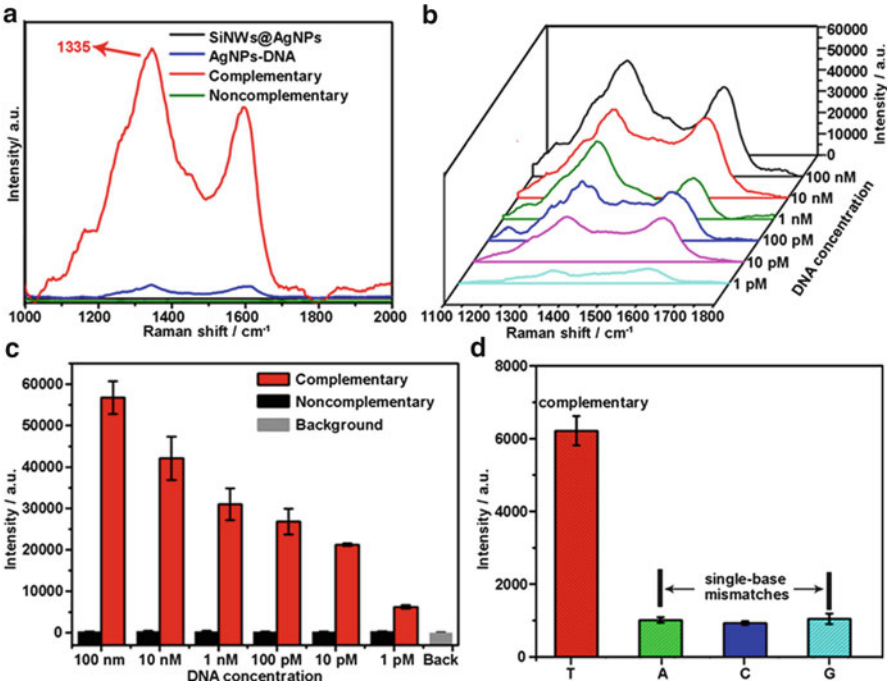

Fig. 6.10 Schematic illustration for the detection of DNA by the SiNWs@AgNPs SERS platform (Reproduced with permission from Ref. [141]. Copyright (2011) Elsevier)

first hybridized in solution with thiolated complementary capture (26-mer) probes and SERS tag-modified reporter (25-mer) oligonucleotide probes, after which the hybridized ternary complex was added to a colloidal suspension of AuNPs and SERS spectra were immediately acquired upon laser excitation.

6.3 Outlook

In this chapter, we have summarized different approaches for optical detection of non-amplified DNA targets. Nanotechnology has shown great advantage in this area. Signal amplification with biomolecular nanoprobes has led to extremely sensitive assays with attomolar detection limit, which represents sensitivity improvement up to six orders of magnitude compared to conventional fluorescent detection (1–10 pM).

Despite such tremendous progress, there remain several challenges for optical DNA detection technologies before they can be widely accepted in clinical or food-related applications. First, DNA hybridization occurring at the nanomaterial surface is inherently low in recognition efficiency and slow in binding kinetics. This is

because the use of inorganic nanomaterials introduces interfaces that bring about the problems of heterogeneous diffusion and convection that are not present in homogenous solution. Second, while some DNA assays have realized ultrahigh sensitivity, they typically require multiple steps that significantly increase the operation complexity. Third, only a few of the mentioned assays show excellent performance in complex biological matrixes (e.g. serum). Thus, non-specific binding is still a major problem that should be studied in order to realize practical applications of these new assay methods.

Notably, the emerging DNA nanotechnology might provide unprecedented opportunities to meet these challenges. For example, the recently developed DNA origami-based chips [150, 151] provide a potentially promising solution to overcome the interface problem since DNA origami structures, in contrast to inorganic nanomaterials, are essentially in aqueous solution. While this method is rather immature in their current form, the combination with high-speed and high throughput readouts as well as automated devices [152], might eventually lead to an unparalleled platform for DNA detection, as well as for the detection of a range of biomolecules.

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Chapter 7
Electrochemical Detection of DNA Using Nanomaterials Based Sensors

Sergio Marín and Arben Merkoçi

Abstract The detection of non-amplified DNA sequences plays a crucial role in the rapid diagnosis of genetic-related diseases especially for early stage treatment. Among the various biosensors that have been used for DNA detection, electrochemical sensors show great promise because they present efficient signal transduction and are capable of precise DNA recognition at a relatively low cost in addition to the broad range of designs with interest to be applied in different kinds of samples. Advancements in micro- and nanotechnologies, specifically fabrication techniques and new nanomaterials, have enabled for the development of highly sensitive, highly specific electrochemical sensors making them attractive for the detection of small sequence variations. Furthermore, the integration of sensors with sample preparation and fluidic processes enables for rapid, multiplexed electrochemical DNA detection essential for point of care clinical diagnostics.

7.1 Introduction

The recent discovery and sequencing of the human genome has provided valuable insight into understanding how genetic factors contribute to the development of diseases. One of the most important factors is the detection of DNA sequence variations that play an important role in the diagnosis of genetic-related diseases, especially for early stage treatment and monitoring [1–3].

Conventional technologies as for example the PCR requires a long time so as to obtain a final diagnostic report. The biosensors in general and especially

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electrochemical DNA sensors can be an interesting alternative. The electrochemical DNA sensors offer simplicity in operation and sample manipulation and provide highly sensitive and specific measurements for a broad spectrum of biomolecules [4–8]. The sample size required is small, ranging several microliters to hundred of nanoliters, which includes the sample pretreatment reagent. Additionally, the detection time is relatively fast, varying from a few minutes to 10 s. However, the most important feature of electrochemical DNA sensors is their potential to be easily transformed from a laboratory-based instrument to a commercializable point of care device. Because of all these advantages, electrochemical biosensing for DNA diagnostics is becoming a very promising area of research and development.

Nowadays, micro- and nanotechnologies have shown emerging potential in electrochemical DNA diagnostics. Electrochemical sensors offer perfect interface for incorporating these technologies, which includes a variety of new materials and fabrication processes. Nanomaterials can be used in various aspects of the detection system including capture probes, reporting molecules, electrode fabrication, and electrode coatings [9–16]. These materials offer improved biocompatibility, additional binding sites and higher signal intensities (via enhanced electrical properties) compared with traditional materials in electrochemical sensors [8, 12, 16–19]. Nanofabrications allow for miniaturization of the sensor, which improve the sensitivity and reduce the samples and reagent volumes, making the detection process more efficient.

A typical electrochemical DNA sensor consists of an electrode, a capture probe and a reporter probe. A capture probe is a DNA strand used to recognize and bind to the target DNA and is usually immobilized onto solid substrate, such as the electrode surface. A reporter probe is a DNA strand that either alone or through a label makes possible the generation of the electrochemical signal as response to the positive hybridization reaction. Both the capture probe and reporter probe are designed so as to show high specificity to the target DNA. Probe, target, and reporter molecules can all be modified or linked with properly integrated nanomaterials. Because of their high surface surface-to-volume ratios and biologic compatibilities, nanomaterials not only increase the signal intensity but also help to accumulate/separate specific DNA molecules during electrochemical reactions, which greatly improve the signal-to-noise ratio, especially for sequence-specific recognition [19]. A wide variety of nanomaterials can be applied, where the most common include metal nanoparticles, quantum dots, carbon nanotubes and nanowires.

7.2 Nanoparticles as Quantification Tags

Nanoparticles can be used in a variety of bioanalytical formats with electrochemical detection. By analogy to fluorescence-based methods, several electrochemical-detection methods have been pursued in which DNA sequences have been labelled with active nanoparticles. The characteristic electrochemical response of nanoparticle reporter signals the hybridizations event.

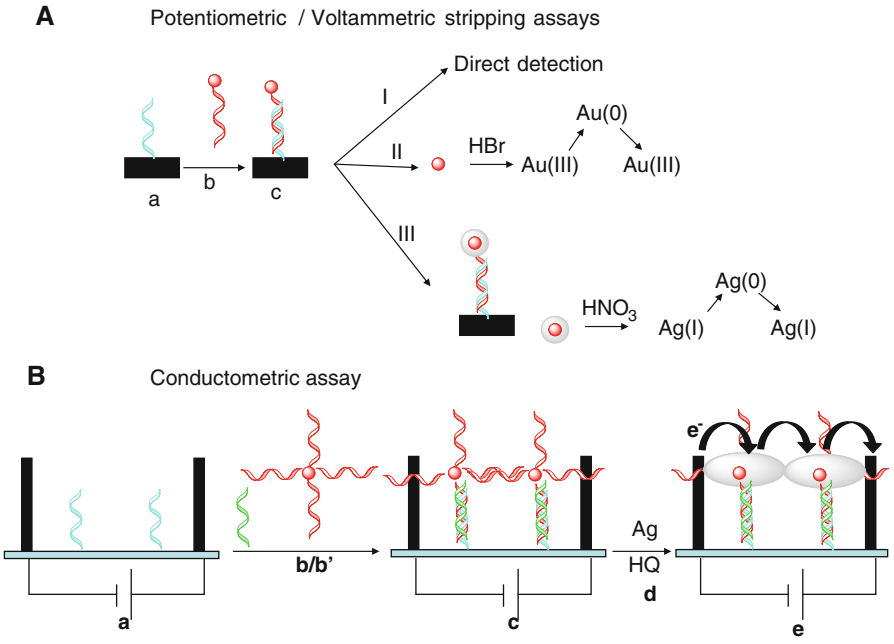


Fig. 7.1 Detection strategies for gold nanoparticles. (**A**) Potentiometric/voltammetric stripping assay. The hybridization event occurs between DNA strand (*a*) and gold-tagged DNA (*b*). The gold-labeled duplex (*c*) formed is then detected according to each strategy: (**I**) direct detection of the nanoparticles onto the bare electrode without the need for tag dissolution (Adapted from reference [20]) (**II**) the gold nanoparticles are dissolved with HBr/Br_2 treatment and then detected by stripping techniques (Adapted from reference [21]) and, (**III**) the gold nanoparticles are first covered with Ag by a deposition treatment and then detected by stripping techniques via silver enhanced signal (Adapted from reference [22]) (**B**). Conductivity assay. Probe DNA immobilized in a small gap between two electrodes (*a*) is hybridized with DNA target (*b*) and then with gold-modified DNA probes (*b′*). Gold is accumulated in the gap (*c*). Silver enhancement (*d*) is performed in the presence of hydroquinone (*HQ*). The silver precipitated onto the gold nanoparticles (*e*) improves the sensitivity of the assay by lowering the resistance across the electrode gap (Adapted from reference [24])

7.2.1 Gold Nanoparticles

Gold nanoparticles are the most popular nanoparticles that have been used as labels to hybridization signals of DNA. Three strategies for DNA detections of gold have been reported (see Fig. 7.1).

According to the first strategy, a direct detection of gold nanoparticle without the previous dissolution is reported [16, 20]. A DNA strand modified with gold nanoparticles is hybridized with DNA probe that is marked with paramagnetic beads. These complexes are concentrated onto the graphite-epoxy composite electrode that incorporates a magnet in its matrix. The differential pulse voltammetry is used for a direct voltammetric detection of resulting gold-DNA-paramagnetic bead complex [20] (see Fig. 7.1AI).

According to the second strategy (see Fig. 7.1AII), the intrinsic electrochemical signal of the nanoparticle can be observed after dissolving it with HBr/Br_2 [21]. The gold (III) ions obtained were preconcentrated by electrochemical reduction onto an electrode and subsequently determined by anodic-stripping voltammetry.

The third strategy used for detection of gold nanoparticles uses the silver deposition on the gold nanoparticles after the hybridization and enhanced electrochemical signal attributed to silver is obtained [22, 23]. The accumulated silver is dissolved and detected afterwards using a potentiometric stripping protocol (Fig. 7.1AIII).

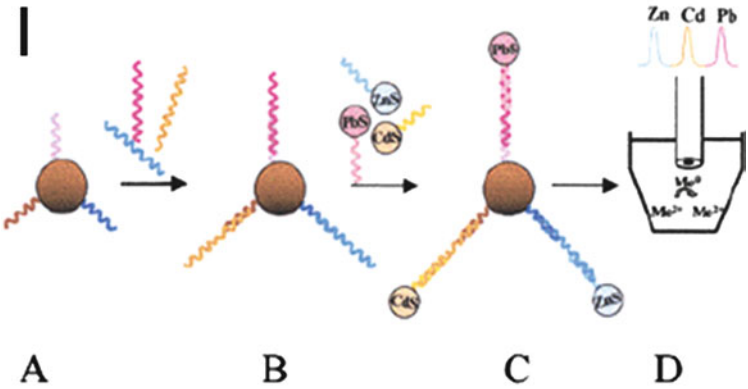
Nevertheless, other interesting methods have been reported. Mirkin et al. [24] have exploited the silver-deposition technique to construct a sensor based on conductivity measurements. In their approach, a small array of microelectrodes with gaps (20 μm) between the electrodes leads is constructed, and probe sequences are immobilized on the substrate between the gaps. Using a three-component sandwich approach, hybridized target DNA is used to recruit gold nanoparticle-tagged reporter probes between the electrode leads. The nanoparticle labels are then developed in the silver-enhancer solution leading to a sharp drop in the resistance of the circuit (Fig. 7.1B).

7.2.2 Quantum Dots

In a similar way as the gold nanoparticles quantum dots (QDs) also are detected using three different strategies (see Fig. 7.2). According to the first strategy, after DNA hybridization quantum dots are dissolved using nitric acid solution and the electrochemical detection of cadmium (II) ions is performed (Fig. 7.2A) [25, 26].

The second strategy is based on nanoparticle-promoted cadmium precipitation (see Fig. 7.2B), using a fresh cadmium solution hydroquinone, used to enlarge the nanoparticle tag and amplify the stripping DNA hybridization signal [27]. In addition to measurements of the dissolved cadmium, it was demonstrated also in direct "solid-state" measurements following a "magnetic" collection of a "magnetic bead/DNA hybrid/CdS tracer" assembly onto a thick-film electrode transducer.

Fig. 7.2 Schematic representation of a DNA biosensor that uses magnetic beads. The formed CdS-labeled duplex is detected according to each strategy: (**I**) Multi-target electrical DNA detection protocol based on different inorganic colloid nanocrystal tracers. (*A*) Introduction of probe-modified magnetic beads. (*B*) Hybridization with the DNA targets. (*C*) Second hybridization with the QD-labeled probes. (*D*) Dissolution of QDs and electrochemical detection. (Adapted from reference [25]) and,(**II**) the CdS QDs are first enhanced with cadmium by a deposition treatment using hydroquinone and afterwards dissolved with HNO_3 and detected by stripping techniques (adapted from reference [29]) (**III**) Schematic representation of sandwich protocol (not toscale): (*A*) immobilization of the biotinylated CF-A probe onto the streptavidin-coated magnetic beads; (*B*) first hybridization between CF-T and CF-A; (*C*) second hybridization between CF-T and CF-B modified thiol; (*D*) addition and capture of the CdS quantum dots (Adapted from reference [30])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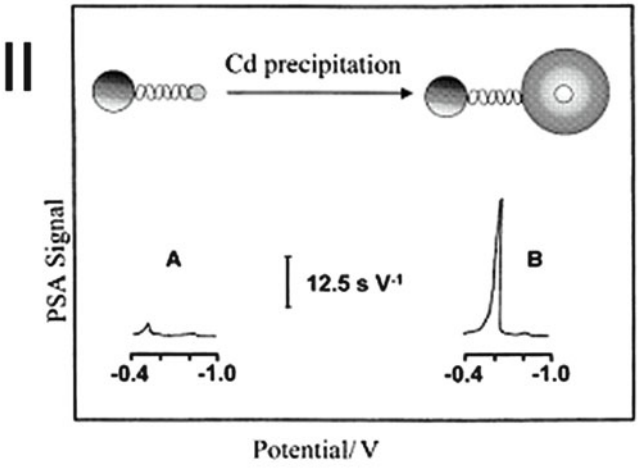
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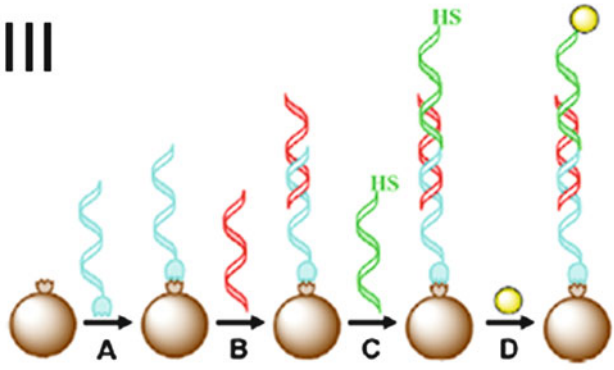
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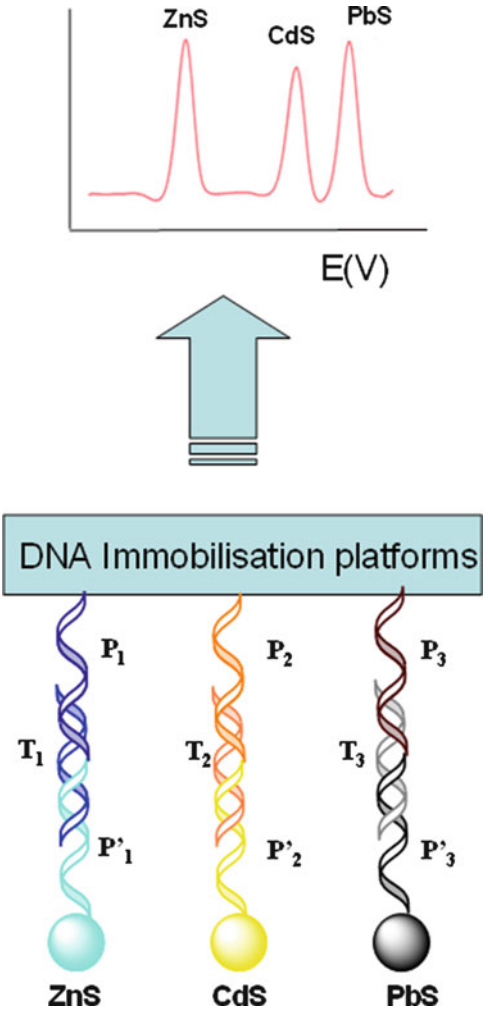


Fig. 7.3 Schematic of multiple detection of DNA. DNA probes (P′1, P′2 and P′3) bearing different DNA sequences with different nanoparticles (ZnS, CdS and PbS, respectively) that enable the simultaneous detection of three DNA targets (T1, T2 and T3) hybridized with corresponding DNA-capturing probes (P1, P2 and P3) immobilized onto a direct or an indirect (magnetic particles) transducing platform/s (Adapted from reference [25])

According to the third strategy, electrochemical detection of a cadmium sulfide quantum dots (CdS QDs)–DNA complex connected to paramagnetic microbeads (MB) was performed without the need for chemical dissolving (Fig. 7.2C) [28].

The labeling of probes bearing different DNA sequences with different nanoparticles enables the simultaneous detection of more than one target in a sample, as shown in Fig. 7.3. The number of targets that can be readily detected simultaneously can be controlled by the number of voltammetrically distinguishable nanoparticle markers.

Following this idea a multi-target sandwich hybridization assay involving a dual hybridization event, with probes linked to three tagged inorganic crystals and to magnetic beads has been reported [25].

The DNA-connected QDs yielded well-defined and resolved stripping peaks at −1.12 V (Zn), −0.68 V (Cd) and −0.53 V (Pb) at the mercury coated glassy carbon electrode (vs. the Ag/AgCl reference-electrode) (Fig. 7.3).

7.2.3 Other Nanoparticles

Other nanoparticles such as silver nanoparticleshave been used in electrochemical DNA sensors to amplify the obtained signal (Fig. 7.4I). As reported by Fu et al. using DNA biosensors based in situ DNA amplification with nanosilver as label and horseradish peroxidase enzyme (HRP) [29]. The thiolated oligomer single-stranded DNA (ssDNA) was initially directly immobilized onto a gold electrode. A competitive format, hybridization reaction was carried out via immersing the DNA biosensor into a stirred hybridization solution containing different concentrations of the complementary ssDNA and constant concentration of nanosilver-labeled ssDNA, followed by binding with HRP. The adsorbed HRP amount on the probe surface decreased with the increment of the target ssDNA in the sample. The hybridization events were monitored by using differential pulse voltammetry (DPV) toward the reduction of H_2O_2 generated by HRP. The reduction current from the enzyme-generated product was related to the number of target ssDNA molecules in the sample.

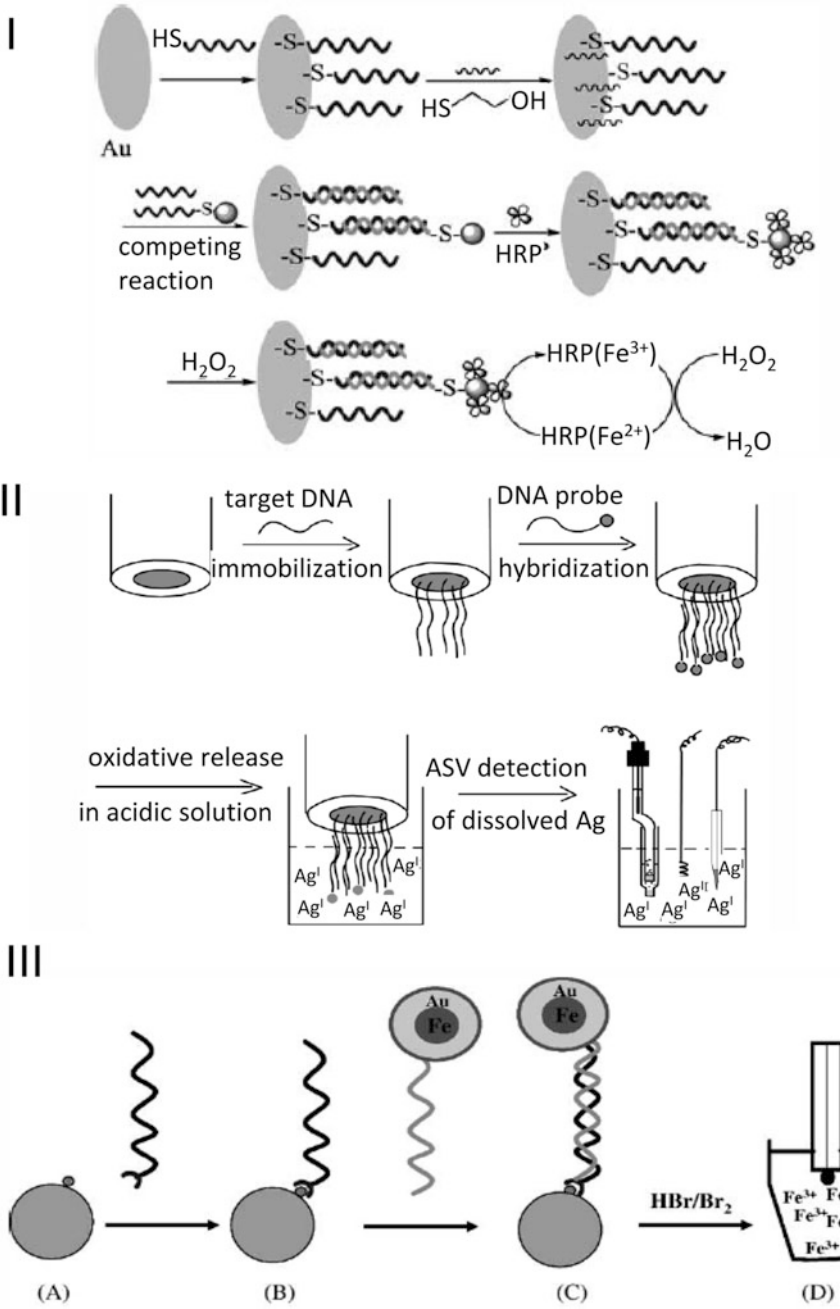
In another assay the hybridization of the target DNA with the silver nanoparticle–oligonucleotide DNA probe, followed by the release of the silver metal atoms anchored on the hybrids by oxidative metal dissolution and the indirect determination of the solubilized Ag^+ by anodic stripping voltammetry (ASV) at a carbon fiber ultramicroelectrode is performed (Fig. 7.4II) [30].

Besides QDs, gold-coated iron nanoparticles also have been used in DNA-detection assays [31]. After hybridization, the captured gold-iron nanoparticles used as DNA labels are dissolved and the released iron is quantified by cathodic stripping voltammetry in the presence of the 1-nitroso-2-naphthol ligand and a bromate catalyst. The DNA-labeling mode developed offers high sensitivity, well-defined dependence on concentration, and minimal contributions from non-complementary nucleic acids (Fig. 7.4III).

7.3 Nanowires and Nanotubes for Labelling and Signal Enhancement

7.3.1 Carbon Nanotubes as Substrates for DNA Attachment

Carbon nanotubes (CNTs) were first noticed and characterized in 1991 by Iijima [32] of NEC Corporation in Japan. CNTs are a new allotrope of carbon originated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HRP(Fe^{2+})
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H_2O
II
target DNA
immobilization
DNA probe
hybridization
oxidative release
in acidic solution
ASV detection
of dissolved Ag
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(A)
(B)
(C)
(D)

from fullerene family, which can be described as a graphite sheet rolled up into a nanoscale-tube (which are single-wall carbon nanotubes, SWCNTs), or with additional graphene tubes around the core of an SWCNT (which are multi-wall CNTs, MWCNTs) [33]. MWCNTs consist of two or more concentric cylindrical shells of graphene sheets coaxially arranged around a central hollow area with spacing between the layers which is close to that of the interlayer separation as in graphite (0.34 nm). In contrast, SWCNT are made of single graphene (one layer of graphite) cylinders and have a very narrow size distribution (1–2 nm). CNTs offer unique electronic and mechanical properties combined with chemical stability. The combination of a biological compound with CNT to monitor a biochemical event seems to be a promising alternative for biosensors designs due to the catalytic effects that this nanomaterial can bring to the detection event.

DNA-end attachment via covalent linkage to the CNTs surface has been demonstrated and further extended for the hybridization of the captured strands to their complementary target sequences. CNTs as sensing surfaces for nucleic acids detection have been extensively demonstrated [34–37].

Tang et al. [34] modified the gold electrodes by carboxylic group-functionalized CNTs activated using N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimidobiotin (NHS) and covalently connected the ssDNA.

Abdullin et al. [35] modified glassy-carbon electrodes (GCEs) with preoxidized CNTs. According to the data of atomic force microscopy, the layers of CNTs on GCEs possess a homogeneous nanostructurized surface. Guanine and deoxyguanosine monophosphate could be strongly adsorbed on GCE/CNT and oxidized at +690 and +930 mV (pH 7.0), respectively. Similar attachment of DNA onto CNTs was also reported by Kerman et al. [36] and Erdem et al. [37].

7.3.2 CNTs as Signal Amplifier for Hybridization

The conductive properties of CNTs suggest that they could mediate electron transfer reactions and enhance the relative electrochemical reactivity with electroactive species in solution when used as the electrode material or modifiers. As electrode or substrates modified materials, CNTs show better electrochemical behavior than traditional glass carbon electrodes, carbon paste electrodes and other carbon electrodes [38].

Fig. 7.4 (**I**) Schematic representation of electrochemical detection of DNA using nanosilver as label and HRP as enhancer (Adapted from reference [31]). (**II**) Schematic representation of electrochemical stripping detection of DNA hybridization based on a silver nanoparticle label (Adapted from reference [32]). (**III**) Schematic representation of the DNA-detection protocol based on gold-coated iron nanoparticle tags: (*A*) introduction of the streptavidin-coated polystyrene beads; (*B*) attachment of the biotinylated target; (*C*) hybridization with the iron/gold particle-labeled probe; (*D*) dissolution of the iron/gold particle tag and stripping voltammetric detection at a hanging mercury drop electrode (Adapted from reference [33])

Therefore, CNTs used as substrates for DNA attachment in electrochemical biosensors, as explained previously [39] could also be regarded as electrochemical signal amplifier. The guanine oxidation signal of double stranded calf-thymus DNA after 3 min accumulation was 20 times higher at a CNTs modified glassy carbon electrodes cross-linked with glutaraldehyde (GTA) than at a bare GCE using differential pulse voltammetry, while the peak potential was around 45 mV less positive. The guanine oxidation signal was demonstrated to be highly reproducible, with 3.4% RSD for five different electrodes [40].

Recently, CNTs have also been utilized as a novel support material to concentrate nanoparticles or enzyme molecules on it as a more powerful DNA hybridization indicator than using a single nanoparticle or enzyme molecule (see Fig. 7.5). Wang et al. [41] described an effective method for amplifying electrical detection of DNA hybridization based on CNTs carrying a large number of CdS particle tracers. Such use of CNT amplification platforms was combined with an ultrasensitive stripping voltammetric detection of the dissolved CdS tags following dual hybridization events of a sandwich assay on a streptavidin modified 96-well microplate.

Anchoring of the monolayer-protected quantum dots to the acetone-activated CNT was accomplished via hydrophobic interactions. SEM images indicated that the nanocrystals were attached along the CNT sidewall, with a loading of around 500 particles per CNT. A substantial (500 fold) lowering of the detection limit was obtained compared to conventional single particle stripping hybridization assays, reflecting the CdS loading on the CNT carrier. A large excess (250 fold) of non-complimentary oligonucleotides had minimal effect on the response. They also demonstrated the use of CNTs for dramatically amplifying enzyme-based bioaffinity electrical sensing DNA [42]. In the new bioaffinity assays, CNTs played a dual amplification role in both the recognition and transduction events, namely as carriers for numerous enzyme tags and for accumulating the product of the enzymatic reaction. Such coupling of several CNT-derived amplification processes led to the low detection limit (Fig. 7.5II). Li et al. [43] developed an ultrasensitive electrogenerated chemiluminescence (ECL) detection method of DNA hybridization based-on SWCNT carrying a large number of ruthenium complex tags. The probe of single strand DNA (ssDNA) and ruthenium complex were loaded at SWCNT, which was taken as an ECL probe (Fig. 7.5III). When the capture ssDNA with a thiol group was self-assembled onto the surface of gold electrode, and then hybridized with target ssDNA and further hybridized with the ECL probe to form DNA sandwich conjugate, a strong ECL response was electrochemically generated.

7.3.3 Nanowires for DNA Attachment

Multisegment nanowires also have been used via a straightforward surface functionalization method for DNA detection [44]. Nanowires comprising CdTe-Au-CdTe segments are fabricated using electrochemical deposition. The electrical characterization indicates a p-type behavior for the multisegment nanostructures,

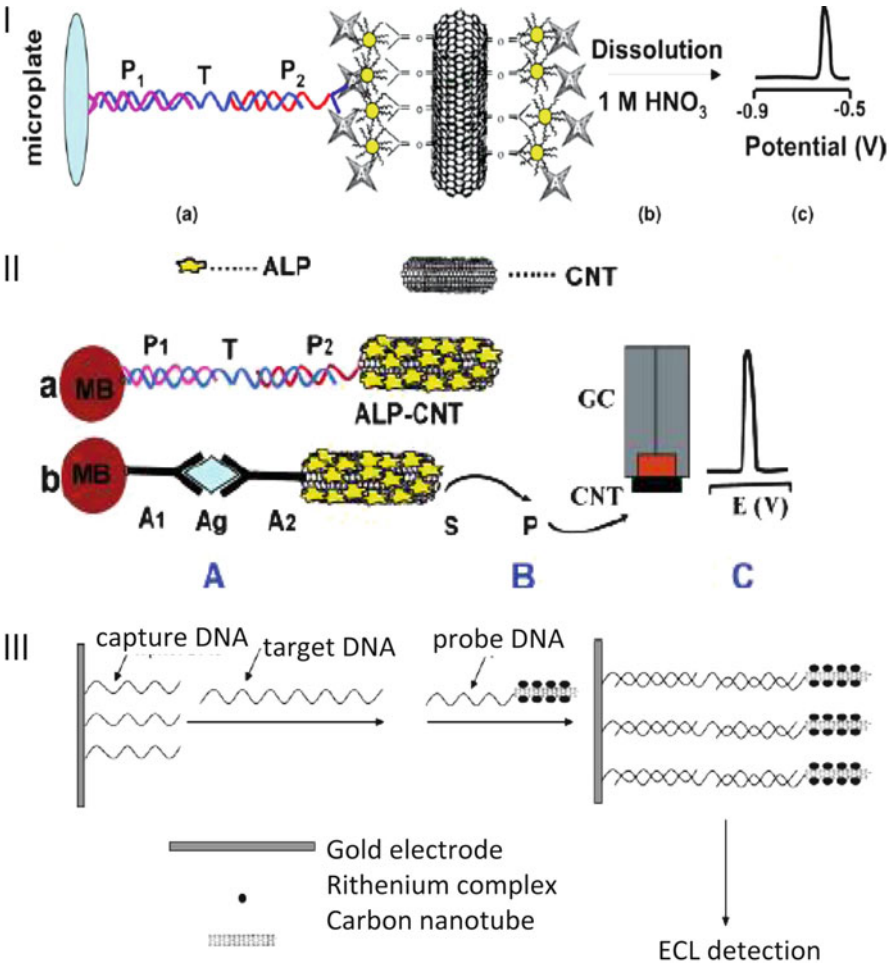


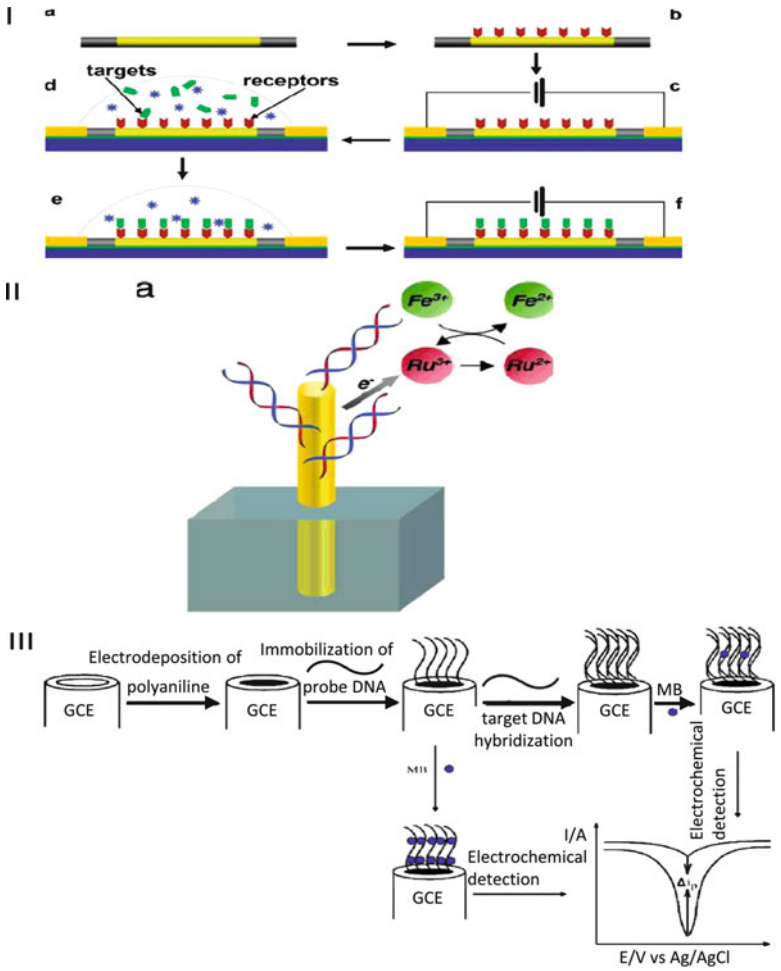
Fig. 7.5 Carbon nanotubes as carriers (**I**) of QDs. Schematic representation of the analytical protocol: (*a*) Dual hybridization event of the sandwich hybridization assay, leading to capturing of the CdS-loaded CNT tags in the microwell; (*b*) dissolution of the CdS tracer; (*c*) stripping voltammetric detection of cadmium at a mercury-coated glassy carbon electrode. P1, DNA probe 1; T, DNA target; P2, DNA probe 2 (Adapted from reference [43]). (**II**) of alcalino phosphatase (*ALP*). Schematic representation of the analytical protocol: (*A*) Capture of the ALP -loaded CNT tags to the streptavidin-modified magnetic beads by a sandwich DNA hybridization (*a*) or Ab-Ag-Ab interaction (*b*). (*B*) Enzymatic reaction. (*C*) Electrochemical detection of the product of the enzymatic reaction at the CNT-modified glassy carbon electrode; *MB* Magnetic beads, *P* DNA probe 1, *T* DNA target, *P2* DNA probe 2, *Ab1* first antibody, *Ag* antigen, *Ab2* secondary antibody, *S* and *P* substrate and product, respectively, of the enzymatic reaction, *GC* glassy carbon electrode, *CNT* carbon nanotube layer (Adapted from reference [44]) (**III**) of Rutenium complex (Ru). Schematic diagram of the electrogenerated chemiluminescence (*ECL*) detection for DNA hybridization using sandwich DNA detection model and the ECL probes of carbon-nanotubes loaded with probe ss-DNA and Ru 1 tags. ECL measurement was performed at a constant potential of +1.30V in 2.0mL of 0.10M PBS (pH 7.4) containing 0.10M TPA (Adapted from reference [45])

in a back-to-back Schottky diode configuration. Such nanostructures modified with thiol-terminated probe DNA fragments could function as sensors for biomolecules at very low concentration. The gold segment is utilized for functionalization and binding of single strand DNA (ssDNA) fragments while the CdTe segments at both ends serve to modulate the equilibrium Fermi level of the heterojunction device upon hybridization of the complementary DNA fragments (cDNA) to the ssDNA over the Au segment. Employing such multisegment nanowires could lead to the fabrication of more sophisticated and high multispecificity biosensors via selective functionalization of individual segments for biowarfare sensing and medical diagnostics applications (Fig. 7.6I).

Arrayed gold nanowires represent a novel and useful platform for electrochemical DNA detection. One factor contributing to the heightened sensitivity is the high signal-to-noise ratio achieved with the large electrocatalytic signals observed at DNA-modified nanowire (Fig. 7.6II). Lapierre-Devlin et al. [45] explain the improved sensitivity of the DNA detection with the fact that the electrocatalysis of nanowire membrane at DNA-modified nanostructures generates amplified signals that are significantly larger than those observed at bulk gold surfaces. The results strongly suggest that the three-dimensional architectures of the nanowires facilitate the electrocatalytic reduction of Ru(III) because of enhanced diffusion occurring around these structures.

Zhu et al. reported a novel and sensitive electrochemical DNA biosensor based on electrochemically fabricated polyaniline nanowire and methylene blue for DNA hybridization detection [46]. Nanowires of conducting polymers were directly synthesized through a three-step electrochemical deposition procedure in an aniline-containing electrolyte solution, by using the glassy carbon electrode (GCE) as the working electrode (Fig. 7.6III). The diameters of the nanowires range from 80 to 100 nm. The polyaniline nanowires-coated electrode exhibited very good electrochemical conductivity. Oligonucleotides with phosphate groups at the 5′ end were covalently linked onto the amino groups of polyaniline nanowires on the electrode. The hybridization events were monitored with differential pulse

Fig. 7.6 (**I**) (*a*) An individual CdTe-Au-CdTe multisegment nanowire, fabricated by electrochemistry, is functionalized on the Au segment with thiol-terminated molecules. (*b*) Fabricated modified nanowire FET device using lithography. (*c*) Immerse the nanowire into the solution containing different biospecies. Specific DNA targets are self-assembled to the receptors at the nanowire surface (*d*) Immerse the nanowire into the solution containing different DNA strands. (*e*) Specific DNA strand are self-assembled to the receptors at the nanowire surface. (*f*) Sensitive conductance responses was obtained after bound with targets (Adapted from reference [46]). (**II**) Schematic illustration of Ru(III)/Fe(III) electrocatalysis at a DNA-modified Au NEE (Adapted from reference [47]). (**III**) Schematic representation of the DNA-sensors. First step the electrochemical deposition of polyaniline nanowires onto GCE was performed, the next step DNA probe was immobilized onto polyaniline nanowire layer; the hybridization of DNA probe with DNA target is performed. The metaline Blue was added (to hybridization complex and the DNA probe step) to carry out the electrochemical detection, obtaining the electrochemical signal when the hybridization reaction was performed (Adapted from reference [48])



voltammetry (DPV) measurement using methylene blue (MB) as an indicator. The approach described here can effectively discriminate complementary from non-complementary DNA sequence, with a detection limit of 1.0×10^{-12} mol l^{-1} of complementary target, suggesting that the polyaniline nanowires hold great promises for sensitive electrochemical biosensor applications.

7.4 Conclusions and Future Perspectives

The integration of nanotechnology with biology and electrochemistry is expected to produce major advances in the field of electrochemical sensors. Recent progress has led to the development of functional nanoparticles that are covalently linked to

biological molecules, such as peptides, proteins and nucleic acids. To be biologically relevant, nanoparticles need to have surface functionality amenable to biological modification, and long-term stability in a range of buffered saline solutions and pH values, and limited non-specific binding.

The future direction of electrochemical DNA sensors is focused on the development of point-of-care systems, which seek to integrate sample handling, fluidic processing, and detection on a portable platform. Although electrochemical biosensors have been widely developed for laboratory-based detection within the past several years, there are very few successful point-of-care devices for clinical diagnostics that are currently commercialized (i.e. glucometers).

Biosensor technology in clinical testing offers several potential advantages over other clinical analysis, including high analytical sensitivity and specificity, increased assay speed and flexibility, automation, capability for multi-target analyses, reduced costs of diagnostic testing and the potential to bring health care delivery closer to the community. Electrochemical devices have traditionally received the major share of the attention in biosensor development. Thick film technology using a screen-printed procedure is a widely used method for the simple and fast mass-production of inexpensive disposable electrochemical sensors.

Clinical applications for electrochemical DNA sensors are still far from reality due to several important issues. Although much work has been done to improve the performance of electrochemical DNA sensors, the sensitivity/specificity is still a key issue. Specifically, the detection of clinical samples requires high sensitivity/ specificity as well as high repeatability/reliability, which is still an unresolved problem. To address these issues, new nanomaterials with effective and stable performance are required along with higher stringency control during manufacturing. Additionally, the accuracy for clinical detection can be enhanced though bio-statistic support based on multiple DNA biomarkers. To improve the application of electrochemical sensors for real clinical tests, a simple detection process is desired, which incorporates automatic sample processing or in situ detection. This can be achieved through using micro/nanotechnologies, which offers new materials and sensor fabrication processes. Furthermore, the safety of nanomaterials is becoming a significant issue, especially as applications of these materials become more widespread.

Several other electrochemical based sensing technologies reported for proteins [47–51] detection using nanoparticles and other nanomaterials can also be applied in the future for DNA sensing. Catalytic methods, applied for example for a model protein (Human IgG) detection based on hydrogen evolution [52] or even nanochannels [53, 54]could be extended in the future in DNA sensing.

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Chapter 8
Piezoelectric Sensing for Sensitive Detection of DNA

Maria Minunni

Abstract Piezoelectric sensing has been widely applied for affinity sensing, and recently sensitive DNA detection has been reported in different matrices for different analytes (i.e. target sequences). In this chapter, the detection principle and the approaches used in DNA-based sensing with focus on detection of microsatellite DNA, present in high number of copy as well as target sequence detection of genes present in one or few copy number per haploid genome will be presented and discussed. Particular attention will be devoted to the pre-analytical steps which may influence the sensor response to the target analyte such as genomic DNA fragmentation and denaturation. Comparison between immobilization chemistries is also presented. In particular, finding in microsatellite detection with both biotinylated and thiolated probes is reported and discussed.

8.1 Introduction

The utilization of piezoelectric quartz crystal oscillators as microbalances (QCM) has been applied in Analytical Chemistry, both in gas and in liquid phases. In literature there is a wide number of papers based on commercially available or in house developed devices, using crystals ranging from 5, up to 10, 30 MHz, using in QCM in the fundamental frequency or in the relative overtones. Applications of QCM's as thin film monitors and controls; in surface science, plasma-assisted etching, analytical chemistry, and space system contamination and for aerosol mass measurements have been reported over the last 20 years. Details of their methodology are scattered widely throughout the literature.

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8.1.1 *Piezoelectric Biosensors: Theory and Applications of Piezoelectric Effect*

Piezoelectric, similarly to Surface Plasmon Resonance (SPR) transduction methods is widely used in biosensors development, mainly because it allows the detection of label-free targets and the kinetic measurement of molecular interactions in real-time. Moreover, piezoelectric crystals as SPR chips can be regenerated, allowing a multi-use of the sensor. Piezoelectric crystals are acoustic transducers generally utilized as microbalance or microviscosimeters. In particular, piezoelectric quartz crystals are the basic elements of quartz crystal microbalance (QCM) device. Quartz resonators are the most used crystals, a crystal variant of Silicon-dioxide (SiO_2).

However, independently from the material used, the term "piezoelectric" derived from the Greek word piezen meaning "to press". The first investigation on the piezoelectricity was performed in 1880 by Jacques and Pierre Curie [1], who observed that a mechanical stress applied to the surfaces of various kinds of crystal caused a corresponding electrical potential across the crystal, whose magnitude was proportional to the applied stress. The Curies also verified the converse piezoelectric effect, in which application of a voltage across these crystals caused a corresponding mechanical strain. This causes a vibrational, or oscillatory, motion in the crystal, resulting in the generation of acoustic standing waves at a characteristic resonant frequency. The wave is called bulk acoustic wave (BAW) or surface acoustic wave (SAW) in the case of propagation through the substrate or on the surface, respectively. A few naturally abundant crystals (i.e. quartz, tourmaline and Rochelle salt) are piezoelectric, but many other materials exhibit this effect, including quartz analogue crystals, such as berlinite ($AlPO_4$), ceramics with perovskite or tungsten-bronze structures ($BaTiO_3$, $KNbO_3$, $LiNbO_3$, $LiTaO_3$, $BiFeO_3$, $NaxWO_3$, $Ba_2NaNb_5O_5$, $Pb_2KNb_5O_{15}$). However, recently piezoelectric crystals based on gallium orthophosphate ($GaPO_4$) have been reported for different application, including biosensing [2, 3]. $GaPO_4$ production has been, first set up by AVL List GmbH, Graz Austria, for applications as pressure monitoring in motors combustion engines, since the thermal coefficient of this material is compatible with the high temperature reached in car engines. Characteristics of this material are, in particular, high sensitivity, stability up to 970 °C, no pyroelectricity, no stress induced twinning, high electric resistivity >1,015 Ωcm. For all these interesting properties different applications have been identified and investigated also within an European project entitled "Growth of Large $GaPO_4$ Single Crystals and their use for Special Sensor Applications", (contract n° G5RD-CT-2002-00709, coordinated by Prof. Dr. Peter Kremple at AVL), who had foreseen in particular some Bulk Wave Applications such as high shear $GaPO_4$ crystal rheometer, $GaPO_4$ crystal particulate microbalance (GCPM) and Nanobalance Affinity Sensor for DNA-based sensing (i.e. GMO) and drug detection. Within this project sensitive DNA detection was studied using conventional quartz crystals and eventually $GaPO_4$.

However, only quartz provides the unique combination of mechanical, electrical, chemical and thermal properties, which allowed its commercial success. Direct

and converse piezoelectricity has a wide range of applications. Piezoelectric quartz crystals are used in quartz watches, computers and in many high-performance devices (such as Atomic Force Microscopy) to apply tiny mechanical displacements on the scale of nanometers. Furthermore, such crystals are employed as quartz crystal microbalance for thickness monitoring.

The quartz crystal microbalance is a bulk acoustic wave device based on the converse piezoelectric effect, in which a quartz crystal is sandwiched between two electrodes. The resonant frequency of the quartz crystal depends on several parameters, such as size, density and cut. The most used devices employ AT-cut quartz crystals, sliced with an angle of 35°10′ respect to the optical z-axis (Fig. 8.1a). AT-cut quartz crystals show a high frequency stability and a temperature coefficient close to zero between 0 and 50 °C [4]. AT-cut crystals oscillate in the thickness shear mode (TSM) [5].

The application of a voltage between the two electrodes causes a shear deformation of the crystal, which is maximized at the crystal faces, making the device sensitive to surface interactions. The resonant condition with the acoustic wave is satisfied by including the crystal into an oscillation circuit, where the frequency of the alternating potential difference applied to the electrodes matches the fundamental frequency of the crystal. The fundamental frequency depends upon the thickness of the wafer, its chemical structure, its shape and its mass [6]. Since the oscillation frequency depends on the crystal mass, deposition of thin films on the crystal surface increases the resonator thickness and decreases the frequency in proportion to the film mass. Measurements of the crystal frequency allow the detection of the film mass, therefore the device operates like a 'microbalance'. The first quantitative investigation of the piezoelectric effect was performed by Sauerbrey [7], who derived the relationship for the change in frequency ΔF (in Hz) caused by the added mass Δm (in g) in vacuum or in air:

$$\Delta F = -\frac{2F_0^2}{A\sqrt{\mu_Q \rho_Q}} \cdot \Delta m \tag{8.1}$$

where F_0 is the fundamental resonant frequency of unloaded quartz, μ_Q is the shear modulus of AT-cut quartz ($2.947 \times 1{,}011\ \mathrm{g\,cm^{-1}\,s^{-2}}$), ρ_Q is the density of the quartz ($2.648\ \mathrm{g\,cm^{-3}}$) and A is the surface area in $\mathrm{cm^2}$. The Sauerbrey equation assumes a uniform distribution of mass on the entire electrode portion of an AT-cut quartz crystal. Mass sensitivity decreases monotonically with the radius, in a Gaussian manner becoming negligible at and beyond the electrode boundary [8]. Another assumption of this equation is that the mass added or lost at the crystal surface does not experience any deformation during the oscillation: this is true for thin, rigid layers in vacuum or in air. For thicker, less rigid layers, as it happens for quartz crystals operating in liquid, a more complex theory is necessary. Many factors such as density, viscosity, conductivity and dielectric constant of the liquid may influence the oscillating behavior. When a quartz crystal oscillates in contact with a liquid, a shear motion on the surface generates motion in the liquid near the interface.

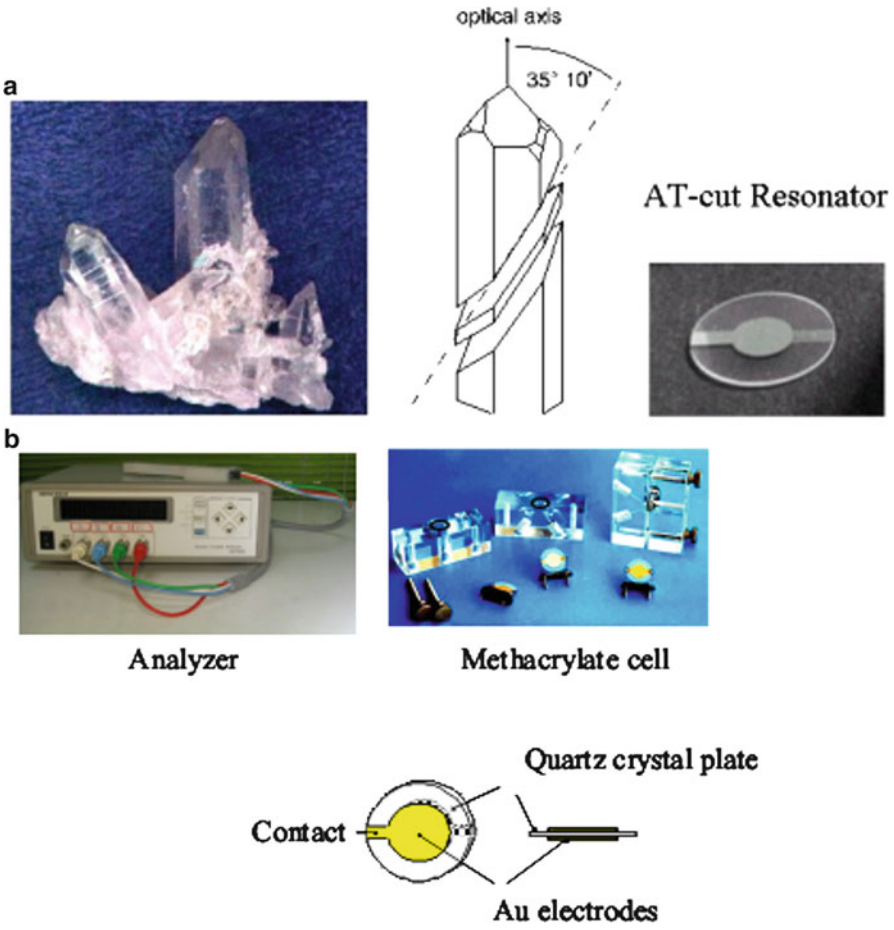

Fig. 8.1 (**a**) The cut-angle with respect to crystal orientation (so-called AT cut) determines the mode of induced mechanical vibration. AT-cut quartz crystals with a cut angle of 35° 10′ with respect to the optical z-axis perform shear displacements perpendicular to the resonator surface [Wegener website]; (**b**) instrumentation: Frequency meter with the oscillator; methacrylate cell where the crystal is housed and scheme of the sensor

The resonant frequency change of a quartz crystal having one face in contact with liquid is described by the Kanazawa and Gordon equation [9]:

$$\Delta F = -F_0^{3/2} \left(\rho_L \eta_L \, / \pi \mu_Q \rho_L \right)^{1/2} \tag{8.2}$$

where ρ_L is the density of the liquid and η_L is the viscosity of the liquid. Piezoelectric crystals have been used as microbalances and as a microviscometer owing to their small size, high sensitivity, simplicity of construction and operation, low cost, lightweight and the low power required [10]. The quartz-crystal microbalance has

traditionally been used in many applications such as thin film deposition control, etching studies, aerosol mass measurements and space system contamination studies. Recently however, the interest in the application of piezoelectric devices in the field of analysis has increased, since it was realized that many opportunities for molecular sensing can be opened up once a suitable recognition layer or molecule is coated on the crystal [11]. Therefore, immobilizing an affinity ligand on the surface of a quartz crystal covered with gold or silver, an affinity mass sensor is realized. When the affinity reaction with the target in solution takes place, the binding ligand can be determined at nanogram levels from the piezoelectric frequency shift. Such piezoelectric biosensors have found a wide range of applications in food [12, 13], environmental [14] and clinical [15, 16] analysis. A typical instrumentation used is displayed in Fig. 8.1b.

8.1.2 Nucleic Acid–Based Piezoelectric Sensing

Among different Affinity Based Biosensors (ABBs), nucleic acid sensing has appeared to be one of the most explored. When developing nucleic acid based approaches, a capturing sequence (probe), responsible for the system selectivity has to be immobilized on the sensing surface. The sequence, complementary to the probe added in solution, binds the surface with consequent frequency decrease, due to surface hybridization reaction and complex (dsDNA) formation. In Fig. 8.2 a typical hybridization signal is displayed. The recorded analytical signal is the frequency shift between the frequency value before sample injection, taken in buffer (base line) and its value after sample injection followed by washing with buffer to remove the sample excess and unbound material, leaving on the surface just hybridized sequences. Ideally, the frequency shift should be different from 0 if the analyte, i.e. the target sequence, is present in the sample and 0 if not. For checking the system selectivity negative control (non complementary sequence) should be also injected on the surface and the recorded should be 0. Negative control should be always used to control eventually present unspecific binding i.e. adsorption, phenomena at the surface. This is extremely important for real analytical system application.

Generally, the complementary sequence to the target analyte (a gene, a fragment or a short oligonucleotide) is first considered during the choice of probe. The probe length is generally set ranging from 15 to 20 bases, with one end usually linked to a functional group to be exploited for the immobilization chemistry. For example, biotinylated probes are used in surface functionalization involving streptavidin; thiolated probes [17], are required for direct probe coupling to gold surfaces via Self Assembled Monolayer (SAM) formation [18]. In Fig. 8.3 is reported a scheme of the relative immobilization protocols.

Some immobilization approaches are summarized in Tombelli et al. [19, 20]. Another criterion taken into account in probe selection is the C-G base content (three hydrogen bonds vs. two with A-T pairing); preferred composition varies from at least 40 to 60 % to stabilize the hybrid on the surface [21]. Finally, to facilitate

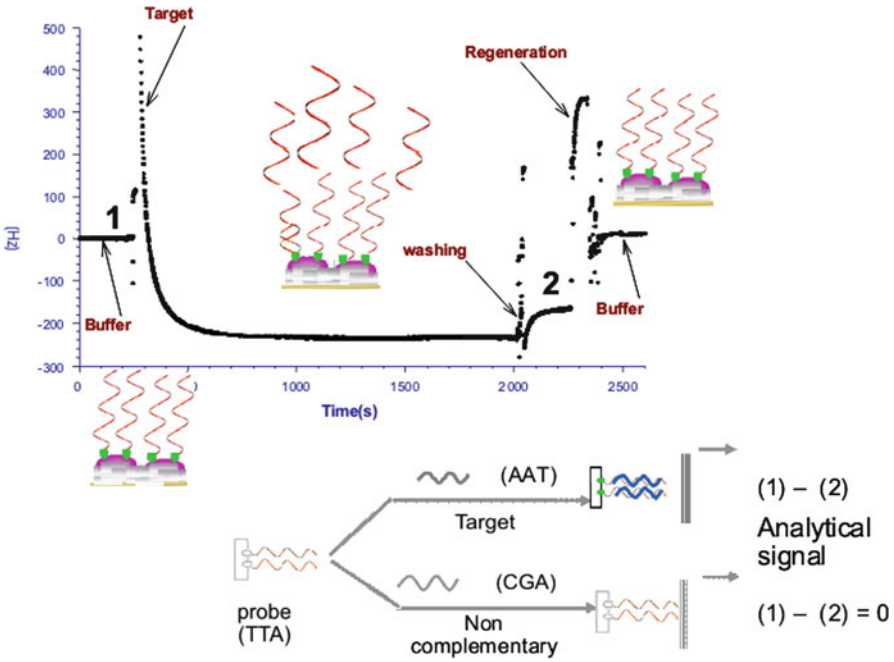

Fig. 8.2 Scheme of the signal recorded by piezolelectric sensing during a measurement cycle (see text). The hybridization reaction between the immobilized probe and the target sequence in solution is displayed

surface hybridization, it is important to avoid probe hybridization on regions that can assume conformations and may obscure the binding site of interest, i.e. by formation of secondary structures such as hairpins or loops.

Recently it has been eventually reported by our group, an optimized and reproducible strategy for probe design for nucleic acid-based sensing based on a free available software computational assisted approach. The *in silico* selection, was validated by experiments conducted using optical transduction for DNA-sensing development, in particular Surface Plasmon Resonance imaging (SPRi), demonstrating that "smart" probe design for DNA-sensing significantly improves the sensor's analytical performances for DNA-DNA hybridization measurements. This approach can be easily transferred to piezoelectric transduction [22].

Behind the probe selection, the immobilization chemistry is strategic for the success of the nucleic acid sensor development. Many immobilization chemistries, using different approaches, have been developed, depending from the sensor surface. Among the available methods, we mainly focused on thiols based approaches, using thiolated probes and dextran modified chip following the chemistry used in the gold chip of the SPR-based Biacore family instrumentation. During the sensor developments the system is first studied using synthetic oligonucleotides to evaluate the system's main analytical parameters such as sensitivity, selectivity, reproducibility expressed as coefficient of variation (CV%), detection limit (DL),

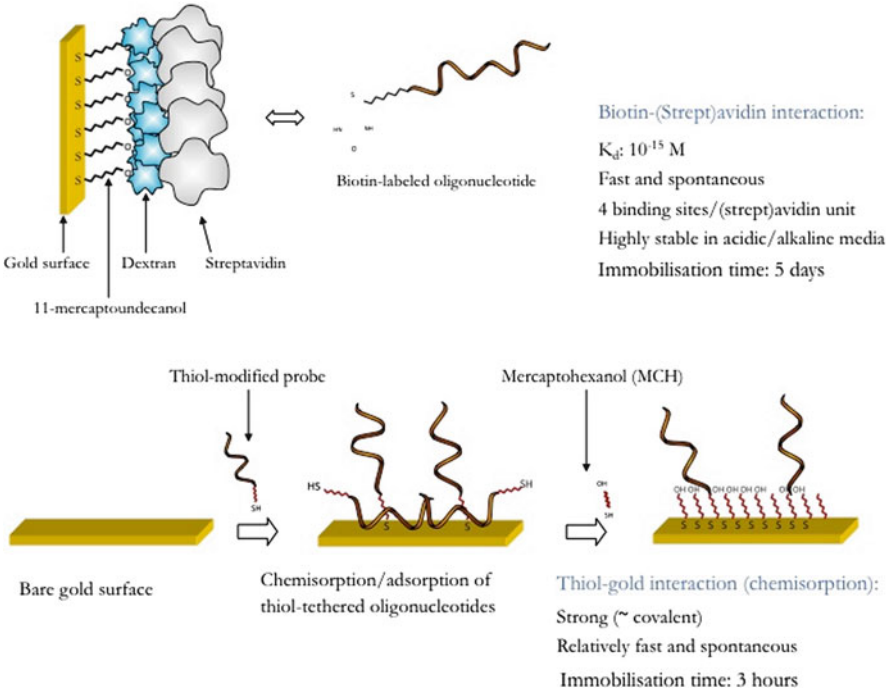

Fig. 8.3 Immobilization chemistry using (**a**) thiol/dextran/streptavidin modified surfaces for biotinylated probe binding; (**b**) direct coupling of thiolated probe with further surface passivation with short thiols

analysis time, etc. Once the assay conditions have been optimized one can move to real sample analysis. For real analysis thus matrix effects have to be evaluated and pre-analytical steps, i.e. sample-pre-treatment, should also be considered. In nucleic acid sensing, the sample pre-treatment is a key step. DNA can be detected in different cellular compartments (nucleic, mitochondrial, etc.) but it should be extracted from the cell. Double stranded DNA is the form in which the sample is obtained after its extraction.

8.2 Development of Piezoelectric Sensing for DNA-Based Sensitive Detection

The extracted DNA can be then purified by precipitation and different widely used protocols are available [23]. Re-suspension of DNA can be achieved in buffer or distilled water. In any case the extracted DNA is a double helix, with the target sequence hybridizing the probe immobilized on the sensor surface, hidden in the double helix. Thus it is clear that the dsDNA should be opened to allow surface hybridization of the target sequence.

8.2.1 Sample Pre-treatment for Target Sequence Analysis

8.2.1.1 DNA Fragmentation

Fragmentation of DNA is achieved using ultrasound generating random fragments or by enzymatic digestion by nucleases that breaks the helix at specific sites, different for the various nucleases, able to originate fragments eventually containing the intact target sequence. In literature related to genomic detection of sequences by DNA-based sensing using ultrasound has been successfully reported [24, 25]. Alternatively fragmentation can be achieved by restriction enzymes, proteins that recognize specific, short oligonucleotides sequences (the "restriction enzyme cutting sites") and cut DNA at those sites and nowhere else [26]. Bacteria contain over 400 such enzymes that cut over 100 different DNA sequences. The restriction enzymes cleave DNA in a very specific fashion. Type II restriction enzymes, most commonly used for DNA analysis and genetic engineering, have a unique nucleotide sequence at which they cut a DNA molecule. The recognition sequence is often a six base pair palindromic sequence (the top DNA strand from 5′ to 3′ is the same as the bottom DNA strand from 5′ to 3′), but some recognize four or even eight base pair sequence. Some sites occur frequently in DNA (e.g. every several hundred of base pairs), other much less frequently (rare-cutter; e.g., every 10,000 base pairs).

There are two kinds of restriction enzymes: restriction "endonuclease" and restriction "exonuclease". Nuclease is the general term for enzymes that catalyse the hydrolysis of nucleic acids by clearing chains of nucleotides into smaller units. The endonuclease is a nuclease that claves nucleic acid at interior bonds and so produces fragments of various size; while exonuclease is a nuclease that releases one nucleotide at a time (serially) starting from a nucleic acid termination. Other restriction enzymes can be used to further characterize a particular DNA molecule. The location of these restriction enzyme cleavage sites on the DNA molecule can be compiled to create a "restriction enzyme map". These maps are very useful for identifying and characterizing a particular DNA plasmid or region. Restriction fragment length polymorphism map (RFLP) are widely employed in clinical diagnostic for point mutation detection as well as in food analysis (see below). The selection of the enzyme to be applied in genomic DNA digestion is performed by the analysis with free available software.

8.2.1.2 Denaturation of dsDNA

In this section we will discuss about the importance of the sample pre-treatment in terms of dissociating the two strands of the DNA double helix (dsDNA) using different strategies. We strongly believe that for sensitive DNA detection, such as direct sequence analysis in unamplified genomic DNA, the dissociation step is of key importance. For this reason we spent quite a lot of time in studying and evaluating different approaches, starting from the most popular denaturing

thermal treatment up to the addition of suitable chemicals or oligonucleotides to the sample to prevent strand re-annealing. Thermal treatment is very simple and for this reason is widely employed, consisting in heating up the dsDNA to 90 °C for 5 min with further cooling and injection into the instrumentation. Many examples demonstrated the success of this procedure, which is particularly suited with amplified DNA material, i.e. by PCR. This procedure, on the contrary, ends in very low reproducible results, not applicable analytically, when target sequence sensitive detection has to be achieved, as in the case of single copy sequences per haploid genomic DNA in systems without any amplification [25]. Thermal denaturation can be eventually applied to satellite DNA, significantly (i.e. in many copies) present in haploid genome [27], as we will discuss later on in this chapter. We had this feeling already with optical detection using Biacore family instrumentation (BIAcore X™) where we demonstrated that in this system re-annealing of the injected sequence occurs with important reflection on the system analytical performances [28]. To be clearer, in the case of this instrumentation or eventually in flow mode systems, we think re-annealing of the strands can occur, preventing hybridization from the analyte (target sequence) with the immobilized probe. As consequence reduced amount of ssDNA analyte are available for hybridization. This reduced amount may became significant and critic in case of genomic DNA, where only few copies of target are available for probe hybridization. If significant target depletion by re-annealing occurs, no significant analytical signal may be registered, unless when signal amplification systems i.e. by gold nanoparticles are used [25].On the base of our initial findings with sample thermal denaturation, we then focused on dedicated sample pre-treatments optimization for improving sensitive DNA sensing performances, eventually starting with amplified DNA to go further into genomic DNA target sequence detection. We initially studied different approaches both at the PCR and post-PCR level aiming to improve sensor signals and reproducibility for further application to sensitive DNA sensing. The developed approaches have a general validity, independently of the transduction principle used. We focused our approaches in particular on two sequences dealing with detection of transgenosis markers contained in the promoter region (P35S) of the cauliflower mosaic virus (CAMV) ribosomal RNA and TNOS terminator, indicated in the official protocols as the target analyte for genetically modified organisms (GMO) detection [29]. The rational we used in these approaches was to evaluate the possibility either to isolate single stranded target DNA or to prolong the half-life of the ssDNA in the denaturation step, by preventing re-annealing. ssDNA can be obtained using magnetic particles for capturing and precipitation or by enzymatic digestion [13]. The approaches used to obtain selective extraction of the single strand, containing the target sequence, are based on the use of proper primers, modified by biotinylation or phosphorylation. In particular streptavidin modified particles were used to bound biotinylated DNA, while one phosphorylated strand was obtained in the amplified DNA, using phosphorylated primers. The modified ssDNA was selectively recognized and digested by a lambda exonuclease. Other researchers, as well as our group, have employed asymmetric PCR with optical sensing to obtain significant amount ssDNA target in solution as with asymmetric

amplification (i.e. SPR, Biacore family instrumentation), by suitable ration of reverse and forward primers, an excess of ssDNA containing the target sequence can be synthetized, preventing re-annealing with the other strand of the dsDNA, now present in much lower amount [30–32]. We will not further comment these just mentioned approaches because they can be applied only to amplified DNA samples, since primers have to be employed. However the full understanding of encountered problems and the developed solutions, stresses the importance of the denaturing step in improving DNA-based sensing performances. Thus further strategies have to be introduced for sensitive sequence detection in genomic DNA. To this aim, behind this PCR-based mentioned approaches, we evaluated other denaturation methods assisted by the use of chemical reagents added to the DNA sample. In particular denaturation treatments can be performed by the combination of a strong alkaline environment and a formamide treatment at 42 °C. Denaturation of dsDNA in strong alkaline conditions is well established. At a pH 13 the charge of the DNA bases changes, thus preventing H-bond formation [33]. On the other hand, the influence of organic compounds such as formamide, urea and formaldehyde on the thermal DNA denaturation process has been well documented [23]. Formamide is a helix destabiliser that replaces the native DNA bases for inter-strand hydrogen bonds, thus inducing the denaturation of dsDNA [34]. Results were obtained again with amplified and genomic DNA [32], using different concentration of chemical, but still the applied treatment was found to be insufficient to generate reproducible and significant SPR sensor signals. These treatments however were eventually applied to piezoelectric DNA to evaluate the ability of sensitive genomic DNA detection [35]. Finally an innovative approach was developed, based on the use of small selective oligonucleotides added to the denaturing mixture, for hybridizing, at a selected annealing temperature, the two ssDNA of the helix. The oligos attachment site can be designed and should not overlap the ssDNA region involved in the surface probe binding. The hybridization of these small oligos prevents re-annealing of the two strands, leaving the target sequences to hybridize the immobilized probe, thus prolongating the single stranded state of the target sequence. For its re-annealing blocking behaviour, the approach was named “denaturation by blocking oligos”.

In Fig. 8.4 is displayed a scheme of the developed approach and the differences with the conventional thermal denaturation. Initially Biacore X™ instrumentation was used [36] in the protocol optimization as proof of principle. Once demonstrated the suitability of the “blocking oligonucleotides approach” for improving sensitive DNA detection of three different sequences in genomic DNA, the latter developed denaturing approaches based on the addition of formamide at different percentages or of blocking oligos were applied to sensitive detection of target DNA present in single or few copies per haploid DNA by piezoelectric sensing [37].

We will here report about the finding obtained initially by our group and more recently appeared in literature relative to sequence detection in unamplified genomic DNA. We will first discuss the detection of specific micro satellite DNA detection with application to food analysis and further detection of sequences present in few copies per haploid genome, identified as marker of transgenosis.

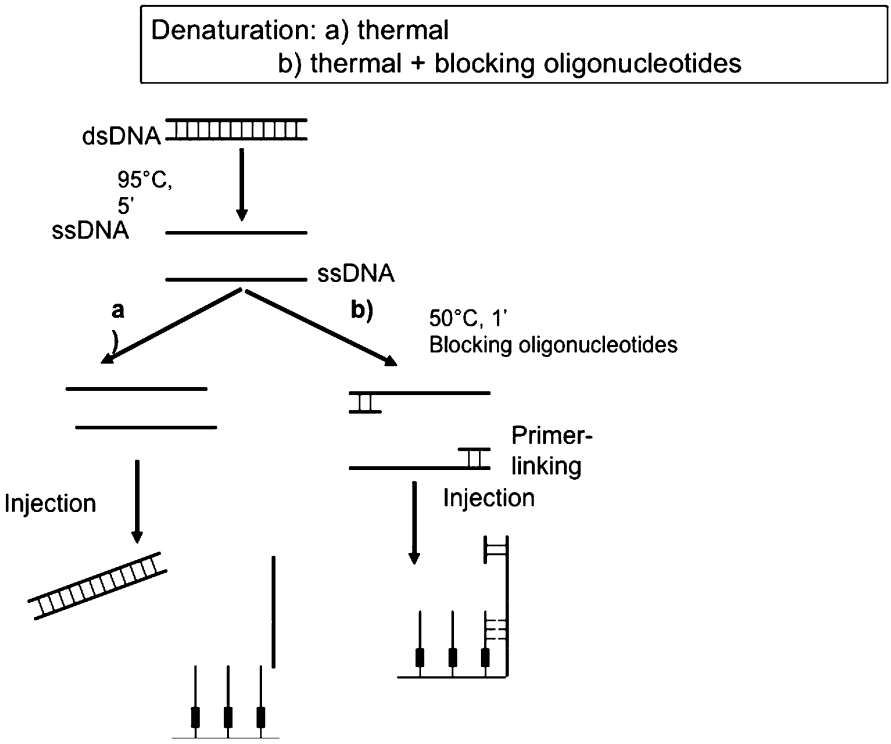

Fig. 8.4 Denaturation of dsDNA (**a**) thermal denaturation; (**b**) thermal with blocking oligonucleotides

8.3 Detection of Microsatellite Sequences in Bovine Genomic DNA

First the detection of microsatellite sequences was achieved to demonstrate the ability of piezoelectric sensing to operate directly in genomic DNA. To approach the problem of direct sequence detection in unamplified genomic DNA, we first focused on target sequences highly repeated in genomes, which are species specific and, in some cases, could represent a significant part of the genome. In particular, detection of the highly repeated sequence called satellite 13, present in *Bos taurus* genome was achieved in genomic DNA, for the first time by piezoelectric sensing. In the bovine genome, eight highly repetitive and several minor repetitive sequences have been detected comprising a total of 27% of the DNA [38]. The target sequence chosen in this case is situated in highly repeated DNA and is therefore present in the genome in a high number of copies, increasing the amount of target sequence available for the hybridization with the probe [39] immobilized on the sensing element. The developed quartz crystal microbalance-based sensor has been applied to the identification of animal species in meat samples, which represents

Table 8.1 Oligonucleotide sequences of immobilized probe, modified with biotin or SH in 5′ end, complementary and non complementary sequences (negative control) used in the optimization assays and blocking oligonucleotide used in the denaturation protocol

Biotinylated and thiolated probe:	Biotin or SH-5′ TCACGCAGCTCAGCAGGCCCT 3′
Complementary target:	5′ AGGGCCTGCTGAGCTGCGTGA 3′
Non-complementary sequence:	5′ GGCAGAGGCATCTTCAACGATGGCC 3′
Blocking oligonucleotide 1:	5′ GTCTGCTCATCTGCTTGACAATTTC 3′
Blocking oligonucleotide 2:	5′ TAATCAAGTAGATGAGCAGGCAG 3

a relevant problem in food analysis for economical, religious or public health concerning reasons. Numerous analytical methods, currently available for species differentiation, rely on protein analysis, such as electrophoresis techniques [40, 41], liquid chromatography [42, 43], and immunoassays [44].

Modern methods for meat identification are instead based on DNA analysis. They allow species-specific DNA sequences identification, which has some advantages over protein analysis [45]. Molecular biology methods allow the determination of DNA also in heat-treated nourishment and are, therefore, suitable for the identification of species-specific DNA in meat and bone meal and concentrate mixtures [46]. Furthermore, they allow the discrimination between related species, such as sheep and goat or chicken and turkey [47].

Earlier DNA sequence analysis was performed using genomic DNA as species-specific probe, which was hybridized to DNA extracted from meat samples [48–51]. Later, probes derived from highly repetitive (satellite) DNA sequences were developed [52].

The analysis of restriction fragment length polymorphism (RFLP) of PCR fragments is an alternative DNA detection system and it has already been successfully applied to species differentiation [53–56]. PCR-RFLP allows the amplification of a conserved region of DNA sequence using PCR, and the detection of the genetic variation between species by digestion of the amplified fragment with restriction enzymes. This technique was used for speciation by exploiting DNA sequence variations within the mitochondrial D-loop region [57], cytochromeB (cytB) gene [52] and satellite DNA sequences [58].

More recently, real-time PCR for meat species identification has also been reported [59].

DNA-based piezoelectric sensing was tested as alternative method in the identification of species-specific DNA sequences for meat analysis, based on determination of a highly repetitive and species-specific DNA sequence present in bovine (*B. taurus*) genomic (non-amplified). The sensor was developed by immobilizing the specific probe on the surface using thiol chemistry with thiolated probes, and surface saturation with short thiols, following the protocol previously developed [27]. Behind thiolated probes, dextran modified surfaces using biotinylated probes were also used. The base sequences of the 5-thiol functionalized probe (21-mer), complementary (21-mer) and non-complementary (25-mer) oligonucleotides and blocking oligonucleotides (25-mer and 23-mer) are reported in Table 8.1. The analytical parameters of the sensor are evaluated first by synthetic oligonucleotides,

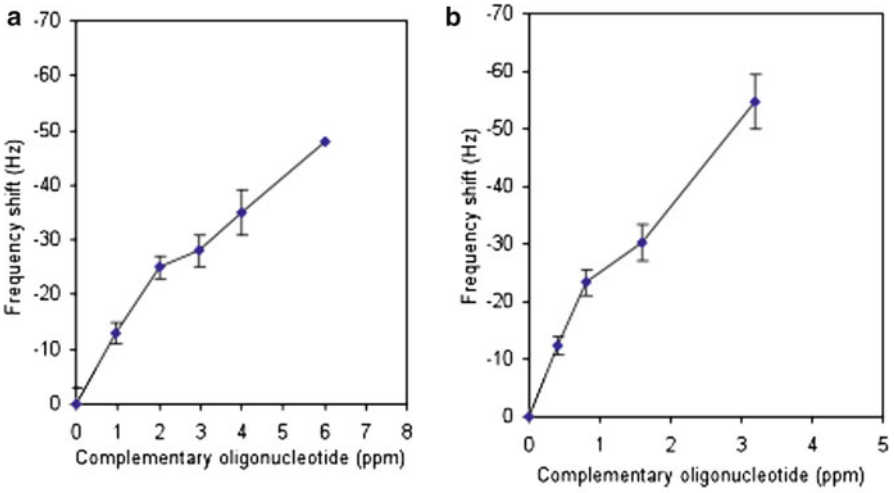

Fig. 8.5 (**a**) Biotinylated oligonucleotide probe. Hybridisation with synthetic oligonucleotide target. (**b**) Thiolated oligonucleotide probe. Hybridisation with synthetic oligonucleotide target

complementary and non-complementary to the 21-mer immobilized probe; once assessed good analytical behavior in terms of selectivity, sensitivity and reproducibility, the system is then applied to real samples.

8.3.1 Sensor Optimization: Biotinylated and Thiolated Probes

A synthetic 21-mer biotinylated and thiolated probes, complementary to a sequence, which is present inside the *Bos taurus* satellite n.13 (247 bp), were used. The selectivity of the systems was tested with the non-complementary sequence. Comparison between the two immobilization chemistries was achieved to select the best performing sensor with standard solutions for further application to sequence detection in genomic, unamplified DNA.

The hybridization between the probe and 21-mer complementary oligonucleotide solutions were tested and the results are shown in Fig. 8.5. In case of sensor modified with biotinylated probe, the CV% has been calculated for all the concentrations and the average is 10 %. For the thiolated probe, the sensor performances are similar to the biotinylated one. The average CV% calculated is 10 % for both. The selectivity of the crystals surfaces just towards the target was confirmed by the absence of frequency shift with non-complementary sequence 1.8 ppm in both modified surfaces. Both sensor were regenerable. The surface was regenerated by treatment with HCl (10 mM, 30 s), which allowed to perform up to 20 hybridization cycles on a single surface, without affecting the sensor surface.

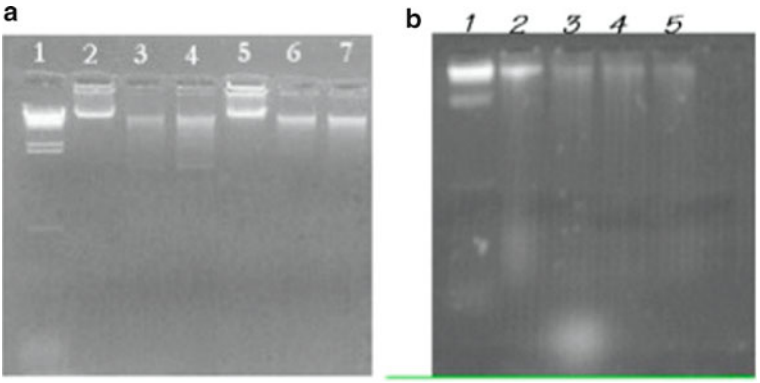

Fig. 8.6 Electrophoretical analysis of digestion reaction. (**a**) Commercially available genomic DNA (Novagen). Lanes: *1* marker l-DNA-HindIII, *2* genomic bovine DNA, *3–4* digested genomic bovine DNA, *5* genomic porcine DNA, *6–7* digested genomic porcine DNA. (**b**) Real sample genomic DNA. Lanes: *1* marker l-DNA-HindIII, *2* genomic bovine DNA, *3*-4-*5* digested genomic bovine DNA

In this case of *Bos taurus* micro satellite detection was achieved first in Genomic bovine (*B. taurus*); Porcine DNA (*Sus scrofa*) was used as negative control. DNA commercially available and finally the sensor were applied to real samples consisting in genomic bovine DNA extracted from animal muscle.

8.3.2 Sample Pre-treatment

8.3.2.1 DNA Fragmentation

To allow detection of target sequence in genomic DNA, fragmentation of the sample is necessary, achieved by enzymatic digestion. Enzymatic digestion was applied here to keep the target sequence in one piece, although this procedure is much longer then fragmentation by ultrasounds. The genomic DNA was digested using the restriction enzyme EcoRI (overnight), to obtain DNA fragments of around 400–500 bp containing the target sequence (available on GenBank accession number no. V00122).

It was verified that the consensus sequence recognized by the enzyme was not present inside the *B. taurus* satellite no. 13 (247 bp) to maintain integer the target sequence thus allowing the maximum amount of target sequence in solution hybridizing the probe. Checking the restriction site of the enzyme respect the target sequence is important to ensure that the fragmentation does not affect the ability of the target sequence to hybridize to the immobilized probe. The enzymatic fragmentation can be then confirmed by electrophoretical analysis, on agarose gel (1 % in TAE electrophoresis buffer) (Fig. 8.6).

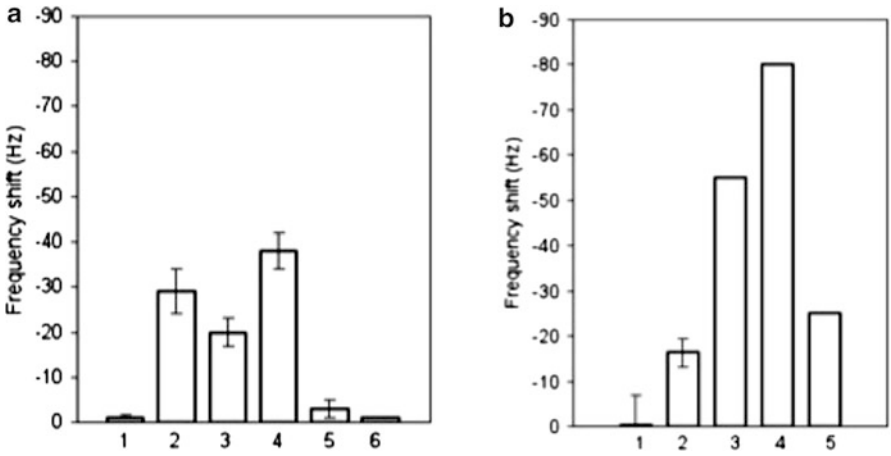

Fig. 8.7 Genomic bovine DNA: thermal denaturation. (**a**) Biotinylated oligonucleotide probe. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 2 ppm (n = 3), *3* Bovine genomic DNA 10 ppm (n = 3), *4* Bovine genomic DNA 20 ppm (n = 3), *5* Porcine genomic DNA 10 ppm (n = 3), *6* Blank solution (n = 1). (**b**) Thiolated oligonucleotide probe. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 1 ppm (n = 3), *3* Bovine genomic DNA 25 ppm (n = 1), *4* Bovine genomic DNA 10 ppm (n = 1), *5* Porcine genomic DNA 10 ppm (n = 1)

8.3.2.2 DNA Denaturation: Thermal Versus Thermal and Blocking Oligos

The genomic fragmented DNA, after precipitation in ethanol and re-suspension, underwent denaturation. Both simple thermal and thermal with blocking oligos were applied to the samples and results compared. In the case of thermal blocking oligo, after the addition of the two oligonucleotides (1 μM), the sample was incubated at 95 °C for 5 min and then 1 min at 50 °C. This second temperature is the appropriate temperature for the annealing of the oligonucleotides to the complementary DNA sequences. The choice of the oligonucleotides depends on the length of the digested fragment and on the position of the target inside the fragment.

8.3.3 Analysis of Genomic Bovine DNA

8.3.3.1 Thermal Denaturation: Biotinylated Versus Thiolated Probe

Hybridization signals obtained after the interaction between both biotinylated and thiolated probes, and the commercially available genomic DNA samples previously treated with the restriction enzyme (EcoRI) and thermally denatured are next discussed.

Figure 8.7 shows the signals relative to the hybridisation of the probes with: non-complementary and complementary oligonucleotide; bovine genomic DNA; porcine

genomic DNA as negative control and blank solution, obtained by applying the digestion treatment and the thermal denaturation to a solution containing all the reagents but DNA [60].

Both sensors are capable to distinguish between the complementary sequence (bovine DNA) and non-complementary solutions (porcine DNA and blank solution). Hybridisation with a double concentration of bovine DNA reports a frequency shift nearly double. In the case of biotinylated surface, the regeneration was fully accomplished with HCl 1 mM.

On the contrary, in the case of thiolated probes, although it was possible to recognize the bovine from the porcine DNA, maybe there is aspecific adsorption that causes a significant signal of the non-complementary sequence. Furthermore, because of this adsorption, the regeneration solution (HCl 1 mM) optimized for oligonucleotides was not sufficient to regenerate the sensor surface and so improved regeneration approach, has to be tested. A different regeneration treatment was also employed. This treatment consists of a first addition of alkaline solution (15 s with NaOH 100 mM), which dissociated the two strands, followed by 30 s with regeneration solution (200 mmol/l Tris·Cl, pH 7, 0.1×SSC, 0.1 % (w/v) SDS). The alkaline pH separates the double strand DNA and then the surfactant, contained in the regeneration solution, removes the target sequence. Several surface washing steps with hybridization buffer were necessary to remove all the surfactant in the regeneration solution.

The surface performances were controlled, using standard solutions of synthetic oligonucleotides, before and after the hybridisation-regeneration cycles. However, after many regeneration cycles, only a partial surface regeneration was achieved, consequently the surface activity results lower. In the case of thiolated probe results were not as satisfying as the ones obtained with the biotinylated one, based on dextran-streptavidin-biotin protocol.

8.3.3.2 Thermal with "Blocking Oligos" Denaturation: Biotinylated Versus Thiolated Probe

The frequency shifts in Fig. 8.8 were obtained after the interaction between the probe and commercially available genomic DNA samples previously treated with the restriction enzyme (EcoRI) and then thermally denatured with blocking oligonucleotides.

Hybridization signals relative to the hybridisation of the probe with: non-complementary and complementary oligonucleotide; bovine genomic DNA; porcine genomic DNA as negative control and blank solution, are shown in Fig. 8.8.

The results are similar to the ones found with thermal denaturation with biotinylated probe (Sect. 8.3.3.1) with regard to the values of frequency shifts and the easiness of regeneration with HCl.

Considering that the results obtained with thermal denaturation (Sect. 8.3.3.1, thiolated probe) do not meet the requirements for analytical applications, the

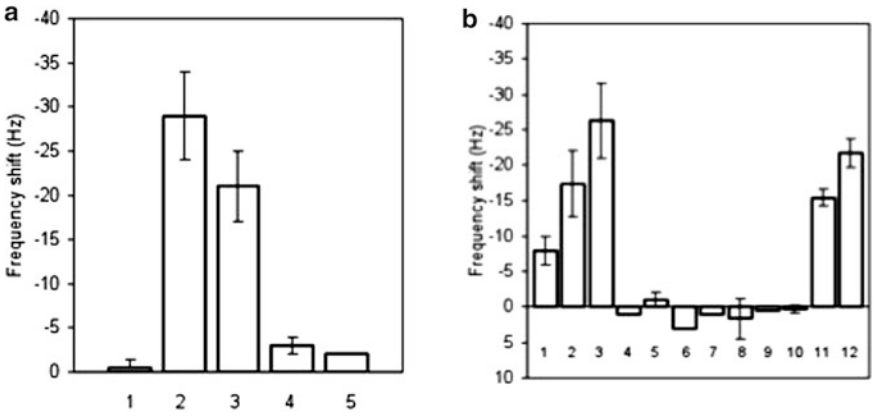

Fig. 8.8 Genomic bovine DNA: thermal with blocking oligonucleotides denaturation. (**a**) Biotinylated oligonucleotide probe. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 2 ppm (n = 3), *3* Bovine genomic DNA 10 ppm (n = 3), *4* Porcine genomic DNA 10 ppm (n = 3), *5* Blank solution (n = 1). (**b**) Thiolated oligonucleotide probe. Signals: *1* Bovine genomic DNA 5 ppm (n = 4), *2* Bovine genomic DNA 10 ppm (n = 6), *3* Bovine genomic DNA 20 ppm (n = 4), *4* Porcine genomic DNA 5 ppm (n = 1), *5* Porcine genomic DNA 10 ppm (n = 3), *6* Porcine genomic DNA 20 ppm (n = 1), *7* Blank solution for 5 ppm (n = 2), *8* Blank solution for 10 ppm (n = 3), *9* Blank solution for 20 ppm (n = 2), *10* Non-complementary oligonucleotide 8 ppm (n = 3), *11* Complementary oligonucleotide 1 ppm (n = 3), *12* Complementary oligonucleotide 2 ppm (n = 3)

denaturation treatment based on the use of "blocking oligo" was combined to the improved regeneration procedure.

Figure 8.8b shows the frequency shifts after the hybridisation reaction between the probe and: non-complementary and complementary oligonucleotides; bovine genomic DNA, porcine genomic DNA and different volumes of blank solution.

The sensor was able to distinguish between complementary and non-complementary sequences both in synthetic oligonucleotidic samples and in digested non-amplified genomic DNA, both with biotinylated and eventually with thiolated probes immobilized on the sensor surfaces.

8.3.3.3 Analysis of Real Genomic Bovine DNA Samples, Extracted from Muscle by "Blocking Oligos" Denaturation; Biotinylated vs Thiolated Probes

Real samples were analysed, with biotinylated probe and with thiolated probe. Both samples were bovine genomic DNA extracted from animal muscle; and they were denatured with the thermal and blocking oligonucleotide treatment since it yielded the best results with both modified surfaces.

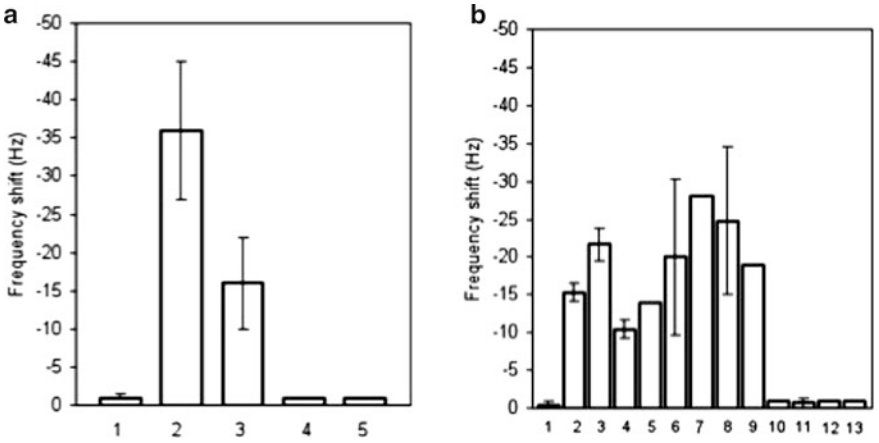

Fig. 8.9 Real genomic bovine DNA sample, extracted from muscle (thermal denaturation + blocking oligonucleotides). (**a**) Biotinylated oligonucleotide probe. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 2 ppm (n = 3), *3* Real sample (bovine genomic DNA) 10 ppm (n = 3), *4* Porcine genomic DNA 10 ppm (n = 1), *5* Blank solution (n = 1). (**b**) Thiolated oligonucleotide probe. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 1 ppm (n = 3), *3* Complementary oligonucleotide 2 ppm (n = 3), *4* Real sample (bovine genomic DNA) 5 ppm, 20 min (n = 4), *5* Real sample (bovine genomic DNA) 5 ppm, 35 min (n = 1), *6* Real sample (bovine genomic DNA) 10 ppm, 20 min (n = 5), *7* Real sample (bovine genomic DNA) 10 ppm, 35 min (n = 1), *8* Real sample (bovine genomic DNA) 20 ppm, 20 min (n = 5), *9* Real sample (bovine genomic DNA) 20 ppm, 35 min (n = 1), *10* Porcine genomic 5 ppm, 20 min (n = 2), *11* Porcine genomic DNA 10 ppm, 20 min (n = 1), *12* Porcine genomic DNA 10 ppm, 35 min (n = 1), *13* Blank solution (n = 2)

Figure 8.9 shows the signals relative to the hybridisation of the probe with: non-complementary and complementary oligonucleotide; the real sample (bovine genomic DNA); porcine genomic DNA as the negative control and blank solution.

The frequency shifts obtained with biotinylated probe (Fig. 8.10a) with the real sample are well comparable with the ones found with commercially available DNA (Sect. 8.3.3.1), with regard to the values of frequency shifts and the easiness of regeneration with HCl.

Figure 8.9b shows the signals relative to the hybridisation of the probe with: non-complementary oligonucleotide, complementary oligonucleotide, real sample (bovine genomic DNA); porcine genomic DNA as negative control and blank solution.

As in the previous case the frequency shifts are comparable with the ones found with commercially available DNA. The signals 4, 6 and 8 have been recorded employing 20 min as contact time; while the 5, 7 and 9 are relative to 35 min contact time. There are no significant differences between the two data series. This is in contrast with the results obtained with the commercially available bovine DNA (see signals 4 and 5 in Fig. 8.9b). A reason of this can rely on the degradation of the

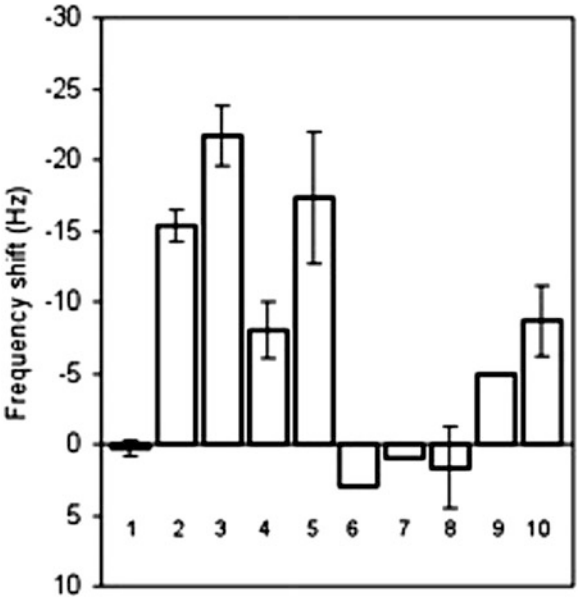

Fig. 8.10 Thiolated oligonucleotide probe. Porcine 20 ppm. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 1 ppm (n = 3), *3* Complementary oligonucleotide 2 ppm (n = 3), *4* Bovine genomic DNA 5 ppm, 20 min (n = 4), *5* Bovine genomic DNA 10 ppm, 20 min (n = 6), *6* Porcine genomic DNA 20 ppm, 20 min (n = 1), *7* Blank solution for 5 ppm, 20 min (n = 2), *8* Blank solution for 10 ppm, 20 min (n = 3), *9* Porcine 20 ppm + Bovine 5 ppm genomic DNA, 20 min (n = 1), *10* Porcine 20 ppm + Bovine 10 ppm genomic DNA, 20 min (n = 3)

real sample, observed in the electrophoresis analysis, that causes a smaller number of non damaged copies. The aspecific adsorbtion was absent, as confirmed by the signals 10, 11, 12 and 13 relative to porcine genomic DNA samples.

8.3.3.4 Analysis of Mixture of Bovine and Porcine Genomic DNA Samples, by "Blocking Oligos" Denaturation

In order to investigate the applicability of this sensor to mixtures composed by DNA of different species, some mixtures with bovine and porcine DNA in different proportion have been prepared and analysed. The bovine and porcine DNA have been first separately digested by restriction enzymes, then mixed together and denatured with the new thermal and blocking oligonucleotides procedure. Considering that both modified surfaces showed similar behaviour, the experiments were performed employing the thiolated probe because of its easiest and quickest preparation.

In Fig. 8.10 results obtained with genomic DNA mixtures, previously digested with the restriction enzyme (EcoRI), and denatured are shown. The samples were prepared adding various bovine DNA concentrations to a fixed porcine DNA concentration.

Comparing the signals of the bovine DNA samples and the ones of the mixtures of bovine and porcine DNA it is evident that the signals obtained with mixtures are specific and comparable to the ones observed with the solution containing just the bovine DNA. No matrix effect has been found, confirming the applicability of this system to sequence detection even in mixed samples, containing DNA from different species. More in detail, the comparison must be made between the signals 13 and 6, 11 and 4, 12 and 5. The contact time for the hybridization was increased from 20 to 35 min, in order to obtain a higher signal from the samples because of their complexity. In fact, the presence, in solution, of many non-complementary sequences hinders the target sequence that can't reach easily the surface. The employment of a longer contact time allowed an increase in the frequency shift while the aspecific adsorbtion did not rise. It is confirmed by the signal 8, obtained with 10 ppm of porcine DNA and a contact time of 35 min. From the comparison of the two denaturing methods we can conclude that although with simple thermal denaturation specific signal is recorded, unspecific bindings occurs and regeneration of the surface is not achieved by simple 1 mM HCl treatment. The sample pretreatment, in term of denaturation step, was here strategic for the sensor re-use and reproducibility. This already in case of highly frequent sequences. We will see how the same step is crucial in case of single of few sequences per haploid genome directly in genomic DNA. In conclusion, the sensor is able to distinguish between complementary and non-complementary sequences both in synthetic oligonucleotide samples and in digested genomic DNA. It is also able to recognize the presence of bovine DNA extracted from muscle (real sample) and also in mixed samples. The only required sample pre-treatment is the enzymatic digestion to fragment the DNA. The system is specific, sensitive, and reusable, with fast analysis time. On the base of our findings, the sensor could represent an alternative method, to the traditional biomolecular techniques, for the identification of species-specific DNA sequences, directly in enzymatically digested DNA, bypassing the PCR step.

8.4 Detection of Target Sequences Present in Single Copy Per Haploid Genome in Plant Genomic DNA

Once assessed the ability of the sensor to detect highly present sequences per haploid DNA we moved further to the detection of target DNA present in single or in few copies in haploid genomes. For this reason we focused on markers of transgenosis sequences, in particular we developed a system for detecting the P35 promoter in Genetically Modified Organisms (GMOs). Here we used a genetically

modified (GM) tobacco plant, *Nicotiana glauca*, an ornamental plant, transformed with pBI121, which contained a gene cassette carrying the P35 form cauliflower virus. A gene cassette consists of a promoter (P), a coding region and a terminator (T). Officially established method on European scale for GMOs control are based on the search of Promoter region (P35S) of the CAMV (cauliflower mosaic virus) ribosomal RNA and on NOS terminator (TNOS) of the nopalin synthase gene from the soil bacterium *Agrobacterium Tumefaciens* [29]. This promoter is used in approved GM soya and maize. Analytical methods for GMOs are based on real-time PCR (required for labelling) or end-point PCR [61]. Our group has been quite active in the development of GMOs DNA-based sensor using different transduction principles with application to GMO Reference Certified Material or real-samples i.e. optical (SPR), piezoelectric and electrochemical. We thus selected the P35S as target analyte for the study of the piezolectric based detection of genomic DNA. The GM plant *Nicotiana glauca* (GR4), was not commercially available, but was transformed at Laboratorio di Genetica, Università di Firenze, Italy. The not transformed plant (wild type) was taken as negative control in all the experiments.

Similarly to the bovine DNA, extraction of the plant DNA (from leaves) was achieved using available protocols [23].

8.4.1 Sample Pre-treatment

8.4.1.1 DNA Fragmentation

Fragmentation was conducted by enzymatic digestion with DNA digestion enzymes Bam HI e Hind III, which ended into a 872 bp fragment containing the P35S target sequence.

The DNA immobilized probe mapped within this fragment. The enzyme selection was achieved by free available software (DNA club). Purification of fragmented DNA by precipitation in ethanol and re-suspension is necessary prior testing on the sensor.

8.4.1.2 DNA Denaturation

Here three different denaturation protocols were tested and compared. In particular we compared chemical denaturation with formamide used in our previous work with optical sensing (SPR) [32] and blocking oligos" denaturation.

The sensor surface was modified by thiol/dextran chemistry and biotinylated probes used for the immobilization via streptavidin anchoring [35].

The oligonucleotides used are reported in Table 8.2.

The P35S sensor has been already optimized and tested on PCR amplified DNA in different real matrices such as soft drinks, crackers, as well as in PCR amplified DNA from *Nicotiana glauca*, thus we will directly report the results on

Table 8.2 Oligonucleotide sequences of immobilized probe, modified with biotin in 5′ end, complementary and non complementary sequences (negative control) used in the optimization assays and blocking oligonucleotide used in the denaturation protocol

Biotinylated probe (35S, 25 mer):	5′ Biotin-GGCCATCGTTGAAGA TGC CTC TGC C-3′
Complementary target (35S, 25 mer):	5′-GGC AGA GGC ATC TTC AAC GAT GGC C-3′
Non complementary (Tnos, 25 mer):	5′-GAT TAG AGT CCC GCA ATT AAT CAT T-3′
Blocking oligonucleotide 1 (35S1, 21 mer):	5′-GCT CCT ACA AAT GCC ATC ATT-3′
Blocking oligonucleotide 2 (35S2, 18 mer):	5′-CTC CAA ATG AAA TGA ACT-3′

the optimized system, using this time genomic DNA samples extracted from GM plant, fragmented by enzymatic reaction, and denatured differently to compare, also in this case, the different sample pre-treatments with the aim to stress the key importance of denaturation steps. In parallel, as control oligonucleotides and PCR sample undergoing to same treatments were used as controls, to study any possible interference with the sensor.

8.4.1.3 Analysis of PCR DNA Samples, by Chemical and "Blocking Oligos" Denaturation

Three approaches were tested: thermal (95 °C for 5 min, cooling in ice for 1 min), chemical (20 % formamide, 0.3 M NaOH at 42 °C for 30 min), and thermal combined with blocking oligonucleotides.

The complementary 35S oligonucleotide (25-mer, 1 ppm), a non complementary oligonucleotide (25-mer, 8 ppm), the 35S PCR fragment (243 bp), a PCR negative control (180 bp), and PCR blanks were analyzed after the three denaturation procedures (Fig. 8.11a, b).

The effect of the chemical denaturation on oligonucleotides resulted in an increase (46 %) of the signal if compared with the one obtained with the thermal treatment. This increase could be due to a non specific effect since it is also observed with the non complementary oligonucleotide. On the contrary, the thermal and blocking oligonucleotides' treatment did not affect the hybridization signal. The same findings were obtained with the same treatments applied to PCR fragments and PCR negative controls.

The same hybridization signal was obtained after treating the 35S samples with the three denaturation methods, but the reproducibility (expressed as coefficient of variation, CV%) (n = 3) was quite different (9 % for the thermal one, 12 % for the thermal and blocking oligonucleotides one, and 25 % for the chemical one). The same denaturation treatments applied to the negative controls gave less homogeneous results since a high signal was observed in the case of the chemical treatment, while negligible results were found in the cases of thermal plus blocking and thermal treatments. The sensor behaviour is shown in Fig. 8.11a.

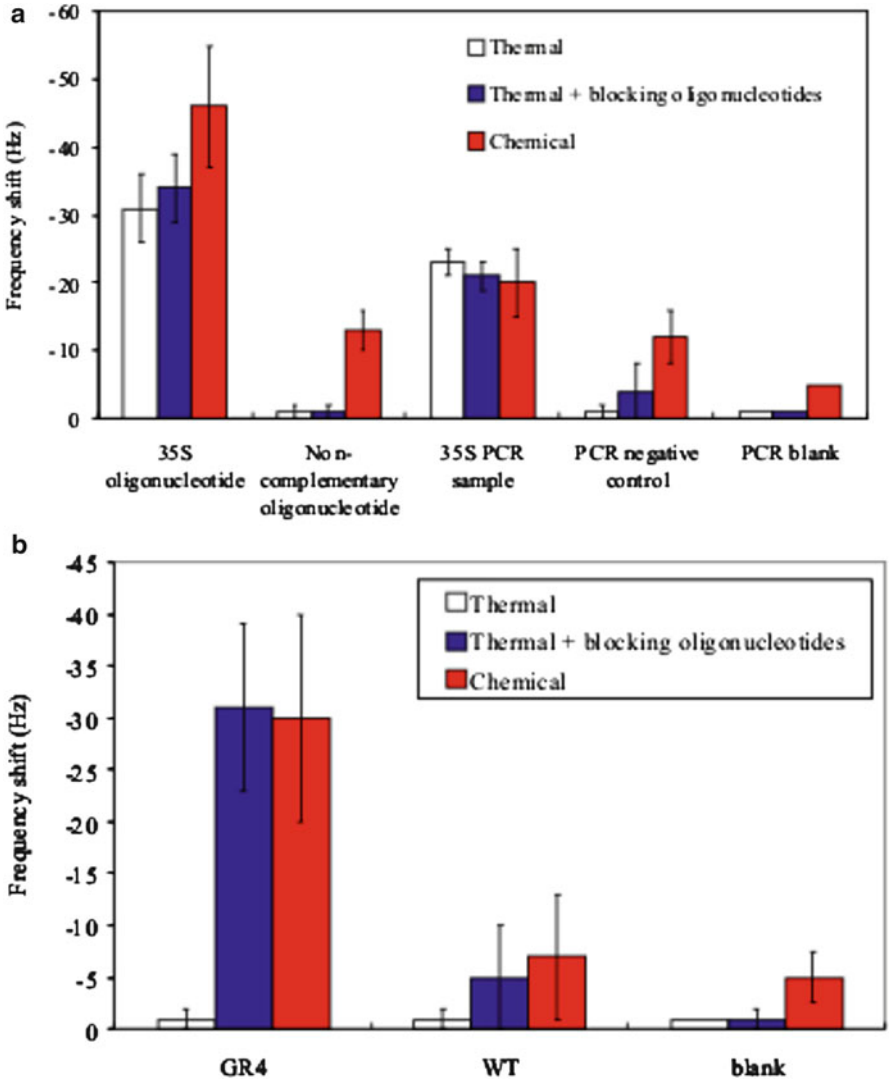

Fig. 8.11 (**a**) Effect of denaturation treatment on oligo and on PCR samples. Samples analyzed: oligonucleotide complementary p35S, oligonucleotide non complementary, fragment PCR 35S (54 ppm), PCR negative control, (35 ppm) and blank (n = 3); (**b**) Analysis of target P35S sequence in fragmented genomic transgenic DNA (10 ppm) GR4 (n = 6), non transgenic WT DNA (n = 6) and blank (n = 3). DNA denatured by three approaches (thermal, thermal with blocking oligonucleotides, and chemical). (Reproduced with permission from Minunni et al. [35]. Copyright (2005) American Chemical Society)

After the initial evaluation of the sample pre-treatments on the sensor behavior, these were finally applied to more complex, unamplified genomic DNA.

8.4.1.4 Analysis of Plant Genomic DNA Samples, by Chemical and "Blocking Oligos" Denaturation

Genomic DNA, previously fragmented by enzymatic digestion by restriction enzymes (BamH), previously checked to leave intact the target sequence. The length of the fragments containing the target sequence, complementary to the immobilized probe, was 872 bp. All of the denaturation procedures were applied to 10 ppm of sample. The results are reported in Fig. 8.11b.

The thermal treatment did not results in a measurable signal. On the contrary the thermal plus blocking oligonucleotides and chemical denaturations allow significant hybridization of the samples. Furthermore, thermal plus oligo oligonucleotide denaturation, results in a better reproducibility then the one obtained by the chemical one. Also the treatment may influence the sensor lifetime, since little regeneration was achieved with chemical denaturation, while with thermal with blocking the surface could be regenerated more then ten times, before losing sensitivity and specificity, thus more then doubling the sensor life-time.

This results, stresses again the importance of selecting the best sample-denaturation.

The piezoelectric sensor detection was very selective, the target sequence was detected in the transgenic sample (GR4) and the signal were significantly different from the negative control (wild type, WT), except when the thermal treatment alone was applied. Moreover, the best results were found when the thermal plus blocking oligonucleotides' treatment was applied, as demonstrated by an evaluation of the reproducibility and the lower nonspecific effect when testing the negative controls, as confirmed by the findings observed with ssDNA and PCR-amplified DNA. This is in line with our previous finding with microsatellite DNA detection in bovine genomic DNA.

This work demonstrated that it is possible to detect the target sequence directly in unamplified genomic DNA, even considering the low concentration of the target in the sample (4×10^5 copies in 10 ppm of sample). PCR-amplified DNA (243 bp) represents an enriched sample where the target sequence is present in a very high number of copies (4×10^{11} copies). To explain the detection of such a low number of copies of target DNA, additional contributions (i.e., viscoelastic effects) to the biosensor signal other than mass loading may be taken into account. Moreover, it must be considered that the signals due to oligonucleotides, to the PCR samples, and to the genomic digested DNA on the surface cannot be compared due to the very different matrix complexity of all these samples and to the different secondary structure once the DNA is hybridized to the probe.

The real-time and label-free DNA sequence detection in non amplified DNA, as reported here, represents an important improvement in DNA analysis. Since the specificity of the system relies on the probe immobilized on the surface, the

applicability of direct genomic sensing is wide, from environmental to food and clinical analysis. Recently some work on sensitive DNA in genomic DNA has been reported by some authors and will be here summarized and discussed.

8.5 State of Art

Recently some groups have reported about sensitive DNA detection by piezoelectric sensing. In Table 8.3, are summarized the approaches. Crystals with different fundamental frequencies, from 5 up to 12 MHz, are used. Different frequency means also different sensitivities as can be deduced by Sauerbrey model, (Eq. 8.1) [7]. Also different operating temperatures (from room temperature up to 55 °C) and flow modes are used, instead, except the case of "Dip and Dry" approach, were the crystal is exposed to the target solution, followed by rinsing to remove the unbound material and then dried. The frequency shift is taken before and after target exposure, when the crystal is dried.

Gene detection directly in genomic DNA is achieved in microorganisms, i.e., *Mycobacterium tuberculosis,* is detected in human specimens (sputum), and the approach used followed our first report, in other words the sample is digested by enzymatic reaction and sample denaturation is achieved by thermal with blocking oligonucleotides [62]. Signal requires amplification and the detection approach is by "dip and dry" method, not directly in liquid. This procedure may be cumbersome and may have important reflections on system reproducibility, at least to our experience The QCM, for its sensitivity to humidity is also used as hygrometer, and in the DNA measurement this effect may be present and difficult to be controlled. However the application reported encourages further development for real clinical application of these devices.

Hepatitis B virus (HBV) genomic DNA is genotyped by hybridization of target sequence to biotinylated Peptide nucleic acids (PNA) probes, which have been proved to be suitable probes especially in mismatch detection [63]. The analysis of the one and two base mismatch is performed without any fragmentation of the DNA molecule and simply by thermal sample denaturation. The hybridization temperature is set at 55 °C. To the dsDNA complex is further bond Rec protein to improve system sensitivity reported to be 8 pg/L in standard solution. Detection of the target sequences with DNA samples extracted from animal feed containing 30 % RR soybean amplified by the PCR and unamplified DNA. The detection limit for genomic DNA was in the range of 4.7·105 numbers of genomic copies contained EPSPS gene in the QCM cell. Mismatch analysis was achieved in DNA extracted from human blood cells. Good correlation ($r = 0.9613$) was found with RT-PCR method, indicating potential real application of the device to detect HBV in clinical diagnostics.

Two papers deal with genetically modified material. In particular detection in Soybean Roundup Ready 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, which is an active component of an insert integrated into RR soybean genome,

Table 8.3 Literature reporting about DNA genomic detection by piezoelectric sensing

Target analyte	T °C	Probe mer	Digestion	Denaturation	Sample	Signal amplification	Ref.
Mycobacterium tuberculosis Gene IS6110	Room	25	Enzymatic BstDSI	Thermal with blocking	Sputum	Streptavidin	[62]
Hepatitis, point mutation in L50 (2562–2613)	55	29 PNA with linkers	No	Thermal	Blood	Rec -protein	[63]
OGM EPSPS gene	Room	21	No	Thermal	R-Ready soybean 30 %	No	[64]
OGM P35S GM tobacco plants	Room	25	EnzymaticBamHI/HindIII vs ultrasounds	Thermal	GM tobacco plants	No	[65]

is reported [64]. EPSPS gene renders plants' resistance against herbicide glyphosate (Roundup Ready). Glyphosate is toxic for plants because it prevents the production of aromatic aminoacids (tryptophan, tyrosine, phenylalanine). A 21-mer single stranded biotinylated oligonucleotide (probes) is immobilized on sensor (8 MHz quartz crystal) surface via avidin binding, previously covalently attached to gold. No sample digestion has been performed and thermal denaturation applied. Very low reproducibility is reported: CV% 20 %, may be due to pre-analytical steps i.e. sample pre-treatment. The sensor proposed in this study was tested for the detection of EPSPS sequence in PCR non-amplified DNA samples extracted from animal feed containing 30 % of the genetically modified soybean Roundup Ready. The sensor was able to distinguish transgene sequence between modified and unmodified soybean DNA (extracted from animal feed) at the following level: 3.6, 4.6 and 5.4 μg of genomic DNA in 200 μl of QCM cell.

The influence of different used probe on the sensor behavior is also reported, confirming the importance of probe optimization in the development of nucleic acid-based sensing as underlined before [22]. The negative control, genomic DNA extracted from unmodified soybean, generated small frequency shift, which might be attributed to the direct adsorption of DNA on gold electrode surface without hybridization to the probe or weak, non-specific interactions between the probe and partially complementary sequences present in very long genomic DNA. The detection limit was in the range of 4.7×10^5 numbers of genome copies with EPSPS gene in the QCM cell with 200 μl of investigated samples.

Finally, detection of P35S has also been reported in pflp (ferredoxin like protein)-gene inserted in *Nicotiana tabacum* plants [65]. Wild type tobacco DNA was used as a control. The extraction protocol applied was the same applied in our laboratory, i.e. CTAB, and BamH/Hind III restriction enzymes were used in the fragmentation by enzymatic digestion, while we used only BamH. Sonication in parallel was used to compare the fragmentation efficacy of the two approaches. It is interesting the comparison between the two fragmentation approaches performed in this work. By the comparison can be observed the same frequency decreases were observed at the same concentrations of both digested and sonicated samples but with different standard deviations, having the sonicated samples the highest. This result was assumed since fragmentation of the DNAs was non-selective during sonication process. However, the sonication even if nonselective is much faster to performed (minutes compared to overnight digestion used in enzymatic fragmentation).

Very sensitive target sequence detection directly in unamplified DNA can be achieved by label free and real-time detection using piezoelectric sensing. The sensor development as well as the preanalytical steps are strategic for the success of the detection.

First the probe immobilization chemistry should allow selecting binding by preventing any unspecific adsorption from the bulk to the surface. For this reason we have selected and compared in the presented application both biotinylated and thiolated probes. Biotinylated probes are bound to the previously modified surface by thiol/dextran/strepatvidin, while thiolated probes are directly bound to the surface. At the same time the probe selection is also a key step. In fact the presence

of loops or hairpins in the probe structure could affect the hybridization with the target sequence, thus careful selection, eventually assisted by *in silico* selection, could be employed. The presence of secondary structures can be also prevented by tuning the denaturation protocols to prevent non only re-annealing but also intra strand bonding.

Fragmentation of genomic DNA is also important, but enzymatic digestion may be too long for use in biosensing. In this sense, once assessed the sensitive DNA detection one could move on to ultrasound fast fragmentation.

The application of these systems to a wide panorama of clinical, food and environmental analysis is the next challenge. In this sense, multi-array approaches based on piezoelectric sensing are welcome, allowing parallel analysis of target sequences. For that different sensor designs can be foreseen. Over years interesting papers dealing with the development of automated instrumentation have been presented.

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Chapter 9
Surface Plasmon Resonance-Based Methods

Roberta D'Agata and Giuseppe Spoto

Abstract Surface plasmon resonance represents a new tool for the simple, fast and cheap nucleic acid detection. Large efforts have been paid during the last decade with the aim to develop even more sensitive and specific SPR-based methods to be used for the direct detection of genomic DNA. This Chapter, after a description of fundamentals of surface plasmon resonance and allied techniques, will review the state-of-the-art of recent platform and assay developments and will provide an overview of recent uses of SPR techniques for the direct detection of non-amplified nucleic acids.

9.1 Introduction

Nucleic acid microarray technologies [1] have revolutionized bioanalytical capabilities in different areas including genomics [2], clinical diagnostics [3], food analysis [4], and environmental control [5].

The most widely adopted nucleic acid microarray platforms rely on nucleic acid amplification procedures and require the introduction of labels for the identification of the target sequence. These requirements are costly, labour-intensive and time-consuming. Moreover, the target amplification and labelling procedures may cause sample contaminations that could interfere with the original interaction event.

The detection and the sequence-identification of nucleic acids are commonly achieved after the hybridization of the target strand to a surface immobilized complementary probe strand. The efficiency of the hybridization reaction depends

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on factors such as ionic strength, temperature, length of the nucleotide sequence, and the degree of mismatch. The surface confinement of the reaction introduces additional factors like surface immobilized probe steric accessibility and orientation, electrostatic repulsion between strands, mass transfer of the target from the bulk of the solution to the solid surface, and probe surface density. Each of them should be optimized in order to control the stability and the kinetic of the probe-target interactions [6].

Surface Plasmon Resonance (SPR) represents the most advanced label-free biosensor technology for detection of chemical and biological analytes [7]. In recent years, SPR biosensors have been applied for the analysis of numerous analytes related to medical diagnostics. In particular, it has been widely used to detect nucleic acids in real-time and by using multiplexed formats. Issues related to the sensitivity of SPR-based methods in detecting nucleic acids have been fully addressed in recent years and will be discussed in following sections.

In this chapter, fundamentals of SPR methods will be introduced and recent advances in the development of SPR-based platforms will be discussed. Particular attention will be paid in discussing new materials and nanostructures that aim at advancing multiplexed and ultrasensitive nucleic acid detection. Capabilities offered by nanostructure-enhanced SPR in detecting non-amplified genomic DNA will be discussed at the end.

9.2 Theory of Surface Plasmon Resonance

After the first observation of the underlying physical phenomenon given by Wood in 1902 [8], the SPR technique has become one of the fastest-growing analytical tool over the last twenty years. Advantages associated with the SPR detection of biomolecular systems (including good sensitivity, label-free and real-time detection), together with the commercial availability of equipments and sensing surfaces, have made SPR the technique-of-choice for interaction analyses and biosensing [9].

Surface plasmons [10] are the quanta associated with periodical oscillations of the electron charge density occurring at the interface between a metal and a dielectric medium.

The concept of plasmon originates in the plasma approach of Maxwell's theory: the free electrons of a metal are treated as an electron liquid (density of about 10^{23} cm^{-3}) called "plasma", ignoring the lattice in a first approximation. Electron density fluctuations propagate through the volume of a metal with a characteristic frequency given by:

$$\hbar\omega_p = h\sqrt{\frac{4\pi ne^2}{m_e}} \tag{9.1}$$

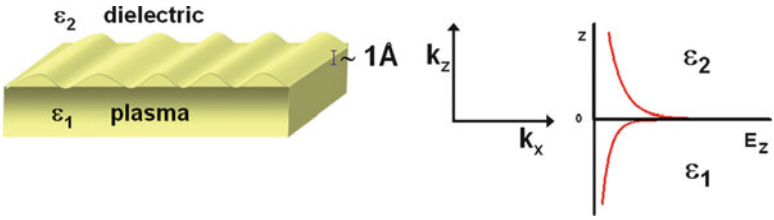

Fig. 9.1 Schematic of surface plasmon waves at the interface between a metal (dielectric constant ε_1) and a dielectric (ε_2). The electromagnetic field of SPs propagating on a surface in the x direction and decaying exponentially into z- direction are also shown schematically. The exponential dependence of the field E_z is seen on the *right*

where ω_p is the frequency associated to plasmon oscillations, n is the free electron density of the material, e is the electron charge, and m_e is the effective mass of an electron. When the plasma is excited by an external source with a frequency equal to ω_p, electrons collectively and coherently oscillate in the metal [11].

Electrons at the boundary between a metallic surface and a medium having a dielectric constant ε_2 can perform coherent fluctuations called surface plasmons (SPs). These fluctuations are confined at the boundary and vanishes both sides of the metal surface. Surface plasmon waves are *p*-polarized and are described by a wave vector $\mathbf{k}_x$ parallel to the x direction (Fig. 9.1)

$$k_x = \frac{\omega}{c}\sqrt{\frac{\varepsilon_1\varepsilon_2}{\varepsilon_1 + \varepsilon_2}} \tag{9.2}$$

where $\varepsilon_1 = \varepsilon_1' + i\varepsilon_2''$ is the complex dielectric constant of the metal and ε_2 is the dielectric constant of the medium.

The above mentioned surface charge fluctuations, which can be localized in the z-direction within the length of about of 1 Å around the metal surface, are accompanied by a mixed transversal and longitudinal electromagnetic field (E) which disappears at $|z| \to \infty$ and has its maximum in the surface $z = 0$ [12]. The frequency ω_p of the surface plasmon longitudinal oscillations is tied to its wave vector $\mathbf{k}_x$ by a dispersion relation $\omega_p(\mathbf{k}_x)$ (Fig. 9.2) [13].

The wave vector ($\mathbf{k}_x$) associated with SPs always remains larger than wave vector of light ($\mathbf{k}_{light}$) having the same energy $\hbar\omega_p$ and traveling through the medium ε_2:

$$k_{light} = \frac{\omega}{c}\sqrt{\varepsilon_2} \tag{9.3}$$

For plasmon excitation by a photon to take place, the energy and the momentum must both be conserved during the photon-plasmon coupling. This requirement is met when the wave vector for the photon $\mathbf{k}_{light}$ and the plasmon $\mathbf{k}_x$ are equal in magnitude and direction for the frequency of both waves. The direction of the wave

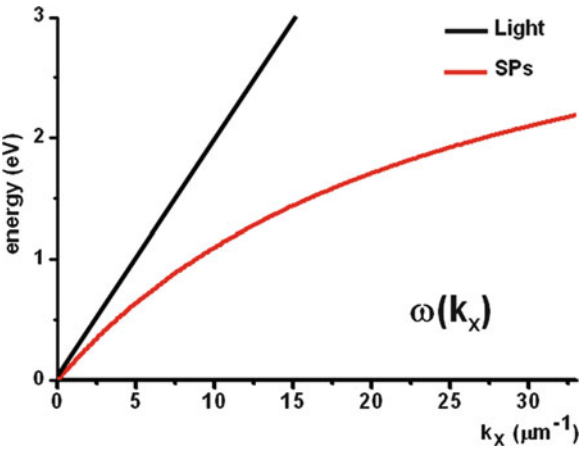

Fig. 9.2 Surface plasmon dispersion $\omega(k_x)$ on gold surface. The vertical axis is scaled as ω(eV). The *straight solid line* in figure shows the *light line* $k_x = \omega/c\sqrt{\varepsilon_2}$

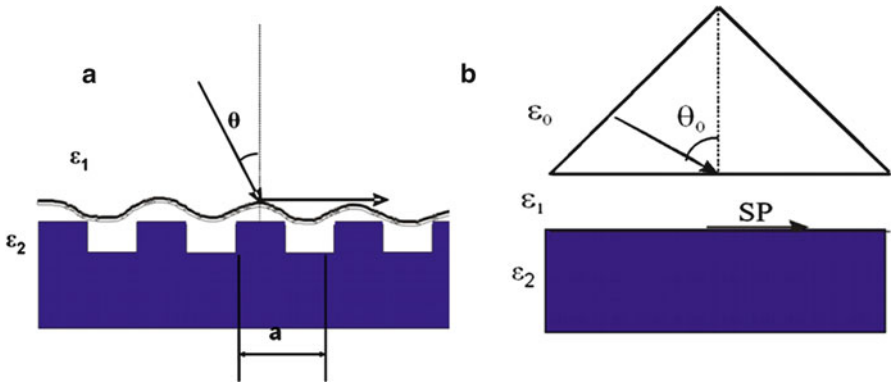

Fig. 9.3 (**a**) Diffraction grating; (**b**) Otto geometry

vector is the direction of the wave propagation, while its magnitude depends on the dielectric constant of media at the interface.

Since the SP's dispersion relation never intersects the dispersion relation of electromagnetic radiation (Fig. 9.2), SPs cannot couple with a freely propagating light beam incident upon the metal surface and for this reason SPs are "non-radiative" fluctuations of the surface electron density. In order to couple photon at a given energy $\hbar\omega$ with SPs the wave vector has to be increased by a $\Delta\mathbf{k}_x$ (Fig. 9.2). A similar increase can be obtained by using specific experimental methods.

Photons can excite plasmons via a grating coupler (Fig. 9.3a) [14]. When light hits a grating with a grating constant a, at a θ angle, the component of the wave vector in the surface is increased by $2\pi/a$:

$$k_x = \frac{\omega}{c}\sin\theta + \frac{2\pi}{a} \tag{9.4}$$

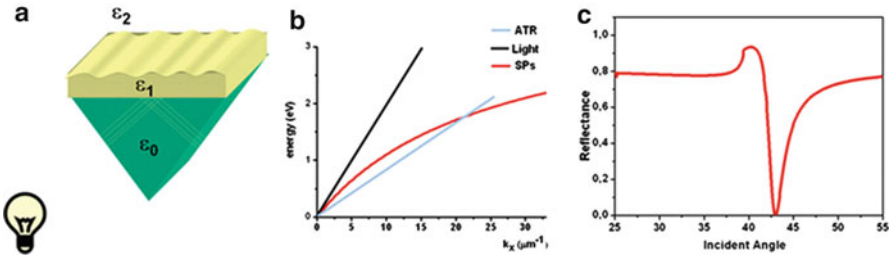

Fig. 9.4 (**a**) Configuration of the Kretschmann geometry; (**b**) the dispersion relation of photons in a coupling prism; (**c**) SPR curve for SF10 (n = 1.723)|gold(50 nm, n1 = 0.1726 + i3.4218)|air(n = 1.0) for collimated white light source (830 nm)

The matching condition for resonance is fulfilled when:

$$\frac{\omega}{c}\sin\theta + \frac{2\pi}{a} = \frac{\omega}{c}\sqrt{\frac{\varepsilon_1\varepsilon_2}{\varepsilon_1+\varepsilon_2}} \tag{9.5}$$

The most commonly used methods for surface plasmon excitation exploit the attenuated total reflection (ATR) effect. The main experimental configurations are based on the Otto (Fig. 9.3b) [15] and the Kretschmann geometries (Fig. 9.4a) [16].

ATR configurations increase the wave vector of the radiation travelling through an optically denser medium (*e.g.* glass prism, dielectric constant $\varepsilon_{\mathrm{pr}}$) by $\sqrt{\varepsilon_{pr}}$:

$$k_x = \frac{\omega}{c}\sqrt{\varepsilon_{pr}}\sin\theta \tag{9.6}$$

The matching condition is obtained when:

$$\frac{\omega}{c}\sqrt{\varepsilon_{pr}}\sin\theta = \frac{\omega}{c}\sqrt{\frac{\varepsilon_1\varepsilon_2}{\varepsilon_1+\varepsilon_2}} \tag{9.7}$$

In the Otto configuration (Fig. 9.3b) the metal surface (ε_2) is separated from the prism (ε_0) by an additional dielectric layer having a dielectric constant value $\varepsilon_1 < \varepsilon_0$ and the SPs resonance occurs at the metal-dielectric interface.

The Kretschmann geometry (Fig. 9.4a) uses a prism (ε_0) with a thin (about 50 nm for gold) metallic layer (ε_1) on one side.

If the match condition (Eq. 9.7) is satisfied when a p-polarized light beam hits the prism on a side opposite to the thin metal layer a drop in intensity of reflected light is observed (Fig. 9.4c) [17]. Any change of the ε_2 value caused by chemical or physical effects modifies the matching condition (Eq. 9.7) and a shift in the energy position of the minimum of the reflected light (reflectance dip) is observed [18]. The SP wave is therefore highly sensitive to changes of the dielectric constant (simply related to the refractive index) of the medium at the metallic interface. Such a distinguishing

property is the basic principle which makes the surface plasmon resonance useful as biosensor [19].

Conditions for surface plasmon resonance are obtained in the infrared and visible wavelength region for air/metal and water/metal interfaces. Absorption in the metal layer influences significantly the width of the reflectance dip. Since the dissipation in metals is determined mainly by the imaginary part of the refractive index, one may choose a metal type with a low imaginary part to reduce the dissipation and, consequently, to obtain a narrower dip. Silver and gold are noble metals that satisfy this condition, while aluminum has a large imaginary part and exhibits wider dips compared to silver and gold.

Other points to be considered for the choice of a metal for SPR are: (i) the chemical purity of the surface: oxides and sulfides formed after the metal interaction with the atmosphere can interfere with the SPR phenomenon; (ii) the compatibility of the metal reactivity with the surface chemistry needed for the SPR experiment. Gold are the substrate of choice for SPR measurements. It is relatively stable in aqueous environments needed for the study of bimolecular interactions and a versatile chemistry-mainly based on the formation of functional self-assembled monolayers (SAMs)-has been developed for this metal.

A surface plasmon resonance biosensor is able to measure the binding between target analyte molecules and receptor molecules immobilized on the gold surface. During the receptor/analyte binding event, the shift of the dip in the spectrum of reflected light is monitored over time and kinetics information regarding biomolecules interactions are gathered.

SPR has the potential to investigate interactions between antigens and antibodies, nucleic acid sequences and their complementary strands, and substrates and enzymes [20] with no need for labeling of the interacting components. The time required for the analysis is typically in the range of a few minutes and the sample consumption is in the nanomole-picomole range.

The kinetic events at the metal surface, displayed as a sensorgram, can be investigated by monitoring the SPR signal change as a function of time. The time interval during which the analyte interacts with the surface bound receptor defines the "association phase" whereas the time interval following the analyte interaction is termed "dissociation phase". In the association phase there is a simultaneous association and dissociation equilibrium. A steady-state condition is reached when the association rate equals the dissociation rate. Under ideal experimental conditions, only dissociation should take place during the dissociation phase.

In order to perform replicate SPR experiments any bound analyte molecule should be removed with no disruption of the receptor structure and activity. So, a typical SPR experiment involves several steps displayed in Fig. 9.5.

The change in the refractive index produced by the capture of biomolecules depends on the concentration of the analyte molecules at the sensor surface. If the binding event occurs within a layer of thickness h, the SPR response is proportional to the binding induced refractive index change, which can be expressed as:

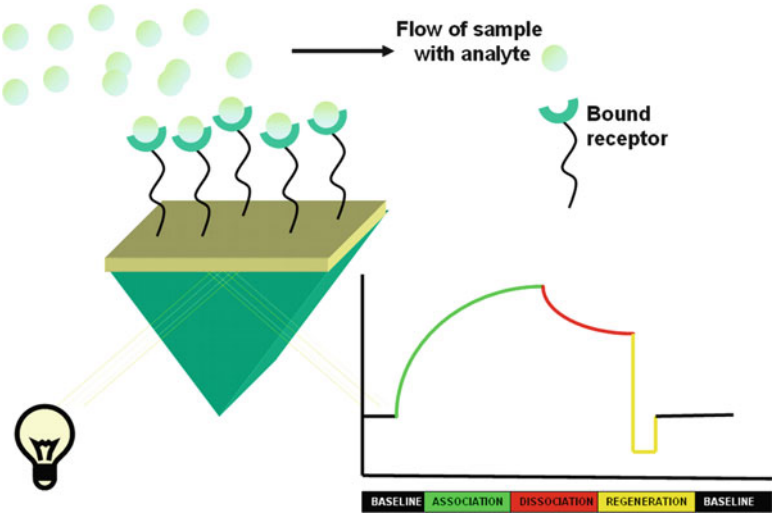

Fig. 9.5 Pictorial description for the receptor-analyte interaction: the analyte is captured by receptors immobilized on the sensor surface. A sensorgram with the steps of an analysis cycle is also shown

$$\Delta n = \left(\frac{dn}{dc}\right)\frac{\Gamma}{h} \tag{9.8}$$

where (dn/dc) denotes the refractive index increment of the analyte molecules and $\mathbf{\Gamma}$ denotes the surface concentration in mass/area. Consequently, quantitative measurements of biomolecules adsorption can be obtained with the SPR technique [21].

Different instrumental formats that can be used to carry out SPR experiments: angle modulation [22], wavelength modulation [23], and intensity modulation also known as SPR imaging [24].

The popularity of the scanning angle SPR, in which the reflectivity of the incident light upon a metal film is monitored as a function of the incident angle, can be attributed to the existence of Pharmacia Biosensor AB company, that in 1990 launched the first commercial SPR platform (Biacore). Both angle and wavelength modulated SPR sensors typically provide few data points at a time. In contrast, SPR imaging uses changes in reflectivity to generate simultaneously binding difference images in a parallel manner. In addition, SPR imaging offers advantages in freedom of design of the liquid handling system.

New aspects of SPR have been explored in recent years concerning the development of combined techniques (SPR-MS [25, 26], SPFS [27]), the use of new materials (glassified substrates [28], carbon-coated metal substrates [29]), and the development of new SPR-based assays (nanoparticle enhancement [30], enzymatic amplification [31]).

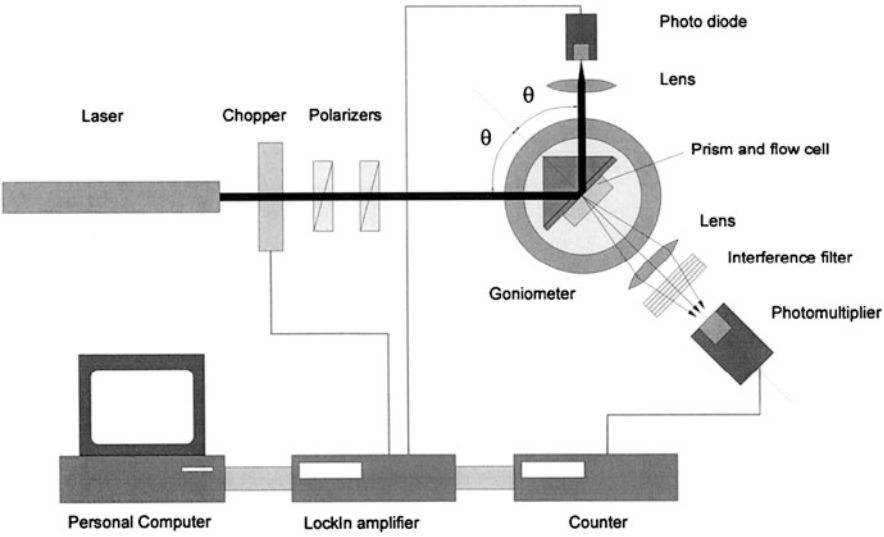

Fig. 9.6 Schematic of a SPFS set-up in the Kretschmann-configuration combined with elements for fluorescence detection (Reprinted with permission from Elsevier: Ref. [38. Copyright (2000))

9.3 Surface Plasmon Fluorescence Spectroscopy (SPFS)

Although SPR provides suitable sensitivity for many label-free applications, it suffers from reduced sensitivity for specific assays when compared to other labelled methods such as electrochemistry or fluorescence [32].

In 1991, Attridge et al. [33] described how the field enhancement from surface-plasmon modes could be used for fluorescence spectroscopy, providing surface-sensitive analysis. However, only a limited number of applications were described before the Schmidt et al. [34] and Neumann et al. [35]. After 2005, the number of papers using SPFS significantly increased [36].

SPFS experiments can be performed simultaneously with SPR measurements. In a basic SPFS setup (Fig. 9.6) a photomultiplier tube (PMT) is placed perpendicular to the plane of the substrate with a suitable filter to reject stray light. A chopper is placed in front of a laser beam in order to prevent photobleaching of fluorophores while measurements are not being taken.

In normal SPR operation, the polarized light is reflected to the photodiode at angles greater than the critical angle for total internal reflection. If a chromophore is in the evanescent field near the metal–dielectric interface, large enhancement of fluorescence excitation can occur at the angle where plasmon resonance reaches its maximum. The resulting fluorescence emission can be measured with high sensitivity in the plane perpendicular to the substrate. Although only fluorophores within the evanescent field can be excited, their fluorescence signal can be significantly quenched by the close metal surface that acts as a very efficient quencher that

absorbs the excitation energy of the fluorophore [37]. Therefore, the separation distance from the metal substrate, at which biomolecular interactions will occur, is a compromise between staying close enough to the peak evanescent field value and getting far enough from the gold surface to prevent de-excitation. An ideal distance has been demonstrated to exist at about 15–25 nm separation from the metallic surface. That distance reduces the optical field by only 10 %, while the fluorescence intensity is more than 80 % [38].

SPFS represents a powerful tool for DNA detection and for this reason extensive DNA hybridization studies have been carried out [39] with the aim to demonstrate the enhanced sensitivity in detecting PCR amplicons (detection limit 100 fmol) [40] and SNPs [41].

The combined use of SPFS and the electrochemical detection offers further possibilities in DNA detection that are discussed in Chap. 10.

9.4 Surface Plasmon Resonance Imaging (SPRI)

SPR imaging, also known as SPR microscopy, was first introduced by Rothenhäusler and Knoll in the 1988 [42]. Since then, SPRI has been proposed as a powerful technique to investigate interactions with biomolecules arrayed onto chemically modified gold surfaces [43]. SPRI combines typical advantages of the traditional SPR in terms of real time analysis, no label requirements and high sensitivity, with those associated to an imaging system such as the easy coupling with microfluidic devices, the high spatial resolution and the possibility to adopt an array-compatible detection approach.

Typical SPRI applications fall into the genomics and proteomics areas. Examples of genomic applications include DNA and RNA detection through the hybridization to RNA and DNA microarrays [44–47], and DNA-protein binding on DNA array [48, 49]. Proteomic applications include the determination of protein film thickness [50], the detection of conformational changes [51], and expression profiling [52]. SPRI has been also used to study kinetics of enzymatic reactions [53, 54] and to evaluate protein interactions with protein [55–57] and carbohydrate microarrays[58, 59].

SPRI measures spatial differences in the intensity of the reflected light (expressed as percent reflectivity %R) at a fixed angle and wavelength [60]. In particular, SPRI platforms based on a Kretschmann geometry use a p-polarized light beam to illuminate a prism/thin gold film assembly at a fixed incident angle. The intensity of the reflected light is detected with a 2D detector (CCD) to obtain an SPR image. The spatially resolved surface functionalization of the metallic SPRI surface allows to detect in real time parallel interaction events.

The spatial contrast in an SPR image comes from the heterogeneity in the dielectric constant due to differences in refractive index or film thickness near the surface at different positions across the surface. If an adsorbate has a refractive index (that is related to its dielectric constant) different from that of the solvent,

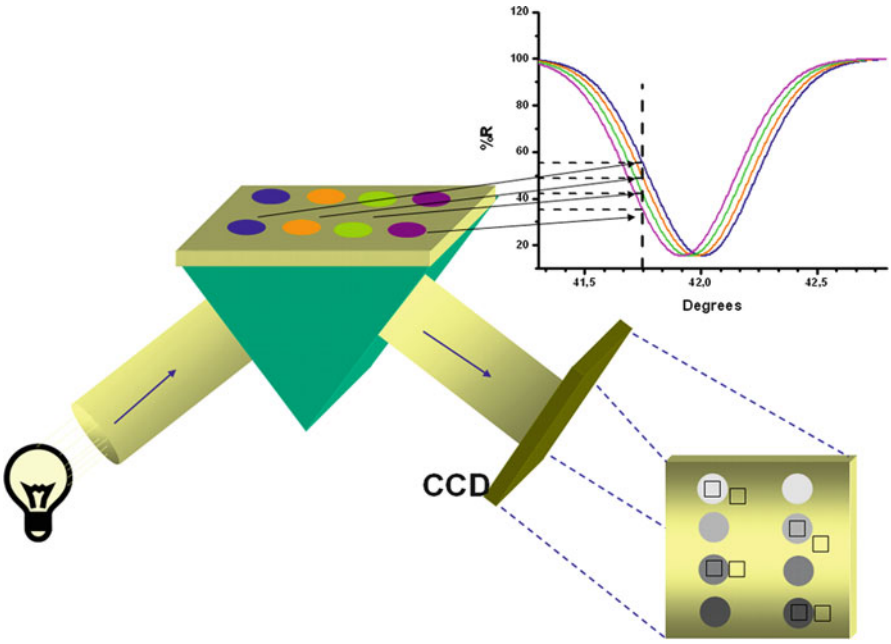

Fig. 9.7 Schematic diagram of SPR imaging

its presence on the surface can be detected with spatially resolution by monitoring changes in the reflected light intensity (Fig. 9.7) [61]. Difference images obtained by the subtraction of a reference image from a post-binding image provide visual confirmation of the binding events.

For small changes in the SPR angle, changes in reflected intensity are proportional to changes in the effective refractive index and effective thickness or surface coverage of an adlayer on the SPR-active surface [62]. The proportionality factor that relates the reflected intensity change to the change in refractive index can be determined by measuring SPRI intensity versus angle curves from different regions of an SPRI surface. Broad, nearly linear regions on either sides of the minimum are observed. These regions where the slope is large and remains constant are referred to as "linear regions" or "high-contrast angles" (Fig. 9.7).

The measured reflected intensity decreases or increases as the minimum shifts to higher angles when the incident angle is set to an angle in the linear region on the left or right side of the curve, respectively. The SPRI response is proportional to the change in bulk refractive index near the sensing surface. The relationship is linear for intensity changes of less than 20 % while the SPRI response becomes nonlinear for larger refractive index changes and the intensity eventually saturates.

The SPRI lateral resolution is limited by the propagation length l_d, and varies for different metals and different source wavelengths. For a 44-nm-thick gold layer, l_d decreases as the excitation wavelength decreases ranging from $l_d = 14$ μm at

676 nm to $l_d = 0.5$ μm at 530 nm [63]. The reduction in the lateral resolution is counterbalanced by the possibility to obtain images with an higher contrast when using near-IR sources [64]. Hence, the source wavelength for SPRI experiment should be optimized as to obtain the highest level of contrast by maintaining a sufficient lateral resolution. Typically, excitation wavelengths ranging from 800 to 1,000 nm provides lateral resolution of about 25 μm and are well suited for imaging an array with micrometric dimensions.

The SPRI system allows simultaneous and independent measurements to be made on different locations of the same sensor surface. In addition, the fact that the sensor surface can be visually observed in real time establishes the strength of SPRI, along with other advantages which are the inherent optical sensitivity and the instrumentation simplicity.

As stated above, there are a number of different reasons why a spatially resolved immobilization of biosystems is desirable:

1. kinetic curves can be monitored simultaneously;
2. when only a low analyte sample volume is available, its solution flows in contact with a number of sensor surface areas at the same time;
3. multi-analyte mixtures can be measured and pattern recognition becomes feasible;
4. reference and duplicate measurements can easily be included and employed to correct for non specific adsorptions and refractive index changes.

The SPRI experimental format increases the analysis throughput. However, to take fully advantage of this approach, a precise control of both the patterning of biomolecules onto the SPRI sensor surface as well as the fluidic of the analyte solution is imposed. In this perspective, the use of microfluidic devices provides an SPRI-compatible convenient mean for both manipulating small amounts of sample solutions as well as for controlling the patterning of a variety of different biomolecules.

Microscale conditions induce the laminar flow of fluids [65] and optimize the way in which liquids are put in contact to the SPRI sensor surface [66]. The use of microfluidic devices significantly reduces the experiment duration and the sample consumption. In addition, diffusion distances are reduced, thus allowing a better control over reaction conditions [67]. Finally, microfluidic platforms allows to manage the interaction experiments in order to decrease the influence of nonspecific interactions and instrument drifts by internal signal referencing [67–69].

9.5 Platforms and Experimental Designs for SPRI Assays

SPRI technology has greatly advanced over the last five years as a consequence of the development of new surface functionalization strategies and new instrumental designs.

Different probe immobilization procedures have been described [70] most of which are based on the use of SAM [71].

DNA arrays can be fabricated by spotting single-stranded DNA (ssDNA) probe solutions on functionally active SPRI sensor surfaces. The spotting procedure suffers from a not uniform probe immobilization and can be performed manually or by using automatic spotters. The manual spotting results in a not uniform spot size distribution and immobilized probe density. The quality of ssDNA spots has been improved by creating mixed ssDNA/oligo(ethylene glycol) (OEG) SAMs [72]. Alternatively, thiolated oligonucleotides can be spotted onto the gold substrate, and thiolated OEG can be used to functionalize the un-modified gold surface [73, 74]. OEG minimizes surface nonspecific adsorption and helps in controlling the ssDNA probe orientation on the surface. DNA arrays offer the possibility to carry out a parallel detection of nucleic acids and proteins by immobilizing antibodies conjugated to ssDNA sequences on DNA arrays carrying a complementary sequence.

The spatially controlled covalent immobilization of amine-terminated ssDNAs has been obtained through the amide bond formation with the NHS/EDC activated poly-L-glutamic acid (pGlu) previously electrostatically adsorbed onto an 11-amino-1-undecanethiol (MUAM) monolayer [75]. The surface architecture assured a high surface hybridization efficiency and minimized non-specific adsorption effects.

The surface chemistry developed for gold exploits the high stability of the gold-sulfur bond. However, the susceptibility of the gold-sulfur bond to oxidation and photodecomposition has hampered SPR sensing uses in areas such as on-surface combinatorial chemistry and photolithography due to the adverse effects of the required chemical condition or ultraviolet radiation on the gold-sulfur bond [76].

Silicon oxide-based materials like glass are standard materials for biosensing. They are inexpensive and benefit the well-developed silane-coupling chemistry for surface functionalization. A number of functionalization strategies developed on glass have already been adapted to gold. However, resulting procedures are complex and require time-consuming syntheses and extensive manipulation of the existing protocols to be effective on the new surface. A simple way to facilitate procedures is obtained by coating the gold SPRI substrate with thin silicon oxide-based materials. Since the SPRI signal decays exponentially within about 200 nm from the metallic surface [77], the coating should be some nanometers thick in order to retain a high detection sensitivity. The potentials of a similar approach have been demonstrated by using glassified gold surfaces to demonstrate the detection of 10–100 attomoles of mesophilic DNA polymerase products by nanoparticle-enhanced SPRI [28]. The approach offers stable and reusable surfaces but is limited by the not fully controlled silane chemistry and by the easy hydrolytic cleavage of Si–O–Si bonds when exposed to harsh environments or to increased temperatures.

Carbon-based surfaces could overcome most of the above mentioned limitations. In this case the ligand immobilization to the surface is obtained after the deposition of carbon [78] or amorphous silicon-carbon alloys [79] on the metallic surface. The biomolecule immobilization is obtained by exploiting the well-developed and robust

chemistry based on the attachment of alkene-containing molecules to the substrate through either UV radiation mediated formation of C-C bonds or Si-C bonds.

Thin layers of amorphous carbon (7.5 nm) can be also deposited by sputtering it onto the SPR gold film [29]. In this case, an alcohol-terminated surface is obtained after a plasma treatment and an UV photo-functionalization with 9-decene-1-ol of the hydrogen terminated carbon surface. A similar interface is useful for the development of SPRI-compatible procedures for the in situ fabrication of oligonucleotide arrays through the use of photochemically protected oligonucleotide building blocks [80]. Photochemical procedures are not compatible with the traditional thiol-based gold surface chemistry because it is affected by the extended exposure to ultraviolet light and oxidizing chemical conditions. Unfortunately, the above mentioned advantages for the in situ oligonucleotide synthesis are partially counterbalanced by a reduced SPRI sensitivity caused by both a decreased photon-plasmon coupling efficiency as well as a broadening of the SPR curve generated by the complex dielectric function of amorphous carbon.

Biointerfaces based on nanomaterials with large surface-to-volume ratio, facile surface modification and overall structural robustness are particularly suitable for the development of improved the DNA detection assays. In particular, nanostructure-based SPR sensors have drawn tremendous interest in recent years as a consequence of the associated enhanced detection sensitivity [81].

The use of nanostructured tags results in a dramatic enhancement of sensitivity due to three main factors: (i) an increase of the absolute mass in each binding event, (ii) an increase in the bulk refractive index of the analyte, and (iii) coupling between the localized surface plasmon (LSP) of metallic nanoparticles and SPR of the sensing film. The role of plasmonic coupling in sensitivity enhancement is still an open question.

LSPs differ from the above discussed extended SP, that are described as a longitudinal electromagnetic wave in a 2D electron gas that exists on the interface between metals and dielectrics, because they are excited in metallic structures with dimensions less than half the wavelength of the exciting electromagnetic wave [82].

Different nanostructures such as nanoparticles, nanowires and nanotubes deposited on the SPR metallic layer have been used in combination with the conventional Kretschmann configuration of SPRI platforms [83–85]. The use of surface relief nanostructures directly built on a metal film has been the topic of recent theoretical and experimental studies. These studies have contributed to demonstrate the enhanced detection of multiple targets achieved by using LSP resonance excited by metallic nanogratings [86]. The experimental results have also shown that the LSPR-based assay may be used for the detection of a variety of different interactions, envisaging the extended feasibility and applicability of these platforms for rapid and efficient screenings.

The widespread application of surface relief nanostructures for SPR experiments is still limited by the inherent uses of nanofabrication procedures such as electron beam lithography, focused ion-beam milling, or photolithography. Only recently, some soft-lithography-based techniques have been used in this context [87]. The future development of nanostructure-based SPR chips is closely related to the

development of rapid and cost-effective nanofabrication methods. In this context, low-cost nanostructured plastic substrates can be used to enhance the sensitivity of conventional SPR imaging [88]. The field enhancement provided by the nanostructures is function of both the composition, dimension and geometry of the nanostructure and of the dielectric environment. It has been shown that the addition of a monolayer at the surface of the nanostructured substrates created a maximum electromagnetic field enhancement in the vicinity of the monolayer, which was larger than that experienced on a bare gold surface.

The integration of SPRI with array systems is quickly advancing. Whether it is made in situ under the microfluidic control or offline by using spotting technologies, there is a vast collection of investigated patterning technique. These procedures are aimed at obtaining surface micropatterning, multiplexed analyte detection and fluids control.

Besides the biointerface architecture, the SPRI biosensor performances are highly dependant on optical, electrical and structural features of the platform. An accurate control of those features have been shown to produce a refractive index resolution as low as 1×10^{-7} RIU (refractive index unit) [89].

Enhanced detection capabilities result also from the exploitation of long range SPR (LRSPR). LRSPR biosensors based on the Kretschmann configuration comprise four basic layers, namely the prism, the dielectric buffer layer, the metal layer, and the analyte. The dielectric buffer layer separates the thin metal layer from the prism. If the dielectric constant of the dielectric buffer layer is similar to that of the analyte, and is lower than that of the prism, a symmetric environment is achieved on either side of the metal thin film. If the thickness of the thin metal layer (d_m) is such that $k_{zm}\, d_m \ll 1$ (where k_{zm} is the z component of the SP wavevector in the metal layer) the resulting symmetric configuration causes the same surface plasmon wave to exist on both sides of the thin metal layer, and interaction then takes place between the two SP waves at both surfaces.

Compared to the conventional SPR biosensor, the propagation length of LRSPR is increased by a factor of approximately ten times and specific combinations of metal thickness and buffer layer thickness can be used to achieved an enhanced sensitivity compared to standard SPR [90].

LRSPR imaging has been obtained by using an inert and optically transparent fluoropolymer with a refractive index very close to that of water (Cytop) as the dielectric buffer layer [91]. In this case, compared with conventional SPR, LRSPR has longer propagation length and longer field penetration. The longer propagation length causes a reduced lateral resolution of the produced image. The LRSPR propagation length is of 200 μm compared to 25 μm measured for SPR modes at 814 nm [63]. Additionally, the penetration depth of the LRSPR mode into the bulk water phase is longer than the 200 nm of conventional SPR at the same wavelength. Potentials offered by LRSPR imaging have been demonstrated by detecting DNA hybridization with enhanced sensitivity compared to SPR imaging [91].

A different SPR imaging platform has been obtained by placing between two crossed polarizers a prism with an SPR chip containing a spatially patterned multilayer structure [92]. The output polarizer blocked the light reflected from the

SPR chip areas outside the sensing areas, generating high-contrast images. Two types of SPR multilayers with opposite sensitivities to changes of refractive index at the surface were employed. The output signal was defined as a ratio of the intensities generated from the two neighboring multilayers. The SPR imaging platform was shown to be able to detect refractive index changes down to 2×10^{-6} RIU and to detect short oligonucleotides at concentrations as low as 100 pM [93].

Grating couplers offer some attractive features for SPRI in comparison to prism couplers [7]. In fact, diffraction-based sensors discriminate against nonspecific adsorption processes to the entire substrate because only patterned bioaffinity adsorption will lead to changes in the diffraction signal. Moreover, in this case the operating wavelength can be readily tuned to a spectral region where the plasmon resonance enhancement generates a maximum optical effect.

Enhanced SPRI sensitivity has been obtained by using a diffraction grating made of Au stripes [94]. In this case, the detection of femtomolar levels of unmodified DNA in a sandwich assay using Au nanoparticles has been obtained. The method termed nanoparticle-enhanced diffraction grating (NEDG) is based on the enhanced diffraction obtained by the optical coupling of planar surface plasmons in the grating to LSPR in the gold nanoparticles. The nanoparticle-enhanced DNA detection has been also investigated by coupling the diffraction grating platform with an *ex situ* nanoparticle assay [95].

An alternative approach for the SPR detection is based on the measurement of the shift in phase of the light wave interacting with SPs at a single angle of incidence and wavelength of the light wave. In fact, p-polarized light shows a very sharp phase shift of 360° in the region of the SPR angle, relative to the phase shift at the critical angle. Changes in this phase shift upon adsorption of biomolecules on the metallic surface have been used in various optical configurations as a highly sensitive method of monitoring surface adsorption processes, with detection limits up to two orders of magnitude lower than amplitude detection approaches. SPR images based on the phase shift measurement have been acquired by using a variable retarder that creates a spatially periodic retardation pattern on the metal interface that is converted into a fringe pattern by a subsequent linear polarizer. The position of the interference fringes defines is related to the p-polarized light phase shift and is used to monitor any phase shift changes due to a molecular adsorption [96]. The SPR phase shift imaging method have been demonstrated to be able to detect ssDNA hybridization with a dynamic range that is comparable to conventional intensity based SPR techniques and a detection limit roughly 100 times lower than traditional intensity-based SPR imaging measurements.

9.6 Detection of Non Amplified Genomic DNA

The successful sequencing of the human genome [97] has improved our understanding of the complex network of gene expression [98] and has prompted towards the development of new methodologies for an even more simple, fast and cheap

detection of nucleic acids to be used for both applicative and research needs [1, 99]. Most of the currently available nucleic acid detection methods require the amplification of the target species and detect the duplex formation by using labels and transducers able to generate signal as a consequence of the specific hybridization event. Both amplification and labelling processes are laborious, can introduce artefacts and may interfere with the hybridization between the complementary strands.

PCR is the most widely used method for nucleic acid amplification. It enhances both the specificity and the sensitivity of the assay by increasing the target concentration. A similar enhancement is balanced by the need to use complex reaction mixtures, by the potential contamination of the genetic sample [100] and by the difficulty in amplifying long target sequences (more than about 40,000 bases) [101]. In addition, sequences different from the target can be erroneously amplified thus producing non-specific amplifications [102].

The identification of single nucleotide polymorphisms (SNPs) [103–105] introduces further complexity in the detection process. In fact, the large number of known SNPs [106] easily allows to evaluate the resulting complexity of the PCR-based detection process since the amplification of each SNP carrying sequence imposes the identification and the synthesis of two different primers to be used to define the genomic DNA portion to be amplified.

The DNA detection is expected to be significantly improved by using simple and economic detection methods which require minimal DNA modifications and provide enhanced signal amplification. In this perspective, the direct detection of non amplified genomic DNA appears an excellent cost-effective alternative the PCR-based approach since extra labour and costs from the amplification procedure are reduced.

Unfortunately, the direct detection brings out constraints caused by the complexity of the genomic DNA that are normally minimized when using PCR amplified targets. The complexity mainly results from the presence of sequences nearly identical to the targeted one and the high melting temperature of the genomic DNA. The melting temperature of the short duplex formed after the probe targeting of a selected DNA sequence could be too low to provide for the stringency needed for a base-level discrimination in the presence of total genomic DNA by resulting in a lack of specificity. An efficient probe hybridization of the target sequence is possible when the latter is available as ssDNA. Simple thermal treatments are sufficient to give a significant amount of ssDNA. However, additional treatments are required when unamplified genomic DNA is going to be analyzed. In fact, a proper genomic DNA fragmentation is required in order to facilitate its detection. Sonication is the preferred method instead of using restriction enzymes in view of the greater randomness of the resulting fragmentation. Sonication is expected to cleaves DNA also according to its composition and sequence instead of only its size [107]. In contrast, the random action of restriction enzymes is compromised by their sequence dependence since their cleaving action is performed only in the presence of a specific base sequence.

9.7 SPR Methods for the Direct Detection of Genomic DNA

The SPR sensitivity for the direct detection of unamplified genomic DNA has been first evaluated by using plant, bovine and human genomic DNAs [108]. Before the SPR detection, unamplified genomic DNAs were fragmented with EcoRI or the BaMH 1 restriction enzymes which were used for bovine and human or plant DNAs respectively. The fragmentation was followed by a thermal denaturation process. In this case, the re-association of the denatured DNA was prevented by means of two blocking oligonucleotides that hybridized to the genomic DNA close to target-probe sequences thus preventing the re-association by steric hindrance. The SPR detection was obtained by immobilizing thiol-capped oligonucleotide probes on the sensor surface and by using only 25 μl of 10 ppm solutions, containing about 10^5 copies of maize or human targets.

The limitations suffered by SPR for the parallel detection of different probe/target interactions are overcome by SPR imaging [109]. The possibility to detect unamplified genomic DNA by using an SPRI-based multiplexed approach has been first demonstrated by detecting the testis-specific protein, Y-encoded, (TSPY) gene located in the Y chromosome of the human genomic DNA [110]. The detection approach was based on an RNA array and a surface enzymatic amplification procedure based on the use of the RNase H enzyme. RNase H specifically hydrolyzes RNA strands in RNA-DNA heteroduplexes. The heteroduplexes were formed on the SPRI sensor surface after the exposure of RNA probes to DNA targets. As a consequence of the enzymatic reaction ssDNA molecules released into the solution can bind to other RNA probes on the surface. New heteroduplexes formed on the SPRI surface are in turn subject to the hydrolytic RNase H action. The final process caused an SPRI signal amplification due to the fact that one target DNA molecule removed approximately 10^4 RNA probe molecules from the surface. The approach produced a 7 fM sensitivity in detecting the TSPY gene from unamplified human genomic DNA.

The enhanced sensitivity required when unamplified genomic DNA is going to be detected has prompted efforts aimed at identifying new strategies for the amplification of signals generated by the transducer after the DNA detection. With this aim the use of nanoparticles has been widely investigated in combination with optical or electrochemical transducers (see Chaps. 3, 6 and 7). The nanoscale dimension of particles gives rise to a high reactivity and to chemically tailorable physical properties. Among the other, gold nanoparticles (AuNPs) have been so far the most versatile and extensively studied for enhanced DNA detection and have been the focus of research for many decades as a result of their intriguing electronic and optical properties [30].

The use of AuNPs for the enhanced SPRI detection of oligonucleotides was first proposed in 2000 by using oligonucleotide probes and a static assay [111]. The method showed a 10 pM sensitivity. A similar strategy, was more recently adopted in combination with the use of peptide nucleic acid (PNA) probes for the detection of DNA sequences and continuous-flow microfluidics [112]. The detection

of oligonucleotide targets was obtained by adopting a sandwich hybridization strategy using AuNPs conjugated to an oligonucleotide complementary to the final tract of the target, which was not involved in the hybridization with the SPRI sensor surface-immobilized PNA probe. The sandwich hybridization strategy allowed discrimination between fully matched and single-base-mismatched sequences, even at a concentration of 1 fM. In addition, the microfluidic management of the fluids allowed multiplexed determination of SPRI responses. The discrimination was obtained using 150 zeptomoles of the DNA target.

An optimized probe design and a proper surface architecture represent fundamental requirements for a selective and specific DNA detection. Probe design is usually optimized on the basis of sequence text analysis and nearest-neighbour and thermodynamic calculations [113] which are aimed at minimizing cross-hybridization with homologous sequences and at achieving higher thermodynamic stability of the complex formed with the target sequence [114].

An SPRI study of a multispot DNA chip fabricated with mixed ssDNA/oligo (ethylene glycol) self-assembled monolayers have demonstrated the influence of hairpins and homodimers on the hybridization process. The presence of secondary structures generate interferences with the DNA hybridization reaction and decrease the efficiency of the detection process [115]. ΔG values are good indicators to predict the effect of secondary structures on the hybridization reaction. Lower ΔG values favour secondary structures and reduce the extent of the hybridization between complementary ssDNA. Interestingly, an increase of the temperature to an appropriate value minimizes the influence of secondary structures and enhances the efficiency of the hybridization reaction.

The replacement of ssDNA probes with suitable synthetic probes such as PNAs has been proved to successfully increase the detection specificity and sensitivity. Unique structural and hybridization features of PNAs make them superior to DNA as sequence-specific hybridization probes. PNAs are DNA mimics in which the negatively phosphate deoxyribose backbone is replaced by a neutral N-(2-aminoethyl) glycine linkage. They interact with their complementary DNA sequence more quickly, with a stronger binding and more specifically than the analogous DNA (see also Sect. 4.2.2) [116]. These properties are largely caused by the absence of the coulombic repulsion established between the two negatively charged strands in DNA. PNA complexes are more thermally stable than DNA complexes and, by virtue of the nature of their backbone, less susceptible to biological degradation by nucleases, proteases, and peptidases [117]. Additionally, their interaction with the complementary strand is almost unaffected by ionic strength and pH changes.

The successful detection of oligonucleotides by using PNA probes has prompted their use for the direct detection of genomic DNA [112, 118–120]. In particular, the nanoparticle-enhanced SPRI approach has been also used to demonstrate the detection of unamplified genomic DNA samples carrying different amounts of genetically modified (GM) sequences (Roundup Ready soybean, RR) [119]. The described approach was able to selectively detect the GM target sequence down to zM concentrations in solutions containing GM and GM-free genomic DNA at aM concentrations, even in the presence of a large excess of non-complementary DNAs.

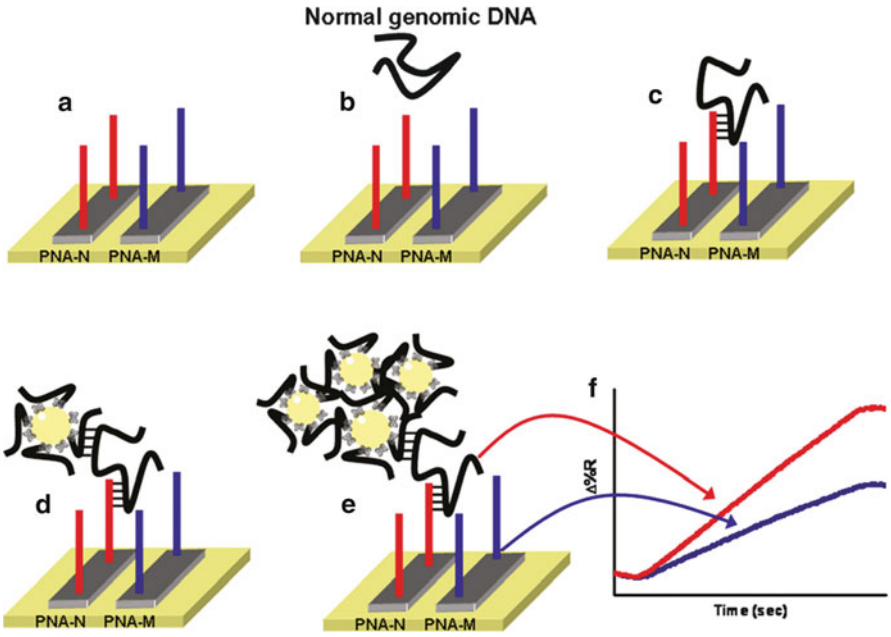

Fig. 9.8 Pictorial description of the nanoparticle-enhanced SPRI strategy used to detect the normal, heterozygous, and homozygous genomic DNAs. In order to simplify the pictorial representation only specifically adsorbed DNA is shown. Nonspecifically adsorbed DNA is also present on the surface and contributes to generate the SPRI-detected signal. (**a**) PNA probes complementary to the normal (PNA-N) and the mutated (PNA-M) DNA target sequence; (**b–c**) Capture of the unamplified normal DNA by the PNA-N probe; (**d**) Absorption of oligonucleotide-conjugated AuNPs; (**e**) Aggregation of AuNPs on the surface; (**f**) Time-dependent SPRI curves obtained after the adsorption of conjugated AuNPs (Reprinted with permission from American Chemical Society: Ref. [120. Copyright (2011))

A similar SPRI-based strategy using PNA probes and microfluidic control was used to detect a point mutation in non-amplified genomic DNA isolated from patients affected by an hereditary genetic disease (Fig. 9.8) [120]. Human genomic DNAs, not subject to costly, time-consuming, and prone to contamination PCR-based amplification procedures, obtained from both healthy individuals and homozygous or heterozygous patients affected by β-thalassemia were used for the experiments. The targeting of genomic DNA was made easier by sonication, generating fragments of approximately 400 bp that are of sufficient size to localize the fragment within the human genome with a good degree of statistical certainty [121], and by denaturation of fragments at high temperature followed by cooling on ice before the introduction of the denatured fragments into the SPRI microfluidics apparatus.

After the above described treatment each sample was fluxed into each of six microchannels of the SPRI fluidic system in order to allow the direct interaction of normal, homozygous and heterozygous genomic DNAs with the PNA probe complementary to the normal DNA sequence (PNA-N) and the PNA probe complementary to DNA sequence carrying the point mutation (PNA-M), respectively

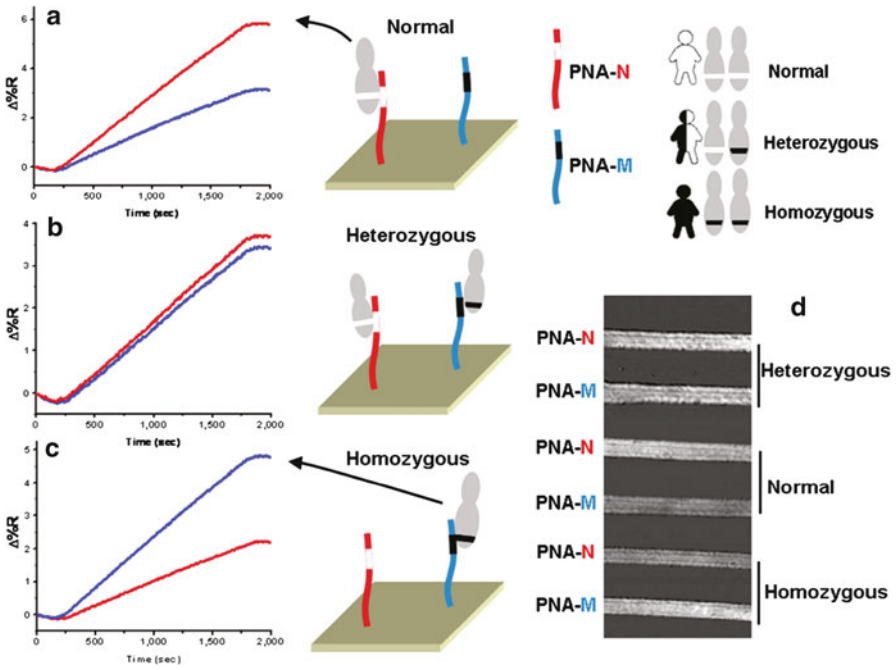

Fig. 9.9 Time-dependent SPRI curves obtained after the adsorption of conjugated AuNPs on normal (**a**), heterozygous (**b**), and homozygous (**c**) DNAs previously adsorbed to the surface immobilized PNA-N and PNA-M probes. Solutions of 5 pg μL^{-1} genomic DNAs were used for the experiments. A representative SPR difference image (**d**) demonstrating the DNA parallel detection is also shown (Reprinted with permission from American Chemical Society: Ref. [120]. Copyright (2011))

(Fig. 9.8a, b). The two different PNA probes were useful both to discriminate between normal, homozygous, and heterozygous DNAs as well as to avoid the use of external controls which were difficult to be obtained for this specific application.

The specific SPRI response patterns obtained when normal, homozygous, or heterozygous DNAs were each allowed to interact with the two different PNA probes provided a robust experiment control (Fig. 9.9). In fact, whereas normal DNA was expected to interact with only the PNA-N probe, different interactions were expected from homozygous and heterozygous DNAs, i.e., interaction between homozygous DNA and only the PNA-M probe and interactions between heterozygous DNA and both PNA-N and PNA-M probes.

The ultrasensitive detection of the genomic DNA was achieved by using AuNPs conjugated to an 11-mer oligonucleotide complementary to an exposed tract of the target DNA not involved in the hybridization with the PNA probe (Fig. 9.8d). After the capture of unamplified genomic DNAs carrying the normal or the mutated gene sequences by the complementary PNA probe(s), the conjugated AuNPs were fluxed into the microchannels in order to enhance detectability. By using this approach, a significant increase of the detected SPRI signals was obtained (Fig. 9.8e, f).

Attomolar concentrations of target genomic DNA were detected, DNAs from healthy individuals and homozygous or heterozygous patients affected by β-thalassemia were discriminated, and only simple manipulations of the genetic samples were required before the analysis (Fig. 9.9).

The simple procedures requiring only sonication and denaturation, minimize the possibility to contaminate the DNA samples to be analyzed. The reduced number of genomic DNA equivalents required for the analysis (about 470) also allows to propose the method as a potential new tool for the analysis of rare cell populations. As an example, the proposed strategy can be used to obtain a non-invasive prenatal genetic diagnosis based on the analysis of circulating free fetal DNA in the blood of the pregnant woman. In particular, the ongoing lowering of costs for massively parallel analyses in a SPRI multiplex format might lead to replacement of most of the other currently used approaches which traditionally require invasive procedures such as amniocentesis, chorionic villous sampling or fetal blood sampling, each associated to a small but finite risk of fetal loss.

SPRI has been also successfully used for the ultrasensitive detection of microRNAs (miRNAs) sequences [122]. miRNAs are involved in the regulation of the gene expression in cell proliferation, fat metabolism, and cell differentiation [123]. A combination of a surface polyadenylation reaction carried out by using poly(A) polymerase enzymes and a gold nanoparticle-based amplification of the SPR signal was employed to detect miRNAs to a concentration of 10 fM. The multiplexed detection of miRNAs with the described methodology is particularly appealing since it can be used for miRNA profiling at attomole levels.

9.8 Conclusion

Enhanced nucleic acid detection imposes the development of even more simple, fast and cheap methods. Those methods are expected to avoid the nucleic acid amplification and labeling. Surface plasmon resonance methods have been shown to offer promising opportunities for the ultrasensitive and direct detection of non amplified DNA. In this perspective, further efforts are expected to be paid with the principal aim to demonstrate the fully applicability of SPR-based methods for the ultrasensitive and specific detection of nucleic acids in complex matrices.

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Chapter 10
Parallel Optical and Electrochemical DNA Detection

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Abstract This contribution introduces strategies for the sensitive detection of oligonucleotides as bio-analytes binding from solution to a variety of probe architectures assembled at the (Au-) sensor surface. Detection principles based on surface plasmon optics and electrochemical techniques are compared. In particular, cyclic- and square wave voltammetry (SWV) are applied for the read-out of ferrocene redox labels conjugated to streptavidin that binds to the (biotinylated) DNA targets after hybridizing to the interfacial probe matrix of either DNA or peptide nucleic acid (PNA) strands. By employing streptavidin modified with fluorophores the identical sensor architecture can be used for the recording of hybridization reactions by surface plasmon fluorescence spectroscopy (SPFS). The Langmuir isotherms determined by both techniques, i.e., by SWV and SPFS, give virtually identical affinity constants K_A, confirming that the mode of detection has no influence on the hybridization reaction. By using semiconducting nanoparticles as luminescence labels that can be tuned in their bandgap energies over a wide range of emission wavelengths surface plasmon fluorescence microscopy allows for the parallel read-out of multiple analyte binding events simultaneously.

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10.1 Introduction

The question as to which detection method – electrical/electrochemical or optical schemes – eventually will win the race for the most powerful transducer principle in bio-sensing is still completely open. The option for higher integration density on chips or in any other array format and the ease of coupling the output signals directly to data analysis electronics certainly points to electrical detection schemes, however, optics is still leading in terms of sensitivity: fluorescence spectroscopy and, in particular, single photon detection are routine methods with unmatched detection limits while the well established electrochemical techniques do not allow for the monitoring of such extremely low currents. And single electron (transistor) recordings are yet to be combined with the aqueous environment needed for bio-affinity studies [1].

A very reasonable experimental approach to further tackle this question is the parallel use of an optical scheme and an electrochemical technique for the simultaneous recording of binding events of bio-analytes from solution to interaction partners covalently attached to the sensor surface via functional architectures assembled at the transducer/analyte solution interface. Surface plasmon resonance spectroscopy [2] and cyclic or differential pulse voltammetry [3] are very well suited for such a combination approach: the (noble) metal substrate needed for the resonant excitation of a propagating surface plasmon mode at the metal/buffer interface can be simultaneously used as the working electrode of a regular three electrode electrochemical set-up. This way, bio-affinity studies can be performed with both techniques in parallel.

A certain limitation to this approach, however, is given by the need to design and assemble the interfacial layer deposited at the sensor surface for an optimized performance of the respective transduction principle. We will demonstrate this for a series of hybridization studies between surface-attached oligonucleotide catcher probes and their (fully complementary or mismatched) target sequences from solution. These target analytes were labeled with streptavidin that was either derivatized with ferrocene for the electrochemical detection or with a chromophore suitable for surface plasmon fluorescence spectroscopic investigations [4].

The use of semiconducting nanoparticles, quantum dots, offers interesting aspects for both schemes: The tunability of the emission wavelength via bandgap engineering of the quantum dots allows for multiple parallel recording of simultaneous binding events on an array sensor by color multiplexing [5]. On the other hand, the manipulation of the energy level of the substrate (by applying an electrical potential) and, hence, the electronic interaction between substrate and nanoparticles – covalently ‘wired’ to the (Au-) electrode – promises interesting coupling schemes [6] and the use of the observed new photo-electrochemical phenomena for completely unconventional biosensor recording principles1.

10.2 Experimental Apparatus

A schematic of the employed combination set-up is given in Fig. 10.1a. The basic instrument is a classical surface plasmon resonance (SPR) spectrometer in the Kretschmann configuration. The thin Au layer evaporated onto the base of the coupling prism is used simultaneously as the active metal/dielectric interface that guides the evanescent surface plasmon mode and can be employed as the working electrode in a typical electrochemical set-up. The two common modes of operation in SPR – the angular scans and the time-dependent kinetic recordings at a fixed angle of incidence – are schematically given in Fig. 10.1b, c, respectively. By attaching, in addition to the working electrode, also a counter and reference electrode to the flow

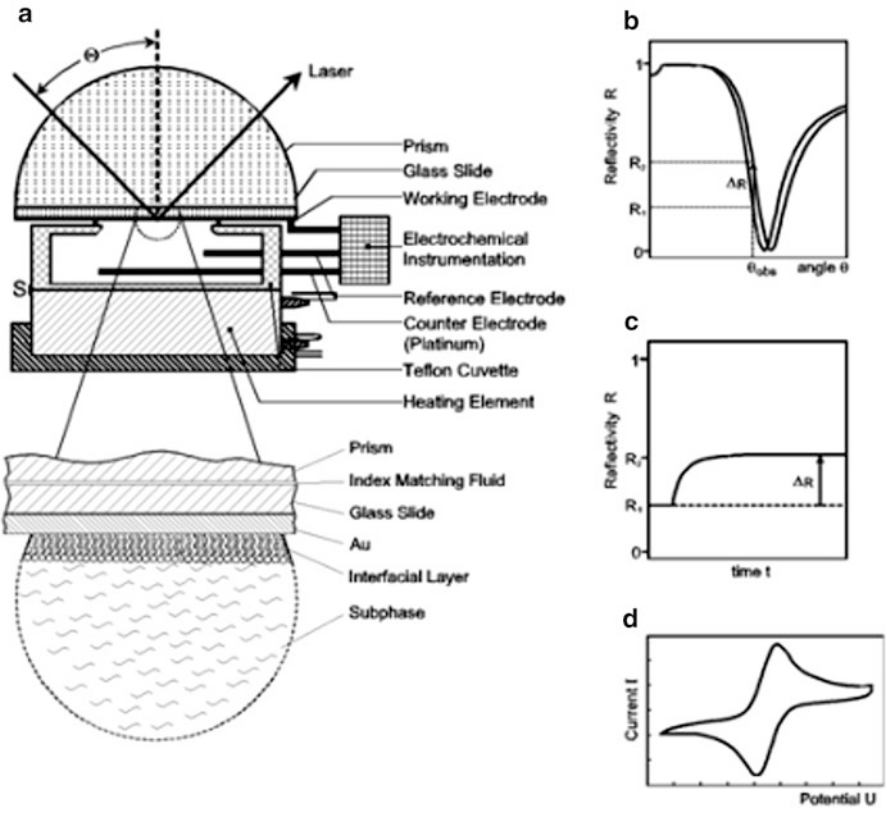


Fig. 10.1 (**a**) Schematic of a surface plasmon spectrometer in the prism-coupled Kretschmann configuration in combination with electrochemical instrumentation; (**b**) angular scan SPR measurements before (*left curve*) and after a thin film deposition, e.g., a layer of capture probes assembled (*right curve*); (**c**) kinetic SPR measurement taken as a function of time at a fixed angle of incidence (and observation), cf. (b); (**d**) schematic of a cyclic voltammogram taken by the electrochemical set-up coupled to the SPR instrument

cell, classical electrochemical techniques, e.g., cyclic voltammetry (cf. Fig. 10.1d), square wave voltammetry (SWV), or impedance spectroscopies [3] can be employed for the functional characterization of interfacial architectures.

A further extension of this combination set-up will be presented in Fig. 10.8: a fluorescence detection unit is attached in a way that the photons emitted by chromophores that are sufficiently close to the interface to be excited by the surface plasmon modes can be detected either as a function of the angle of incidence (tuning the resonance) or as a function of time for kinetic recordings [7].

Depending on whether electrochemical or surface plasmon optical measurements are to be performed the interfacial architectures assembled at the sensor surface need to fulfil different requirements: in order to optimize the electron transfer from a redox label to the electrode that is – in one way or another – the basis for electrochemical biosensing the redox-active unit has to be wired to the electrode interface in a most efficient way. The assembly of the sensor coating that were used for surface hybridization studies is schematically given in Fig. 10.2: onto the bare Au substrate a binary mixture of thiolated PNA oligonucleotides (i.e., the synthetic mimics of natural oligonucleotides with a neutral pseudo-peptide backbone [8]) of a particular sequence (HS-PNA) are co-adsorbed with a diluent molecule, mercaptohexanol (MCH), that optimizes the lateral packing of the probe oligonucleotides for the hybridization reactions (Fig. 10.2a). After hybridization of biotinylated target strands from solution to the probe matrix at the sensor surface (Fig. 10.2b), the biotin groups are 'decorated' in a 1:1 stoichiometric ratio by streptavidin molecules that carry a number of ferrocene molecules covalently attached to the protein through a spacer (Fig. 10.2c). A schematic of the protein structure and one ferrocene spacer unit are given in Fig. 10.2d.

Table 10.1 summarizes details of the employed molecular structures and the specific sequences of probe and target PNAs and DNAs [9]. In addition to the probe HS-PNA-P1 with a 15-mer recognition sequence coupled via the spacer groups to the thiol unit for the linking to the Au substrate the equivalent DNA analogue with the same recognition sequence P1, however, with a spacer unit of 15 thymines and a biotin anchor that allows the molecule to be assembled onto a streptavidin monolayer employed in the optical experiments as a generic binding matrix (cf. also Fig. 10.10) was used. For studies on mismatch discrimination another probe DNA, i.e., the biotin-DNA-P2 was used. This recognition sequence differs by a single base (underlined and in *italics* in Table 10.1).

The target strands follow two different molecular construction principles: for the electrochemical detection the DNA targets used are all modified with a biotin group at the 5′ end. This will allow for the coupling of the ferrocene-labeled streptavidin as redox-markers after hybridization (cf. the scheme given in Fig. 10.2b, c). For the surface plasmon fluorescence spectroscopic experiments the targets in one case were also modified with a biotin which after hybridization, however, were detected via the binding of a fluorophore-labeled streptavidin molecule. For the color multiplexing experiments QDs emitting at different wavelengths (cf. Table 10.1) were directly coupled to the various targets.

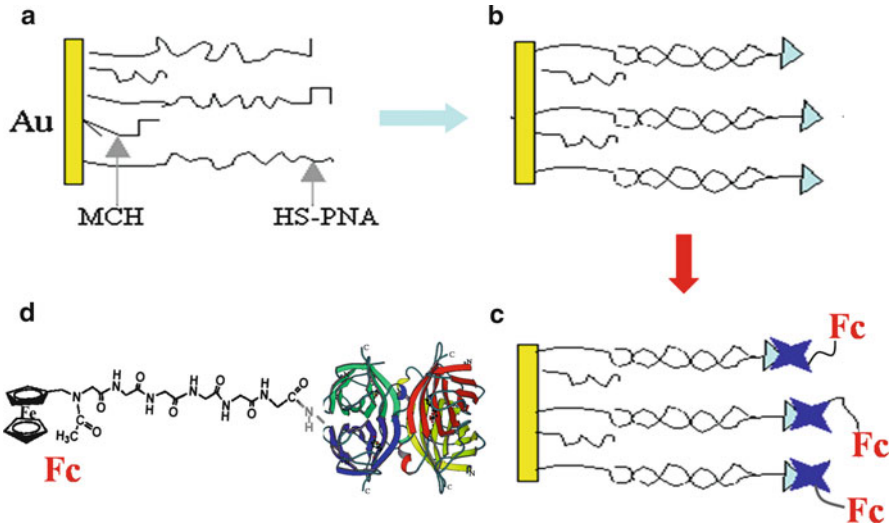
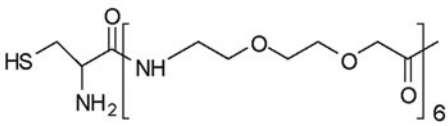


Fig. 10.2 Interfacial architecture self-assembled at the sensor surface for electrochemical read-out of hybridization reactions: (**a**) onto the Au substrate used for SPR- and electrochemical measurements a binary mixture of thiolated PNA strands and mercaptohexanol (MCH) as diluent molecules are forming a functional monolayer; (**b**) after hybridization with biotinylated target strands resulting in DNA duplex formation, ferrocene-labeled streptavidin can be bound to the surface layer (**c**). Some structural details of the ferrocene-labeled streptavidin Fc-Stv are shown in (**d**). Typically, 9 ferrocenes are attached to one streptavidin resulting in a corresponding amplification of the binding signal

Table 10.1 Probe and target sequences used in the experiments

Name	Nucleotide sequence
Probes	
HS-PNA - P1	Cys-[eg1]$_6$ - TGT ACA TCA CAA CTA - NH2
	Cys-[eg1]$_6$: 
Biotin - DNA - P1	5'-biotin - $(TTT)_5$ - TGT ACA TCA CAA CTA - 3'
Biotin - DNA - P2	5'-biotin - $(TTT)_5$ - TGT AC*G* TCA CAA CTA - 3'
Targets	
T1 - biotin	3' - ACA TGT AGT GTT GAT - biotin - 5'
T1 - QD_{565}	3' - ACA TGT AGT GTT GAT - **QD_{565}** - 5'
T2 - QD_{655}	3' - ACA TG<u>C</u> AGT GTT GAT - **QD_{655}** - 5'

10.3 Electrochemical Sensing of Hybridization Reactions

The combination of the electrochemical cell with a surface plasmon spectrometer allows for the structural characterization of the sequential assembly of the interfacial architecture by surface plasmon optical (angular and/or time-dependent) scans followed by the functional assessment of the resulting sensor surface coating by, e.g., cyclic voltammetry. This is documented in Figs. 10.3 and 10.4, respectively, for surface architectures based on the assembly of thiolated molecules directly onto the Au substrate. This results in the formation of a functional monolayer exposing a specific nucleotide sequence to the target strands injected subsequently into the sample cell. The minute shift of the SPR resonance curve upon binding of the targets to the surface probe layer (cf. Fig. 10.3, dots to solid curve) indicates the limited sensitivity of SPR in this case of a small analyte molecule (small molecular mass compared to a typical protein) bound to the surface at a moderate surface density. Only upon decoration of the hybrid monolayer with a layer of streptavidin bound via the target-attached biotin-groups results in a significant shift of the SPR angular scan curve that could be used for a more quantitative analysis of the affinity of this hybridization reaction. Effectively, the streptavidin acts as a mass label in SPR detection. From the angular shift of $\Delta\theta = 0.25°$ one calculates a thickness of the streptavidin layer of c. $\Delta d = 2.1$ nm (assuming a refractive index of $n = 1.45$), corresponding to a protein density of about $1.3 \cdot 10^{12}$ cm^{-2}.

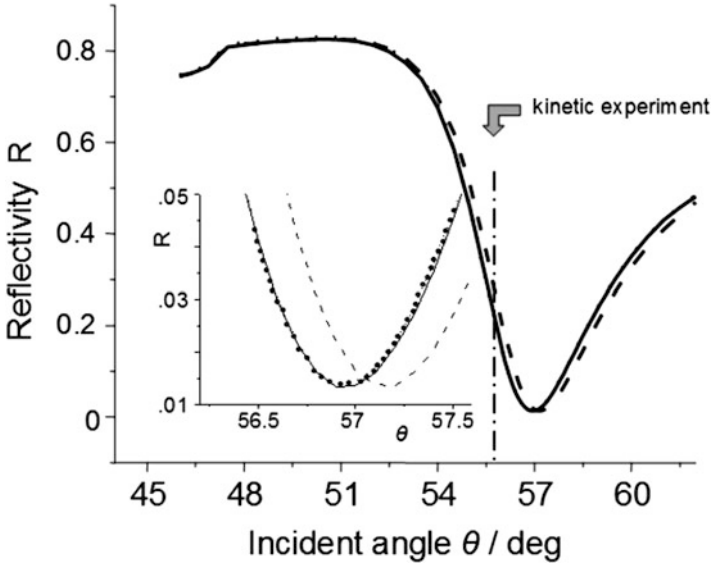


Fig. 10.3 SPR curves recorded in 20 mM PB buffer with 0.005% Tween 20 after PNA/MCH immobilization (*dots*), after hybridization with 500 nM target (*solid curve*) and after Fc-Stv adsorption (200 nM, *dashed curve*). The *inset* is an expanded view of the SPR minima part

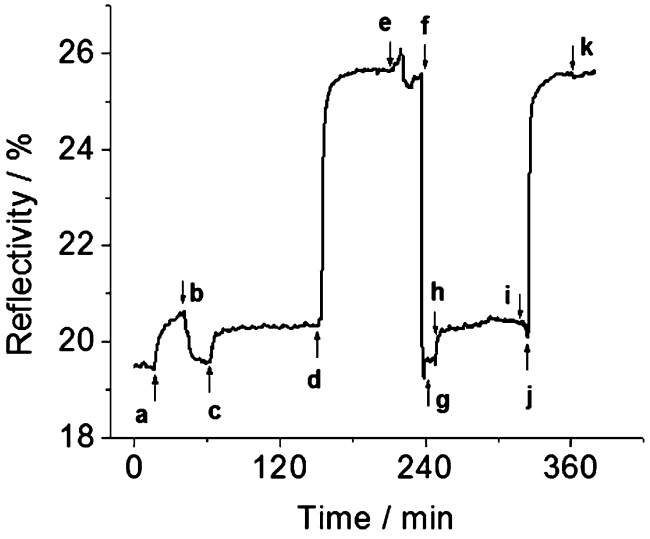


Fig. 10.4 SPR kinetic curve measured at a fixed incident angle ($\theta = 55.7^{o}$): (*a*) injection of 200 nM Fc-Stv after rinsing a 1 μM non-complementary biotinylated target solution through the cell; (*b*) rinsing with buffer; (*c*) injection of 500 nM fully complementary biotinylated target; (*d*) after rinsing and then injection of 200 nM Fc-Stv; (*e*) rinsing with buffer; (*f*) rinsing with 50 mM NaOH in order to regenerate the probe matrix; (*g*)–(*k*) repeating the steps (*b*)–(*e*)

The equivalent kinetic experiment and a few more reference scans are shown in Fig. 10.4. After assembling the probe matrix and injecting an analyte solution with fully mismatched target strands (a), rinsing with neat buffer results in the complete return of the reflected intensity at this fixed angle of observation to the reference level (b). Injection of the complementary, biotinylated target results in a small increase of the reflectivity (c) which – upon injection and binding of the ferrocene-labeled streptavidin (Fc-Stv) – shows the strongly amplified reflectivity increase expected from the angular scans displayed in Fig. 10.3 (d). Regeneration of the probe matrix by injecting a 50 mM NaOH solution through the flow cell (f) results in the return of the reflected intensity to the level measured prior to any hybridization. This observation and the displayed fully repeatable hybridization, i.e., association/dissociation (regeneration) cycle added in Fig. 10.4 indicates the excellent control over the supramolecular functional architecture and the stability of the attached components upon repeated hybridization cycles (Fig. 10.4g–k).

Figure 10.5 shows a series of cyclic voltammograms taken with a sample that was prepared by hybridizing the fully matched target T1-biotin from a 500 nM solution to the surface-bound probe matrix and decorating the hybrids by a monolayer of Fc-Stv [10]. The flexibility of the double strand and the six glycine residues of the linker used to couple the ferrocene to the streptavidin allow for a rather effective electron transfer from the redox site to the electrode and *vice-versa*. The CV curves recorded at different scan rates are perfectly symmetrical, indicating a fully reversible electron transfer reaction. The peak currents are proportional to

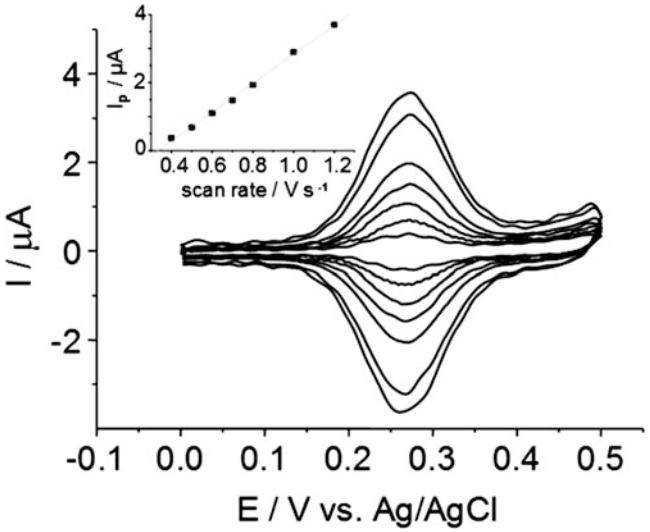


Fig. 10.5 Cyclic voltammograms of Au/PNA/fully complementary target/Fc-Stv measured in buffer with different scan rates, i.e., 0.4, 0.5, 0.6, 0.7, 0.8, 1.0 and 1.2 V/s from inside to outside, respectively. The *inset* shows the linear relationship between peak currents and scan rates

the scan rates, confirming a surface-controlled redox reaction only (cf. the insert in Fig. 10.5). The slightly broadened peak width (i.e. 120 mV compared to the ideal value of 90.6 mV for a reversible one electron surface transfer reaction) possibly indicates some ferrocene interactions and a random distribution of the redox labels within the film. Assuming that one biotinylated target can bind one streptavidin and that all Fc sites are electroactive the amount of bound DNA target can be calculated by integrating the current under the anodic (or cathodic) charge transfer wave in the CV. The obtained value of $\Gamma = 1.1 \cdot 10^{12}$ cm^{-2} corresponds well to the value obtained by SPR ($1.3 \cdot 10^{12}$ cm^{-2}, see above), indicating that, indeed, most of the ferrocenes are electroactive.

The number of bound DNA molecules, hybridized to the PNA matrix depends on the target concentration in the bulk solution, c_0. In particular, at low concentration only a fraction of the maximum PNA/DNA duplexes at the interface can be formed. With increasing concentration of c_0 more and more hybrids are formed until eventually a complete monolayer is asymptotically reached. Within the Langmuir model which requires certain conditions to be met, e.g., that all binding sites are equivalent and that the association and dissociation rate constants do not depend on the coverage, a simple relation for the coverage, Γ, at the sensor surface and the bulk concentration c_0 holds:

$$\theta = K_A c_0 / (1 + K_A c_0) \tag{10.1}$$

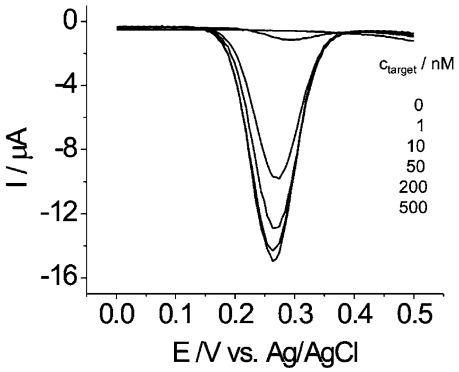


Fig. 10.6 Square wave voltammograms taken after the hybridization reactions of surface-grafted PNA oligonucleotide strands (cf. Fig. 10.2a) and fully complementary biotinylated target strands of different concentrations, as indicated, after Fc-Stv binding to saturation. Frequency: 100 Hz, amplitude: 20 mV

where θ is the fractional coverage defined as $\theta = \Gamma/\Gamma_{max}$ and Γ_{max} is the maximum coverage given by the number density of PNA probe strands at the sensor surface.

With the sensor architecture presented in Fig. 10.2 we can assume that one biotinylated target strand is able to bind to one Fc-labeled streptavidin molecule. Hence, the recorded current should be proportional to the DNA target coverage and will allow for the determination of the affinity constant K_A:

$$I(c_0) = I_m K_A c_0 / (1 + K_A c_0) \tag{10.2}$$

Figure 10.6 displays a series of SWV curves taken after Fc-Stv was bound to the biotinylated target strands after they hybridized to the sensor matrix from bulk solutions of different concentrations, as indicated. Clearly, with increasing target concentration the corresponding redox peak currents increase. If these peak currents are plotted as a function of the bulk target concentration c_0, the saturation behavior is found (cf. Fig. 10.7, (-◇-)) as expected from the Langmuir model that has been demonstrated by optical techniques to describe this process at the sensor interface very well. If fitted to the Langmuir model according to Eq. 10.2, an affinity constant of $K_A = 1.5 \cdot 10^8\ M^{-1}$ is found (Fig. 10.7, blue curve). This value agrees well with other reports based, e.g., on the use fluorescently labeled targets, in particular, if differences in the interfacial architecture, i.e., the coupling chemistry, the spacer length, the probe density, etc., are taken into account.

10.4 Fluorescence Spectroscopic Sensing of Hybridization Reactions

As it has been introduced recently, fluorescence detection schemes in combination with the optical field enhancement mechanisms operating at resonant surface plasmon excitation result in significant sensitivity gains for biosensing applications: for proteins binding to a quasi-3D matrix a limit of detection (LOD) of

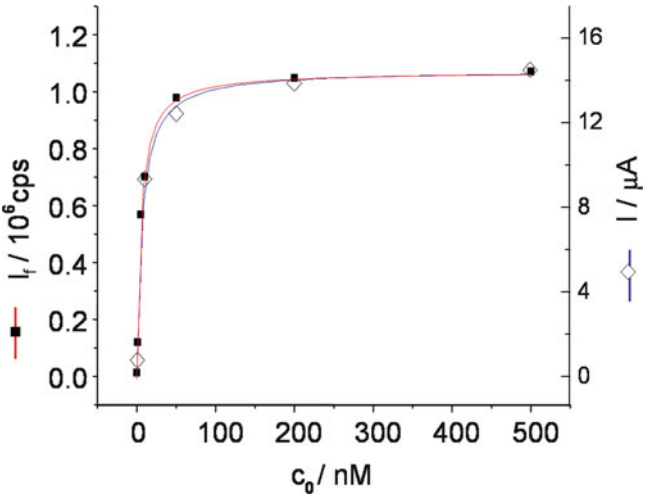


Fig. 10.7 Langmuir isotherms of PNA/DNA hybridization constructed from the electrochemical data presented in Fig. 10.6 (-◇-) and from the surface plasmon fluorescence data shown in Fig. 10.9 (-■-) and fitting curves (*red* for SPFS data, *blue* for electrochemical data)

$c_0 = 500$ aM [11], and for PCR hybridization to a 2D probe layer a LOD of $c_0 = 100$ fM [8] have been reported. As mentioned before the attachment to a regular SPR set-up required for surface plasmon fluorescence spectroscopy (SPFS) is rather trivial: a lens in front of the metal-coated prism base that guides the surface plasmon wave collects the fluorescence photons emitted from chromophores that are within the range of the evanescent surface mode. After passing an attenuator (in order to restrict the count rates to the linear response range of the detector) and a bandpass filter (for discrimination of the fluorescence emission against merely scattered light) this photoluminescence is then recorded by a photomultiplier tube or – in case of the microscopic mode of operation (cf. below) – by a color CCD camera. The whole arrangement is mounted to the SPR instrument in such a way that it rotates in the angular mode with the prism thus recording fluorescence photons emitted normal to the interface. The scheme of this instrumental setup is given in Fig. 10.8.

The possibility to employ fluorescently-labeled streptavidin (Fluor-Stv) (instead of the ferrocene-labeled analogue) for the decoration of surface-hybridized biotinylated target strands allows for a direct comparison of SPFC data with the electrochemical results presented in Figs. 10.6 and 10.7, respectively [10]. To this end, a series of angular SPFS curves were recorded after hybridization of targets from solutions of different concentrations and after the binding of Fluor-Stv. The results are summarized in Fig. 10.9. Similar to the current in the case of the SWV scans, here, the fluorescence intensity increases as one increases the bulk concentration of the target solutions, c_0. Note the slight shift of the angular peak position of the maximum intensity (which is also reflected in the slight shift of the position of the reflectivity minima) upon the increase in surface coverage of the

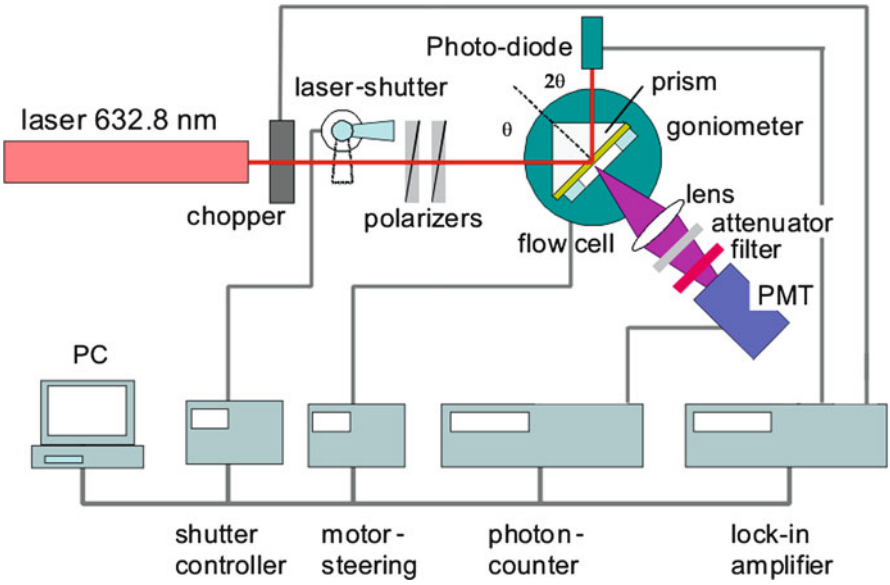


Fig. 10.8 Schematics of a surface plasmon fluorescence spectrometer, based on a normal SPR instrument with an attached fluorescence detection module. A photomultiplier tube (*PMT*) is used as the detector in the spectroscopic mode; however, in the imaging mode (cf. Fig. 10.11) a color CCD camera can be attached

bound streptavidin. Similar to the peak currents of the SWV scans the plot of the peak fluorescence intensities as a function of c_0 (cf. Fig. 10.7, -■-) shows the typical Langmuir isotherm behavior with the saturation at higher concentration (Fig.10.7, blue curve).

As one can see by the almost perfect superposition of the SWV and the SPFS data both methods give nearly identical K_A-values and, hence, confirm that both methods detecting their respective, yet very similar labels (Fc-Stv vs. Fluor-Stv) and the quantitative analysis of the data is not affected methodologically. As far as the sensitivity of the two techniques is concerned one should also expect similar limits of detection because the S/N levels in both data sets are rather equivalent (cf. Figs. 10.6 and 10.9).

The general interest in sensor platforms that allow for the parallel detection of multiple binding events typically requires a chip format in which the individually sensing elements functionalized for a particular analyte are arranged in an array matrix [12]. Read-out can then be achieved either in a serial manner (e.g., by a fluorescence scanner) or in a parallel way by a (microscopic) imaging mode [13].

The use of semiconducting nanoparticles, quantum dots (QDs), offers an alternative approach based on the possibility to engineer their emission wavelength by size (quantum confinement) or compositional control of their bandgap energy [14]. This way, very similar labels that all can be excited by the same laser wavelength

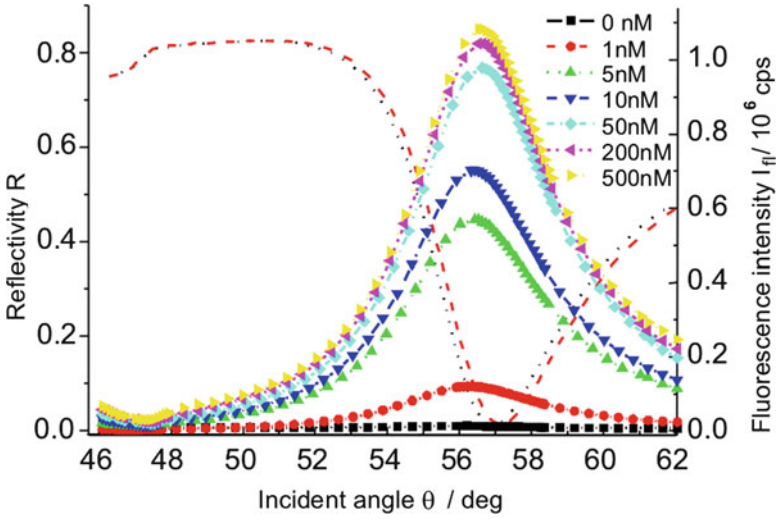


Fig. 10.9 A series of angular reflectivity (*dotted* and *dashed*) curves and SPFS scans (*curves* with *symbols*) taken after the hybridization of biotinylated, fully complementary target strands from solutions of different concentrations and the decoration of the duplex layer by fluorophore-derivatized streptavidin (*Fluor-Stv*). Note the slight shift of the angular peak position due to the increasing surface density of bound streptavidin; *dotted curve*: before hybridization, *dashed curve*: after hybridization with 500 nM target followed by binding of Fluor-Stv

but which emit in different luminescence colors can be synthesized and attached to the respective bio-analytes. Color multiplexing thus allows for the simultaneous recording of the binding signals of a whole set of analyte molecules in a mixture [5].

This concept and its implementation for surface hybridization studies is schematically given in Fig. 10.10. Here, the initial concept for the functional assembly of the sensor coating is modified by first assembling a monolayer of a binary mixture of 10% of a biotinylated thiol derivative laterally diluted by 90% of mercaptohexanol. Onto this coating a streptavidin monolayer as a generic binding matrix is assembled to which other biotinylated units can be attached, e.g., different oligonucleotide probe strands. Their base sequences are complementary to different DNA targets in the analyte solution which, in turn, are coded on different colors by carrying QDs of different emission wavelengths. Their simultaneous recording after hybridization allows for a multiple detection of a variety of analytes in parallel.

An example along this concept is demonstrated in Fig. 10.11. The flow cell was equipped with four Au electrodes that could be used as SPR/SPFS substrates [15]. After the general assembly of a protective coating to all electrodes (i.e., a poly (ethylene oxide) thiol SAM (PEO-SAM)) the flow cell was mounted to the spectrometer. The individual potential control of the four electrodes could then be used for the sequential functionalization of each Au area separately. Firstly, one of the electrodes was subject to a cathodic scan thus reductively desorbing its PEO-SAM, generating a bare, clean Au surface. Injecting sequentially (i) the binary

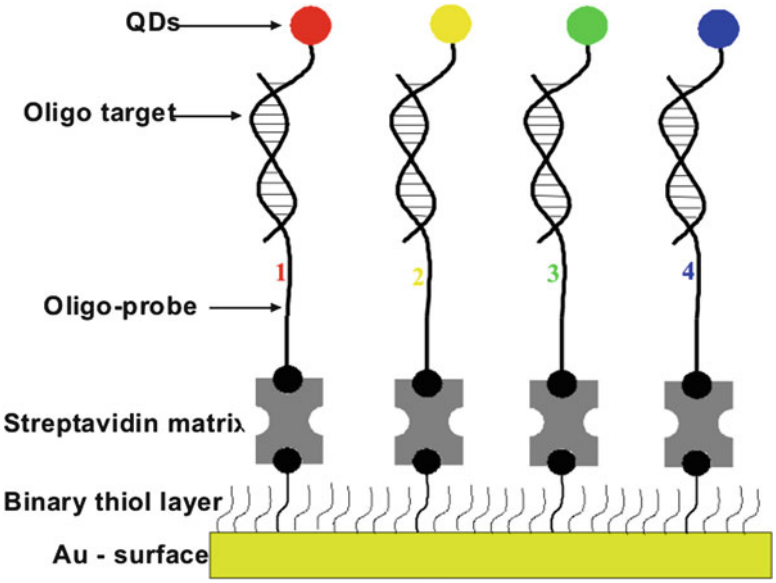


Fig. 10.10 Interfacial architecture optimized for multiple parallel surface hybridization studies in the color multiplexing mode with semiconducting nanoparticles emitting fluorescence light at different wavelengths: Onto the Au substrate a binary monolayer of biotinylated thiols mixed with OH-terminated thiols as diluent molecules is self-assembled, followed by the binding of a monolayer of streptavidin. To this generic binding matrix a mixture of different biotinylated probe oligonucleotides are assembled. They can specifically hybridize to their respective complementary target strands which are sequence-coded by quantum dots of different colors

(biotin-/OH-) thiol mixture, (ii) streptavidin and then (iii) a specific biotinylated PNA oligonucleotide solution (e.g., the Biotin-DNA-T1, cf. Table 10.1) results in the selective functionalization of this electrode only, while the other ones still remain protected by their PEO-SAM. By a step-and-repeat procedure eventually all electrodes are functionalized with no cross-contamination from one area to the neighboring electrode.

This way, the electrode array in Fig. 10.11 was functionalized with Biotin-DNA-P1 (P1), Biotin-DNA-P2 (P2), a mixture of both probes for the color multiplexing experiment, and a PEO-SAM used as an inert reference (cf. Fig.10.11a).

After injection of a 200 nM solution of the T2 target, coded with QDs emitting at $\lambda = 655$ nm, the surface plasmon fluorescence microscopic image displays the expected red color on the second and forth electrode (from top to bottom, cf. Fig. 10.11b) that both expose to the analyte solution the probe strand P2 which is fully complementary to T2 (cf. Table 10.1). The third electrode shows only a very faint reddish color corresponding to only a minute surface binding according to the significantly reduced affinity for T2 hybridizing to P1 which represents a mismatch 1 (MM1) situation with an affinity constant about two orders of magnitude lower than MM0. The reference electrode covered with the PEO-SAM remains completely dark.

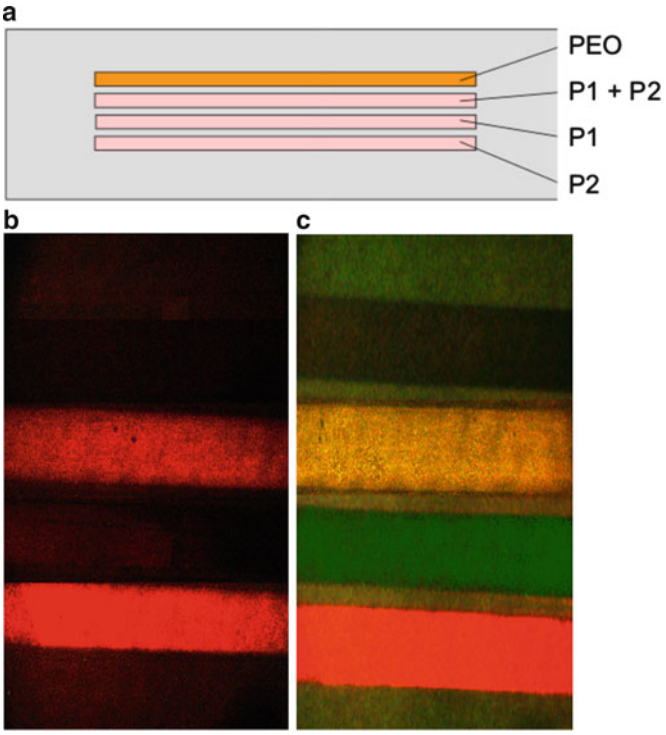


Fig. 10.11 Surface plasmon fluorescence microscopy: (**a**) Schematic arrangement (*top view*) of the probe oligonucleotides P1 and P2 and a mixture of both, as indicated, on a four-electrode chip for hybridization studies with target oligonucleotide strands labeled with quantum dots; (**b**) SPFM image showing the *red color* emitted after the hybridization of the target T2 which is complementary to P2 and is carrying a QD emitting at $\lambda = 655$ nm; (**c**) SPFM taken after the hybridization of a second injected target sequence T1, fully complementary to P1 and coded with a QD emitting at $\lambda = 565$ nm. Note the (artificial) mixture of *red* and *green* in the CCD camera to result in a *yellow color*

After rinsing and injecting the T1 solution the result shown in Fig. 10.11c is obtained after the hybridization reaction was completed. The third electrode exposing the MM0 probe P1 turns green corresponding to the binding of the targets T1 with their QDs emitting at $\lambda = 565$ nm. The forth electrode remains red (the emission appears to be more intense only because the integration time was increased in order to account for the lower quantum yield of the green emitting QDs), while the PEO-SAM also in this case appears to be completely inert.

The second electrode area now looks yellow. However, this is only a (well-known) artefact of the color CCD camera that mixes red and green to yellow. The same result was obtained upon the injection of a solution mixture of the target analytes rather than their sequential injection. Analysing the emitted light via a spectrometer clearly demonstrates that only light originating from green and red

emitting QDs is recorded. This experiment demonstrates that color multiplexing is an option for multiple analyte sensing; however, it requires the spectral analysis by a spectrometer for the unambiguous identification of the emitted colors.

10.5 Conclusions

The presented results document that both, fluorescence-optical and electrochemical principles, are well suited to monitor bio-analyte binding from the aqueous phase to specific functional sites assembled at the sensor surface. Although the read-out of redox labels doesn't quite reach the sensitivity of fluorescence recordings electrochemical techniques offer the advantage of giving direct electrical signals that can be used for data analysis.

Very attractive for both methods are semiconducting nanoparticles. The possibility of tuning their emission wavelength makes them very attractive labels in photoluminescence studies, e.g., for color multiplexing in parallel schemes for multiple analyte detection. One must bear in mind, however, that complications arise from the still unsolved problem of the luminescence blinking of QDs [16].

Particularly promising are schemes that couple the electronic orbitals of QDs to the Fermi-level of the metallic substrate. This way a completely new principle for the interplay between QDs as light emitting fluorophores and their electron donating or accepting properties are conceivable and should give rise to novel ways for the sensitive detection of bio-analyte binding [17].

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Part IV
Detection Strategies – Sequencing

Chapter 11
Recent Advances in Sequencing Technology

John F. Thompson, Fatih Ozsolak, and Patrice M. Milos

Abstract As we celebrate the tenth anniversary of the sequencing of the first human genome, we recognize the remarkable technological innovation that now provides the ability to resequence thousands of human genomes a year. While the current methods of choice utilize amplification-based methods and the corresponding challenges of sample preparation that accompany these methods, new technologies that do not require amplification have emerged. Single-molecule sequencing methods have the potential to dramatically shape the next 10 years of technological progress driven by the continuing interest of driving the cost of whole genome sequencing below the $1000 cost threshold. Yet while whole genome sequencing remains of interest, sequencing technologies also enable new approaches for genome exploration and experimentation including direct RNA sequencing, complete transcript sequencing and real time methods for both nucleic acid and enzyme kinetics.

11.1 Introduction

The development of Maxam and Gilbert, and separately Sanger, sequencing methodologies in the mid-to late 1970s opened a new era for cataloging the genome of a multitude of organisms [1, 2]. While much of the early sequencing included bacteriophage, cDNA's, ribosomal RNAs and viral genomes using these newly developed methods, the Maxam and Gilbert technology was quickly replaced with

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the safer and more expeditious method of Sanger chain termination chemistries. In Sanger sequencing, a primer is used to hybridize to the template DNA and synthesis of the complementary DNA strand is initiated by the DNA polymerase. Initially, four separate reactions were used to distinguish each dNTP incorporation as well as the chain terminating ddNTP with termination occurring due to the lack of a hydroxyl moiety on the ddNTP. Altering the ratio of dNTP's and ddNTP's results in chain termination along the length of the molecule and the use of radioactive nucleotides allowed the electrophoretic and autoradiographic analysis of the newly formed DNA strands providing the sequence of the synthesized strand in four separate electrophoretic gel lanes. The technical advance of using fluorescently labelled ddNTP's, each A, C, G or T ddNTP containing a unique fluorescent dye with distinguishing spectral properties facilitated single tube chemistry as well as automated sequencing instrumentation using the differential fluorescence of the individual nucleotides to record the incorporation event along the DNA strand through a laser detection system [3].

The commercial opportunity for DNA sequencing was quickly realized when in 1981 two engineers from Hewlett-Packard started Applied Biosystems to manufacture and sell sequencing instrumentation to enable large scale sequencing in genome research laboratories across the globe. Continued improvements in instrumentation allowing ever increasing speed and high-throughput capacity using the same basic Sanger chain terminator chemistry culminated in the release of the ABI Prism® 3700 Genetic Analyzer which became the work horse for the Human Genome Project delivering sequence data at a volume never imagined possible. A consistent theme throughout the revolution of DNA sequencing we are experiencing involves scientists envisioning the need and technologists finding new, innovative solutions to meet those needs.

Initial uses of the sequencing technology focused on cDNAs and the genomes of small organisms, including the first complete 4.6-Mb *Escherichia coli* K-12 genome [4] representing the work of more than 250 people that required a 6 year effort. The draft human genome sequence was published concurrently by the publicly funded Human Genome Project [5] and the commercial venture Celera [6]. The government sponsored project took some 15 years and three Billion dollars yet prompted scientists and technologists alike to begin considering the potential for expanding the number of genomes beyond the initial draft genome sequences. In 2004, following the completion of the human genome sequence [7], the National Human Genome Research Institute under the guidance of Dr. Francis Collins, leader of the HGP, announced a major 70 million dollar funding initiative to fuel development of new DNA sequencing technologies with a goal of achieving the $1000 genome by 2014. In now looking back, the initial round of funding shaped the field of both amplified and unamplified DNA next-generation sequencing as it exists today (Table 11.1). Indeed the goal of achieving the $1000 genome by the year 2014 once again reiterates the notion that clarity of vision allows the developments necessary to achieve the desired objectives, often more quickly than projected.

Today, we have taken the basic principles of DNA sequencing chemistry described above and moved far beyond the throughput and yield envisioned by the early pioneers in sequencing. Further we have extended the bulk DNA methodology

Table 11.1 The initial genome technology grants funded by the National Human Genome Research Institute in 2004

Grantee	Funding amount	Company/Institution	Title
$100,000 Genome Grants: NHGRI's "Near-Term Development for Genome Sequencing"			
Stevan B. Jovanovich, Ph.D.	$6.1 million (3 years)	Microchip Biotechnologies Inc.	Microbead Integrated DNA Sequencer (MINDS) System
Gina L. Costa, Ph.D.	$5.4 million (3 years)	Agencourt Bioscience Corp.	Bead-based Polony Sequencing
Kenton Lohman, Ph.D.	$2 million (2 years)	454 Life Sciences Corp.	Massively Parallel High Throughput, Low Cost Sequencing
Marcel Margulies, Ph.D.	5 million (3 years)	454 Life Sciences Corp.	454 Life Sciences Massively Parallel System DNA Sequencing
John Williams, Ph.D.	$2.5 million (3 years)	Li-COR Inc.	Single-Molecule DNA Sequencing Using Charge-Switch dNTPs
Michael L. Metzker, Ph.D.	$2 million (3 years)	Human Genome Sequencing Center, Baylor College of Medicine	Ultrafast SBS (Sequencing by Synthesis) Method for Large-Scale Human Resequencing
Stephen R. Quake, Ph.D.	$1.8 million (3 years)	Stanford University	High-Throughput, Single-Molecule DNA Sequencing
Mostafa Ronaghi, Ph.D.	$1.8 million (3 years)	Stanford Genome Technology Center	Pyrosequencing Array for DNA Sequencing
Jingyue Ju, Ph.D.	$1.8 million (3 years)	Columbia University	An Integrated System for DNA Sequencing by Synthesis
Peter Williams, Ph.D.	$1.7 million (3 years)	Arizona State University	Multiplexed Reactive Sequencing of DNA
Steven A. Benner, Ph.D.	$800,000 (3 years)	University of Florida	Polymerases for Sequencing by Synthesis
Amit Meller, Ph.D.	$600,000 (2 years)	Rowland Institute at Harvard, Harvard University	Ultra-fast Nanopore Readout Platform for Designed DNA's
"$1000 Genome" Grants NHGRI's "Revolutionary Genome Sequencing Technologies"			
J. Michael Ramsey, Ph.D.	$2 million (2 years)	University of North Carolina, Chapel Hill	Nanotechnology for the Structural Interrogation of DNA
James Weifu Lee, Ph.D.	$700,000 (3 years)	Oak Ridge National Laboratory	"Computational Research & Development for Rapid Sequencing Nanotechnology"
James Weifu Lee, Ph.D.	$750,000 (3 years)	Oak Ridge National Laboratory	"Experimental Research & Development for Rapid Sequencing Nanotechnology"
Scott D. Collins, Ph.D.	$850,000 (2 years)	University of Maine	High-speed Nanopore Gene Sequencing
Steven A. Benner, Ph.D.	$800,000 (3 years)	University of Florida	DNA Sequencing Using Nanopores
Andre Marziali, Ph.D.	$650,000 (3 years)	University of British Columbia, Vancouver	Nanopores for Trans-Membrane Bio-Molecule Detection
Stuart Lindsay, Ph.D.	$550,000 (3 years)	Arizona State University	Molecular Reading Head for Single-Molecule DNA Sequencing
Ronald W. Davis, Ph.D.	$450,000 (2 years)	Stanford University	Single Molecule Nucleic Acid Detection with Nanopipettes

Data Compiled from the National Human Genome Research Institute release announcing these awards (http://www.genome.gov/12513210)

utilized in these early technologies to now allow the detection and sequencing of single molecules of DNA and RNA. This chapter describes the rapid pace of amplified sequencing technology developments and the emergence of non-amplified, single molecule DNA and RNA sequencing, all of which have resulted in dramatic increases in the generation of genomic information including thousands of complete human genome sequences that will help to unravel the complexity of numerous diseases, as well as the use of sequencing for basic biological measurements including cDNA sequencing and quantitation, direct RNA sequencing and quantitation, and new insight into ancient genomes which are also highlighted in this chapter. The field of non-amplified sequencing now offers the opportunity for direct measurements of both DNA and RNA, providing a true measurement of cellular biology.

11.2 Emergence of Amplification Based Short Read Sequencing

Through the recognition of the important role "shot-gun" sequencing played in the commercial venture led by Craig Venter, the important concepts emerged for the "second generation" technologies – if one could simply sequence short fragments of DNA, the problem of assembling the genomes of small organisms with an eye to the whole human genome, seemed entirely possible. 454 Life Sciences emerged as the first entrant into the field of new non-Sanger based sequencing technologies publishing data demonstrating an increase in sequencing throughput enabling some 25 million bases of sequence data or some 100 fold greater than traditional Sanger technology in a period of 4 h [8]. This advance was enabled by the massively parallel synthesis of DNA templates by polymerase chain amplification to provide sufficient substrate for sequencing. This technology relies on the basic principles of sequencing by synthesis applied to pyrosequencing in which the emission of light upon incorporation of the labeled nucleotide for subsequent detection and visualization of the incorporation event, the landscape of DNA sequencing was changed forever. Commercial introduction of the Genome Sequencer 20/FLX in 2005 enabled researchers to achieve the complete sequence of the first human genome subsequent to that published from the Human Genome Project. This genome however was completed in just two months time, using the 454 technology with average read lengths approaching 250 nucleotides, and provided a genome coverage of $\sim$7.5$\times$ allowing redundancy of the reads to ensure both near complete coverage and accuracy of sequence data [9].

Solexa, a company founded in Cambridge, England in the early 2000s was one of the first commercial companies interested in the pursuit of single-molecule sequencing along with Helicos BioSciences founded at about the same time in Cambridge, MA. While both companies started as single-molecule sequencing by synthesis companies, Solexa abandoned the single-molecule approach, was acquired by Illumina in 2006 and their initial commercial platform, the GA1 was introduced in the 2007 timeframe. The GA1, an amplification based second generation system

of short reads, provided the customer with a new level of sequence throughput with 36 nucleotide reads. Today, the sequencing by synthesis approach practiced by Illumina has been continually improved through continued investment in the technology surrounding the chemistry, image analysis, engineering hardware and image analysis software to the point that the current HiSeq instrument allows researchers to sequence two complete human genomes per run at 30× coverage for approximately $10,000, closely approaching the goal of the $1000 genome.

11.2.1 Emerging Low Cost, High Throughput Technologies

While the increasing capacity of instruments, such as the Illumina HiSeq and Life Tech Solid, provide genome centers across the globe with the capacity to sequence hundreds to thousands of genomes per year, access to lower cost platforms to allow genomic scientists at smaller research institutions and translational research centers not well served by the ultra-high-throughput capacity has been limited. Former 454 founder, Jonathan Rothberg meanwhile recognized the limitations of the sequencing-by-synthesis chemistries dependence on ultra-fast imaging requirements. Ion Torrent was founded on the principles of image-independent chemistry in which the nucleotide incorporation event could simply be monitored by local changes in pH evident when a hydrogen atom is released upon nucleotide incorporation, promising a future where scale is only dependent on the ability to create a semiconductor surface which gets smaller and smaller to allow ever increasing numbers of molecular events to be monitored. Ion Torrent, purchased by Life Technologies in 2010, has its eyes set on the ultimate prize of the $1000 genome, however, along that path, the company has introduced the first low cost Personal Genome Analyzer (PGM^{TM}) to address the need of scientific researchers requiring low cost and moderate throughout to enable genomic experimentation. With a throughput today of 100 megabases, and promised improvements of ten-fold every 6 months the Ion Torrent technology appears ready for placing DNA sequencing within the realm of every biological researcher [10]. To exemplify, while this platform today is not able to sequence a complete genome in a single run, this technology enables comprehensive exon sequencing for important genes of biological relevance with the potential for diagnostic applications and when combined with bar-coding for each individual sample, hundreds to thousands of samples could be analyzed at the same time. Like the other sequencing technologies, a broad array of applications are available with the PGM machine.

11.3 Sequencing Applications

While all the technologies mentioned above rely on DNA or cDNA amplification to obtain sequence information, numerous applications are enabled by these methodologies and have revolutionized the manner by which biological measurements are

possible. These applications are briefly highlighted below and will be discussed in more depth as we describe the applications for non-amplified DNA and RNA sequencing later in the Chapter.

11.3.1 Whole Genome Sequencing

Whole genome sequencing costs have dropped dramatically over the last 10 years to the current cost estimate of 12 cents per megabase of DNA sequence as demonstrated in Table 11.2 (www.genome.gov/sequencingcosts). As the technologies have improved read length and paired-read capabilities, the major application driven by the Genome Centers has been genomic sequencing. From viral species and bacterial genome sequencing for purposes of strain identification to the human microbiome to the baboon, chimpanzee and 1,000s of normal human genomes and tumor genomes remains, these technologies have become the workhorse for genome sequencing. While the scope of this chapter is focused on sequencing technology, a brief mention of the informatics developments accompanying the technological advances described herein is appropriate as these developments have been integral in continued sequencing improvements.

Whole genome sequencing in particular has been dramatically improved by new methods and algorithms to enable *de novo* assembly of genomes and are nicely highlighted in recent publications [11, 12].

11.3.2 cDNA Sequencing

For more than a decade much of our exploration of the transcriptome has been conducted using microarrays or hybridization based methods that allow one to reliably detect the relative abundance of the known transcripts which hybridize to known probes on the array surface. This field, much like the field of DNA sequencing, has exploded with the use of next-generation sequencing. Termed RNA-Sequencing or RNA-Seq, scientists have pushed the technology to conduct biological experimentation at a scale not previously imagined and further, in a hypothesis free manner. By generating cDNA molecules through traditional reverse transcriptase methods and including the ligation of adapters that allow for amplification of the corresponding molecules, one has the ability to interrogate the transcriptome to ask important biological questions including information on the relative quantitation of RNA transcripts [13]. Since the initial publications describing the RNA-Seq methods appeared in 2008, important biological insight on stems cells [14], the complexity of the cancer transcriptome [13] and more recently the beginnings of the analysis of single cells [15] has allowed scientists to gain new insight into the RNA which plays such an integral role in cellular biology and disease states.

Table 11.2 The National Human Genome Research Institutes calculated costs for whole genome sequencing

Date	Cost per Mb of DNA sequence	Cost per genome
September-2001	$5,292.39	$95,263,072
March-2002	$3,898.64	$70,175,437
September-2002	$3,413.80	$61,448,422
March-2003	$2,986.20	$53,751,684
October-2003	$2,230.98	$40,157,554
January-2004	$1,598.91	$28,780,376
April-2004	$1,135.70	$20,442,576
July-2004	$1,107.46	$19,934,346
October-2004	$1,028.85	$18,519,312
January-2005	$974.16	$17,534,970
April-2005	$897.76	$16,159,699
July-2005	$898.90	$16,180,224
October-2005	$766.73	$13,801,124
January-2006	$699.20	$12,585,659
April-2006	$651.81	$11,732,535
July-2006	$636.41	$11,455,315
October-2006	$581.92	$10,474,556
January-2007	$522.71	$9,408,739
April-2007	$502.61	$9,047,003
July-2007	$495.96	$8,927,342
October-2007	$397.09	$7,147,571
January-2008	$102.13	$3,063,820
April-2008	$15.03	$1,352,982
July-2008	$8.36	$752,080
October-2008	$3.81	$342,502
January-2009	$2.59	$232,735
April-2009	$1.72	$154,714
July-2009	$1.20	$108,065
October-2009	$0.78	$70,333
January-2010	$0.52	$46,774
April-2010	$0.35	$31,512
July-2010	$0.35	$31,125
October-2010	$0.32	$29,092
January-2011	$0.23	$20,963
April-2011	$0.19	$16,712
July-2011	$0.12	$10,497

Reprinted from Wetterstrand KA. DNA Sequencing Costs: Data from the NHGRI Large-Scale Genome Sequencing Program. Available at: www.genome.gov/sequencingcosts. Accessed 12 Nov 2011

11.3.3 Chromatin Immunoprecipitation Studies

While DNA sequence is clearly the foundation for much of our understanding of the human genome, the additional insight provided by studying the structure of the

DNA in its native chromatin state has been significantly aided by next-generation sequencing. Chromatin immunoprecipitation studies (ChIP) allow one to study the interaction of the DNA sequence with any DNA-binding protein including histone proteins and their various modified forms in the nucleosome structure of the DNA found in the nucleus. Additional proteins including transcription factors which regulate the dynamic state of gene transcription form key interactions with the chromatin. The state of these protein:DNA interactions are studied genome-wide through a snapshot of antibody immunoprecipitation specific to the various proteins followed by isolation and sequencing of the specific DNA region to which the protein-antibody complex had bound. New insight into development [16], hematopoiesis [17] clinical outcome in ovarian cancer [18] has been possible through use of ChIP-Sequencing at the whole genome level.

11.3.4 Limitations of Amplification Technology

While amplification-based sequencing has led to tremendous advances across a range of biological questions, it remains limited or subject to artifacts in a variety of specific areas. Challenges still remain with respect to genomic rearrangements of large scale, accurate quantitation of both DNA and RNA as well as severe bias in regions of the genome with extreme G+C content. The new methods involving non-amplified DNA and RNA sequencing offer significant opportunity in all these areas as the technologies continue to mature [19]. Additionally, the various single-molecule approaches offer other technology-specific advantages including the ability to generate very long reads, to directly detect modified bases, and to directly sequence RNA.

11.4 Emergence of Non-amplified DNA Technologies

Researchers had long been interested in single molecule, non-amplified measurements of DNA. Non-amplified methods offered the potential to eliminate many of the challenges associated with complex sample preparation, PCR amplification, and the ability to interrogate single-cell nucleic acid as well as the potential for real-time interrogation to allow faster and cheaper detection of the sequence information. A landmark publication demonstrating single molecule fluorescence imaging to monitor the turnover of ATP molecules by single muscle myosin molecules using total internal reflection microscope [20] provided insight into the potential for such single-molecule measurements. This was rapidly followed by key developments in this field of non-amplified DNA sequencing which can be divided into three different areas which are sufficiently advanced for the purpose of this review: (1) direct imaging for single DNA molecules to allow both mapping and sequencing of large DNA molecules important for describing higher order structure of the

DNA sequence; (2) Optical sequencing by synthesis technologies and (3) nanopore sequencing technologies. What follows is a summary of these various technologies as well as detailed examples of sequencing applications available today.

11.4.1 Optical Mapping Technologies

As the field of DNA sequencing was maturing and the Human Genome Project was in full swing, many questions remained about the ability of shot-gun sequencing to recapitulate the accurate sequence of a genome the size of humans. Optical mapping techniques emerged in the 1990s as a potential solution to create an ordered structural map of the human genome and was one of the first single molecule methods for visualization of the higher order structure of the genome. Following the isolation of high molecular weight DNA upwards to some 10–20 megabases, DNA molecules are fixed on a glass surface, liquid flow elongates the single DNA molecules and restriction digestion of the DNA is performed directly on the surface. Figure 11.1 illustrates an optical image obtained from human genomic DNA to allow visualization of the single DNA fragments and illustrates a schematic representation of the potential ability to assemble long stretches of DNA along a contiguous stretch thus recapitulating the higher order structure of the human genome [21].

More recently scientists at companies such as Halcyon Molecular (http://halcyonmolecular.com/) and ZS Genetics (http://www.zsgenetics.com/index.html) have turned to the transmission electron microscopy as a new tool to investigate individual DNA molecules at the atomic level to allow visualization of the DNA sequence along the length of the molecule offering the potential to directly image and visualize at the DNA sequence level [22]. These methods while still under development offer the potential to provide sequence data on long stretches of DNA to overcome the limitations of current sequencing technologies which at present are limited by read length and throughput.

11.4.2 Optical Non-amplified Sequencing Technologies and Their Applications

11.4.2.1 Optical Sequencing Technologies

An initial demonstration of single-molecule sequencing-by-synthesis using DNA polymerase and fluorescent nucleotides to monitor the complementary nucleotide incorporation enabled DNA sequence data to be obtained from individual DNA molecules [23]. These initial studies led to the founding of Helicos BioSciences which further developed and commercialized the world's first single-molecule sequencing-by-synthesis technology.

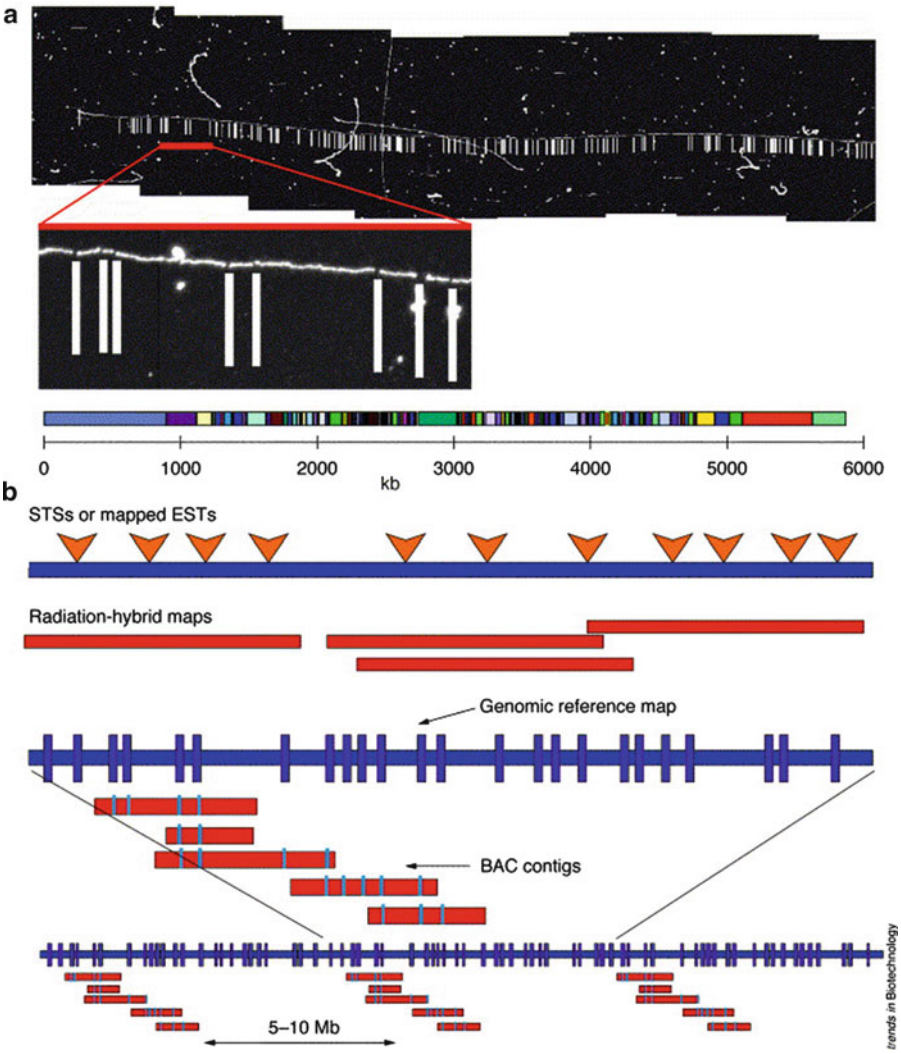
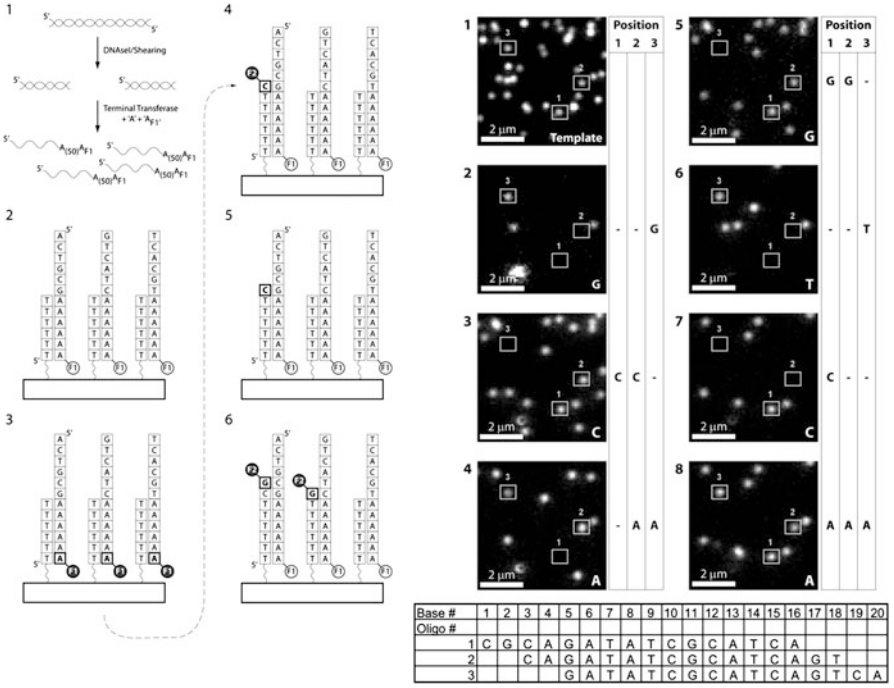


Fig. 11.1 Single molecule optical mapping depiction of the human genome. (**a**) An image of a DNA molecule 2 mm long covering 6 megabases of DNA, digested with PacI represented by overlapping microscope images. *White bars* represent the cutting sites on the DNA molecule. (**b**) Schematic representation depicting the linking of a whole genome optical map with that of physical maps (Reprinted with permission from Trends in Biotechnology, Ref. [21] Copyright 1999, Elsevier Sciences)

The Helicos technology utilizes non-amplified fragments of DNA or RNA for direct capture of the nucleic acid on the glass flow cell surface to which either universal capture primers or gene specific capture primers are covalently affixed. The depiction of the DNA sequencing process, which is also utilized for direct RNA sequencing, as well as the actual images captured during the sequencing process are shown in Fig. 11.2 [24, 25].



Base #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Oligo #																				
1	C	G	C	A	G	A	T	A	T	C	G	C	A	T	C	A				
2			C	A	G	A	T	A	T	C	G	C	A	T	C	A	G	T		
3					G	A	T	A	T	C	G	C	A	T	C	A	G	T	C	A

Fig. 11.2 Helicos BioSciences single molecule sequencing sample preparation and imaging of single molecules of DNA (*Left*) Single-molecule sequencing by synthesis process. (**1**) Genomic DNA is fragmented and a 3′ poly(A) tail added, labeled, and blocked using terminal transferase. (**2**) The DNA templates are captured on the surface with covalently bound oligo- dT(50). (**3**) Imaging of the captured templates to template sites on the surface. (**4**) One labeled nucleotide and polymerase mixture are added, followed by rinsing of the synthesis mixture and direct imaging. (**5**) Chemical cleavage of the dye–nucleotide linker to release the dye label. (**6**) Addition of the next nucleotide and polymerase mixture. (*Right*) Image series illustrating template-specific base addition, successful rinsing, and successful linker cleavage. A mix of three templates is used to allow visual sequence assignment. Template complementary sequences are shown in the table (*bottom*). One example of each template is outlined in the figure. Each frame is a 6.6-µm square image of the same sample position, and shows ∼35 of the 1.8×10^6 imaged templates in this experiment. Frame 1 is the image of the template labels. Template activity in three positions is shown in the columns to the right. Frame 2 is after the first synthesis and rinse cycle. Frames 3 to 8 show the effect of six more consecutive cleave, synthesis, and image cycles, using the base identity shown in the lower right corner of the frame (Reprinted with permission from Science, Ref. [24] Copyright 2008, American Association for the Advancement of Sciences)

The universal surface consists of an Oligo-dT50 surface to allow the researcher to add a polyA tail to the individual DNA molecules with terminal transferase for subsequent hybridization on the flow cell surface. When sequencing RNA, the natural polyA tail of the RNA molecules are captured on the surface or alternatively a polyA tail can be added to RNA molecules via polyA polymerase. Following hybridization to the flow cell, fluorescently labeled nucleotides, termed "Virtual

Terminators" and polymerase are added sequentially to the flow cell to allow the incorporation of complementary nucleotides into the growing strands of DNA or RNA. Once incorporated, laser excitation of the fluorophore present on the individual molecules leads to fluorescence that is captured with a charge coupled device (CCD) camera and converted to sequence reads using specialized imaging software. Following 120 cycles of this sequencing-by-synthesis and optical capture of the sequence data, more than one billion individual DNA sequence reads are generated from the DNAs held on the flow cell surface area and available for the variety of research applications of interest to the scientist. The Helicos sequencing technology provides an average read length of 35 nucleotides for each DNA molecule with a raw error profile between 3 and 5% with the predominant error form being a 'dark base' due to the incorporation of a nucleotide which is not visualized during the sequencing by synthesis process. Key is the alignment algorithm, IndexDP Genomic which allows for accurate alignment of DNA sequence data taking into account potential single nucleotide gaps in the DNA sequence [26]. Various applications of the sequencing technology are described in later sections of this Chapter.

Meanwhile, scientists at Cornell University were pursuing the real-time incorporation of fluorescent nucleotides via DNA polymerase into growing strands of DNA using a zero-mode waveguide (ZMW) technology [27]. Commericalized recently by Pacific Biosciences, the sequencing technology utilizes a SMRT™ cell or chip consisting of thousands of ZMW guides which are tiny microwells, nanometers in diameter, created in a metal film on a glass surface (shown in a schematic form in Fig. 11.3). Here the DNA polymerase is affixed to the bottom glass surface of the well. Laser illumination of the bottom 30 nm of the ZMW guide allows detection only of molecules which are near the bottom of the well. Because the DNA polymerase is attached to the bottom of the ZMWs, only labeled molecules bound to the polymerase remain in the illumination region long enough to be detected. DNA molecules are flowed across the surface to allow single molecules of DNA to bind to the polymerase and reside in the ZMW followed by addition of fluorescently-labeled nucleotides. Through diffusion, the nucleotides find their way into the ZMW where incorporation via the DNA polymerase occurs on the growing strands of DNA. Prior to each incorporation event, the fluorescent nucleotide must remain bound to the polymerase prior to incorporation and this results in fluorescence and corresponding detection of the color indicative of the nucleotide fluor. Following incorporation, the signal returns to low background level until the next nucleotide finds its way to the growing DNA strand. With the initial SMRT cell configuration consisting of some 75,000 ZMW, the incorporation events are monitored in real-time allowing the researcher to interrogate thousands of strands of individual DNA molecules in real time. In addition, the natural processivity of the DNA polymerase enzyme as well as the cleavage of the dye molecule attached to the phosphate chain of the nucleotide leaving a natural nucleotide in the growing DNA strand, has the potential to deliver read lengths well exceeding all other amplification and non-amplification based methods. Currently the average read lengths can approach 1,000 bases but continued efforts on both chemistry and detection offer the opportunity to surpass the current read length.

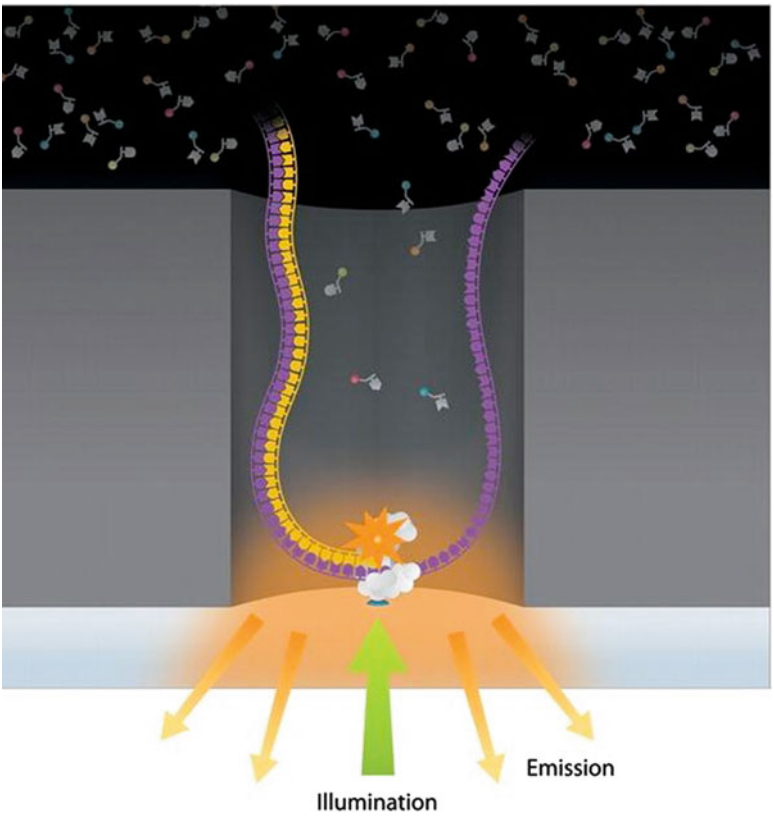


Fig. 11.3 Depiction of Pacific Biosciences Zero Mode Waveguide. The ZMW microwell is depicted here with a polymerase molecule to which DNA is bound. Synthesis of the DNA strand occurs through incorporation of the appropriate labeled nucleotide which will be detected upon laser excitation and emission to the recorded below the ZMW surface (Adapted with Permission from Pacific Biosciences)

Helicos and Pacific Biosciences non-amplified DNA sequencing technology have been used for genome sequencing. The publication describing the first single molecule sequencing of a human genome using the Helicos sequencing technology was indeed a remarkable achievement in the field of non-amplified DNA [28]. Requiring minimal sample preparation and three weeks time for sequencing followed by several weeks of analysis time, the field of non-amplified sequencing was established. Additionally, new insights into ancient genomes have been provided by recent studies of DNA from a Pleistocene-era horse [29] and thus the unique attributes of this ability are highlighted in a later section. Pacific Biosciences and colleagues at Harvard Medical School followed with another single-molecule sequencing study demonstrating the ability of SMRT sequencing to decipher the origin of the cholera strain responsible for the outbreak in Haiti in 2010 [30].

Yet, importantly, these two technologies each provide unique features that differentiate them from the existing amplification-based sequencing technologies. The current ability and future potential of the SMRT sequencing technology to obtain read lengths that far surpass the current technologies combined with the sheer speed of the DNA read-out offer new opportunity for more complete characterization of the genome, allowing us to address the many rearrangements and repetitive regions not possible with current technologies. In addition the ability to obtain full length cDNA transcripts to fully elucidate the complex structure of the transcriptome is entirely within the realm of the technology as throughput improvements occur.

The accuracy of quantitation with the Helicos single molecule sequencing technology is unparalleled as the ability to examine upwards of one billion molecules which have not been amplified or ligated. Thus the inherent bias caused by these molecular manipulations are avoided and the unamplified methodology provides the scientist with the purest quantitative measurement for nucleic acid from a biological source of interest [31] and enables new insight into important new biology revealing new RNA species not previously detected with amplification based technologies [32]. Additionally, the Helicos technology allows the direct sequencing of RNA. Molecules which have not been manipulated via copying with reverse transcriptase can thus be examined in a massively parallel fashion, something never before possible [33]. A more in-depth characterization of these unique applications is detailed below.

11.4.2.2 Gene Expression Measurements: Tag-Based and RNA Seq

Gene expression measurements provide a powerful window into how cells and organisms behave normally as well as in response to various stimuli. Both the number of genes that can be analyzed and the sensitivity with which those genes can be detected has increased substantially as the technology of choice moved from Northern blots and S1 assays to microarrays and qPCR and most recently to RNA Seq. Each of the methodologies has its own strengths and weaknesses with respect to identification of exon structure, crosstalk with similar genes, sensitivity, quantitative accuracy, ability to detect poorly expressed or uncharacterized transcripts and susceptibility to artifacts and errors. No technology is able to provide a complete, quantitative picture of which transcripts are expressed combined with a detailed view of their exon structure and 5′/3′ ends. As such, experimenters need to decide which aspects of the true expression profile are most important for their purposes so the technology can be chosen which is best suited to the needed information.

Most early technologies were able to monitor only a small number of genes for their expression levels so are limited if one wishes to do a complete transcriptome characterization. A more thorough view on transcription became possible with the advent of various types of microarrays and the ability to analyze thousands of different genes simultaneously. However, microarrays suffer from poor sensitivity for genes that are expressed at low levels, can suffer from significant crosstalk with genes that are closely related, has difficulty determining splicing patterns,

and is not generally useful for uncharacterized transcripts. To varying extents, next generation sequencing technologies have overcome all of these difficulties. For NGS technologies with a very high read count, very precise expression patterns can be generated. However, all sequencing methods also have limitations and these must be recognized so that experimental results can be properly interpreted.

Sequencing-based gene expression methods can be divided into two types: tag-based methods and methods that interrogate the entire RNA molecule. Tag-based methods include CAGE (Cap Analysis of Gene Expression) [34], SAGE (Serial Analysis of Gene Expression) [35, 36], PET-Seq (Paired End Tag Seq) [37], DGE (Digital Gene Expression) [38, 39], and DRS (Direct RNA Sequencing) [33]. These techniques capture a specific sequence at either the 5′ end (CAGE and DGE) or 3′ end (SAGE and DRS) or both (PET Seq) and count those molecules for determining gene expression profiles. Little or no information is captured on splicing or the opposite end of the molecule. Thus, these techniques are ideal for assessing and comparing gene expression levels. Additionally, the tag-based systems typically incorporate a selection step in which a specific feature of mRNA (5′ cap or 3′ polyA tail) that allows preferential sequencing of the RNAs of interest with less sequencing of ribosomal RNA that is generally of less importance for expression.

RNA Seq, in contrast to tag-based methods, captures reads from throughout each RNA molecule. This provides information about the entire RNA but, unlike microarrays and tag-based sequencing, this introduces an artifact into RNA Seq data in that the results depend on the length of the RNA being interrogated. If the RNA is long, more reads will arise from that RNA even if expressed at the same number of molecules as a short RNA. Frequently, the raw expression levels are corrected for length but these corrections are imperfect for many reasons [40] and can lead to analysis issues [41]. RNAs with extremes of GC content are less likely to be amplified and thus will appear less often than they should based on actual expression levels [42]. Efforts to eliminate GC bias in library construction have been partially successful [43] but some of these biases remain and are exacerbated by the amplification that is required during sequencing. Even after removing much of the library-induced amplification bias, genomic coverage patterns are still far from what is predicted based on known sequence content [43]. Figure 11.4 demonstrates this principle with data derived from RNA Seq experimentation in which amplification based methods are compared directly to single molecule methods, both involving cDNA analyses. Single molecule sequence data demonstrates more uniform coverage across the gene transcripts depicted [19].

The paired-end protocols used to generate long-range data for splicing analysis can introduce artifacts as well. If the raw reads from RNA Seq libraries are examined, 5–10% of all reads have paired ends that do not match the same gene [44]. Thus, one could expect that some artifacts could also be present in matched pairs, suggesting that rare splice variants should be verified using an independent technology. Additionally, the random hexamers typically used for synthesis of cDNA introduce another level of bias into the process [45], independent of sequencing platform.

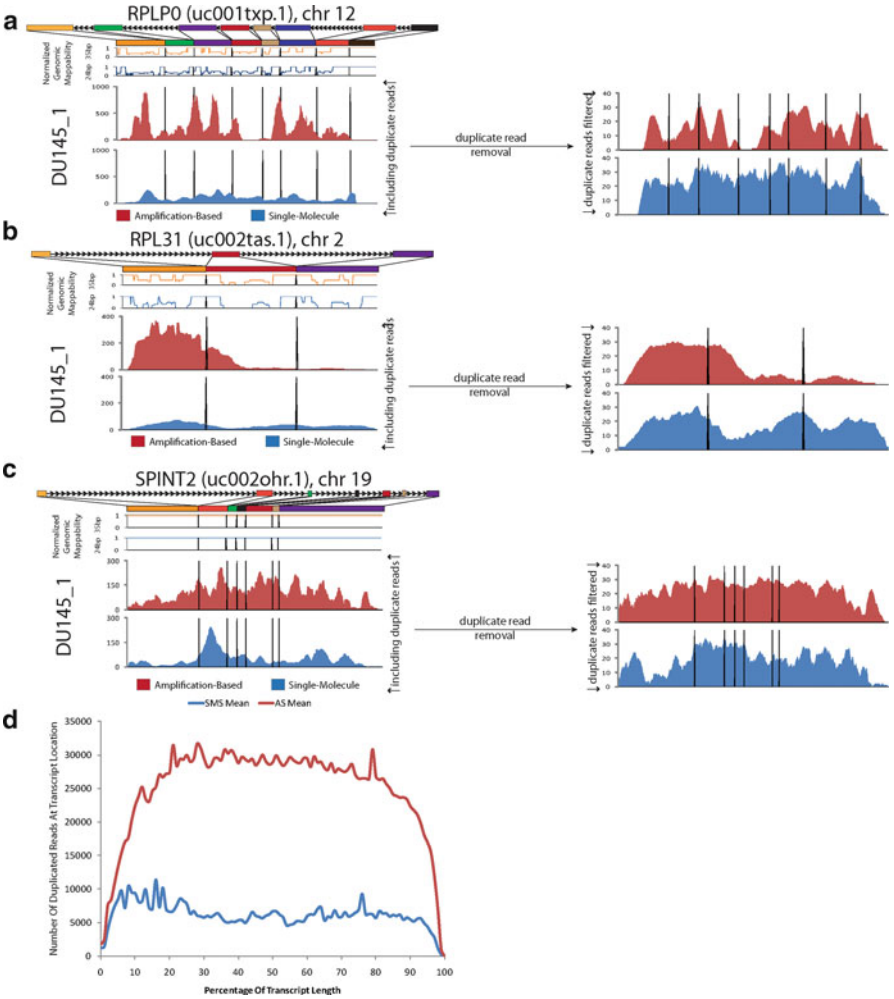


Fig. 11.4 Comparison of amplification and non-amplification RNA Seq data sets derived from cancer cell line. Amplification-based sequencing leads to a bias in high-concentration transcripts. Coverage maps from amplification-based and single molecule sequencing demonstrate significantly greater coverage of (**a**) RPLP0, (**b**) RPL31, and (**c**) SPINT2. Removal of reads with the same start positions significantly reduces the "spikiness" seen in these cases. (**d**) Duplicate reads are relatively evenly distributed along the length of observed transcripts across all samples and artificially inflate the apparent expression of those genes in amplification-based sequencing but not in single-molecule sequencing (Reproduced with Permission from PLoS ONE: Ref. [19])

While amplification-based sequencing has been used extensively for high quality expression profiles [46], there are situations in which single-molecule RNA Seq methods can provide superior results for some aspects of gene expression studies. For example, the Pacific Biosciences system is capable of long reads [47] so offers the potential of a direct view of exon content and start/stop sites for different

transcript isoforms. Unfortunately, the read count with this system is far too low to be generally useful for accurate expression profiles. A reasonable splicing picture of very highly expressed genes probably could be generated but the frequency of reads from most genes will leave them with no coverage or insufficient coverage to assess splicing and start/stop sites. For that reason, no RNA Seq profiles have yet been published with this system.

In contrast to Pacific Biosciences, the Helicos system generates a very high read count and thus can provide a very accurate quantitative assessment of gene expression [38]. Because amplification is not necessary, quantitative accuracy is maintained to a much higher extent than with amplification-based systems.

Any artifacts that arise from reverse transcriptase and cDNA synthesis may still skew the true gene expression measurements unless Direct RNA Sequencing (DRS) is carried out (see below) but biased amplification during library construction and sequencing is not an issue. The Helicos read lengths are generally, though not always, sufficient for unambiguous assignment to a particular gene. With highly homologous genes, some crosstalk may occur so there will be uncertainty in the expression level of such genes. Additionally, though some information can be gleaned about splicing, complex splice patterns are not detectable. Nonetheless, very precise RNA Seq profiles have been obtained [48] and technical replication far superior to amplification-based systems has been described [40].

To a large extent, the differential expression patterns obtained with single-molecule versus amplified sequencing are very similar but there are key differences. In particular, genes that are poorly expressed are found much less often with amplification-based sequencing [19], likely due to issues of limiting library diversity but other issues with PCR cannot be ruled out. In addition to limited visibility of poorly expressed RNAs and those with extreme GC contents, very short RNAs can also be problematic [32]. Many short RNAs are not well amplified during library construction and are too short to be amplified using bridge PCR required in the Illumina platform.

11.4.2.3 Gene Expression Measurements: Direct RNA Sequencing Technology

In addition to the frequent use of amplification, all of the gene expression methods described above rely on conversion of RNA to cDNA prior to sequencing. However, cDNA synthesis is known to be plagued by many artifacts including template switching, primer-independent first and second strand synthesis, and biased cDNA synthesis [49–51]. DRS technology can alleviate many limitations inherent in the transcriptomics methods in use today, and provide new avenues of research and applications in diagnostics. DRS not only eliminates the reverse transcription step, but also the other sample manipulation steps such as ligation and amplification, thus resulting in minimal distortion in the representation of RNA templates. The natural strand-specificity of DRS and its requirement for only femtomole quantities of RNA are advantageous for all aspects of RNA research.

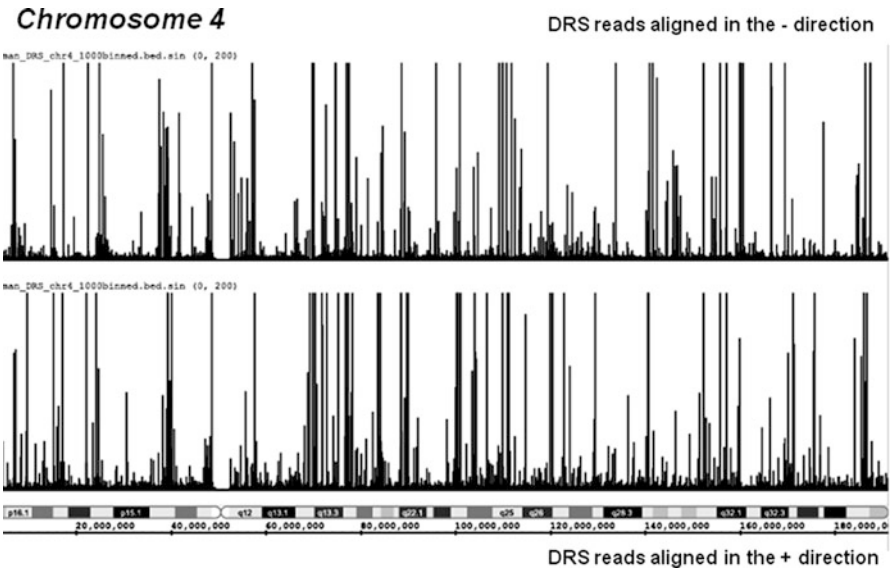


Fig. 11.5 Mapping of liver-derived direct RNA sequencing reads to human Chromosome 4.Total liver RNA was sequencing using the DRS technology. Sequence reads were mapped with high stringency to the human genome reference. Reads mapping to Chromosome 4 are shown (Unpublished data used with Permission from Helicos BioSciences Corporation)

DRS offers a simple route for polyadenylation site mapping [52]. Given its nature of capturing polyadenylated RNAs on poly(dT)-coated surfaces and sequencing after a "fill & lock" step, DRS reads emerge immediately upstream of the polyA-tail. Thus, the 5′ end of DRS reads signify polyadenylation addition locations. The DRS procedure is capable of capturing the polyA + mRNA population from total RNAs or cell lysates directly after blocking only the 3′ hydroxyl. The data generated is quantitative, thus for the first time allows genome-wide study of alternative polyadenylation states in both quantitative and qualitative manner across biological settings of interest. Figure 11.5 demonstrates direct RNA sequence reads obtained from liver total RNA mapped to human chromosome 4. Peaks across the chromosome demonstrate the diversity of RNA reads at low resolution. This data can also be used to generate a tag-based gene expression profile of polyA + mRNAs within cells.

DRS can also be adapted for all RNA analyses being performed today. Whole transcriptome profiling can be done with RNA fragmentation with standard methods, followed by polyadenylation of the RNAs. One advantage of DRS is the universality of sample preparation steps for different applications. In other words, unlike cDNA methods which require different cDNA synthesis and sample manipulation steps for short and long RNAs, DRS requires only 3′ polyadenylated templates. Thus, both short and long RNAs can be sequenced together in a single experiment.

11.4.2.4 Ancient and Degraded DNA

In most situations, long sequence reads are an advantage. However, when the DNA is degraded due to age, chemical fixation, or damaged by other deleterious conditions, it may actually be advantageous to use short read technologies. For example, ancient DNA is frequently contaminated with DNA from other species which is sometimes of more recent origin and hence potentially longer and of higher quality [53]. When such mixtures are amplified, the longer and less damaged modern DNA is preferentially replicated and hence increases its fractional composition and could potentially swamp out the desired signal from the ancient or damaged DNA. This is evident from work with DNA from a Pleistocene-era horse bone in which the same sample sequenced with an amplified versus non-amplified system yielded very different results for the per cent horse versus non-horse sequence reads [29]. On average, $>3\times$ more reads were from horse using the non-amplified sequencing. This difference was substantially increased when the sample preparation for the ancient DNA was modified slightly to remove blocking $3'$ phosphates from the ancient DNA [54]. The extent to which single-molecule sequencing is superior is highly dependent on the quality of the DNA sample of interest. The more degraded the sample, the higher degree of improvement can be obtained. However, it is not necessary for DNA to be Pleistocene era in order to be too degraded for analysis. For example, some remains buried at the National Memorial Cemetery of the Pacific that had been exposed to highly damaging conditions during embalming and which, even after extensive amplification, had not previously provided more than a few dozen base pairs of usable human sequence using amplification-based sequencing was able to be effectively sequenced using the Helicos system [55]. Similarly, many clinical samples are formaldehyde-treated and preserved in paraffin and thus can be significantly damaged or degraded. More recently preserved samples tend to be higher quality as experimenters have realized the importance of mild conditions to allow subsequent sequencing but many samples have not been as carefully handled and thus are problematic with sequencing systems that require longer templates. Again, single-molecule sequencing has been able to provide good sequence data for even RNA samples extracted from FFPE clinical specimens [56].

11.4.3 Nanopore Sequencing Methods

Classical next-generation sequencing techniques have dramatically dropped the price of sequencing while opening up numerous new avenues of scientific investigations. These tremendous advances have served to whet the appetite for even greater capacity at lower costs. However, the current commercial technologies are unlikely to yield orders of magnitude cheaper sequence or provide markedly different capabilities relative to what is already available. Consistent with the initial goals laid out by NHGRI for the $1000 genome and in contrast to the existing technologies, there are a variety of nanopore technologies, though not yet

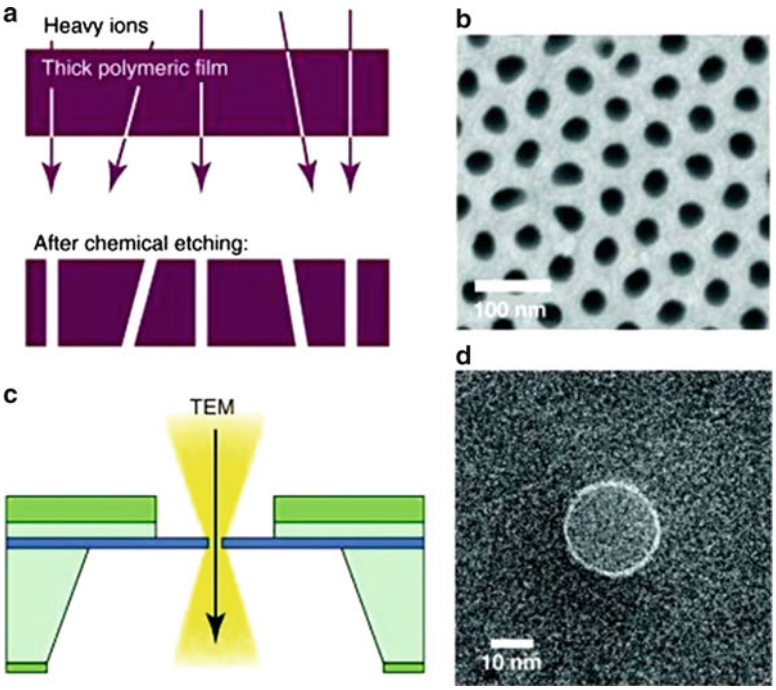


Fig. 11.6 Schematic representation of nanopore fabrication. artificial nanopores and nanochannels can be made by heavy ion and chemical ecthing (**a** and **b**) or by drilling holes through a silicon wafer with a focused electron beam (**c** and **d**). A more detailed description is provided in Ref. [60] (Reprinted with permission from Trends in Biotechnology, Ref. [60] Copyright 2011, Elsevier Sciences)

technically and/or commercially viable, that could provide dramatically different or cheaper output. Nanopore technology is attractive because, in theory, extremely long reads could be generated on single molecules with little or no reagent costs, simple sample preparation in a highly parallel fashion and with a very short time to results. However, realizing all these goals or even some of them, with a single technology will be challenging. To be commercially successful, the output from a technology will either have to provide a qualitatively different result or substantial cost/time savings relative to the already high bar created by existing technologies. Reviews dedicated to nanopores and nanopore sequencing are available and describe the wide variety of approaches being taken in this area [57–61]. Healey [62] has reviewed nanopore sequencing from a more historical perspective.

Nanopore approaches can be categorized in a number of ways based on the nature of the nanopore with the most frequent classification being biological or solid state. Biological nanopores use a protein channel with a pore of the necessary dimensions to allow DNA to pass through [63]. While early biological nanopores were in lipid membranes, some more recent versions are hybrid, using a solid-state scaffold with a protein pore. Solid-state pores (Fig. 11.6) were originally derived from silicon and

its derivatives though recent efforts have also explored graphene as an alternative. In addition to the nature of the pore, another useful categorization is the mode of sequence detection. Most nanopores detect variations in electrical signals (voltage or current) induced by blocking an ionic current between chambers but other methods, including optical detection and differences in transverse electrical currents, can also be used. The theoretical background and modeling of blocking ionic currents have been described in detail [64, 65]. Similarly, the theory and signals expected from transverse currents detected across a tunneling gap have also been described [66, 67] and confirmed experimentally [68–70].

Most efforts directed at biological nanopores have employed either α-hemolysin or MspA as the protein of choice [63, 71, 72] though other proteins may also be used [73]. All of these proteins have a pore with a diameter greater than 1 nm needed to allow single-stranded DNA (ssDNA) to pass through. Some proteins have an even larger pore allowing double-stranded DNA to pass. However, the variation in the lengths, widths and charge distributions within the pores of these proteins is not ideal for sequence detection so efforts have been directed at improving their properties. α-hemolysin and MspA have been successfully mutated to improve a variety of sequencing properties [72, 74, 75]. These mutated pore proteins have been shown to be capable of distinguishing the electrical signals from all four natural nucleosides/nucleotides [76–78]. However, distinguishing free nucleotides is not the same as sequencing extended lengths of DNA. To generate usable sequence data, the DNA must be translocated through the nanopore with sufficient force that movement is unidirectional but not so quickly that the signal from the individual bases cannot be distinguished. The length and width of the pore must be such that the base being sequenced is the primary contributor to the signal or a very complex set of signals will result. These conditions are not easily met so a variety of methods have been used in an attempt to generate sequence data. The speed of DNA translocation through protein pores has been slowed by altering viscosity [79] and by varying salt concentrations [80]. These manipulations have not yet proved sufficient for reading sequence so various proteins have been used to assist in the process.

For example, DNA polymerase has been used to detect sequence incorporations while attached to α-hemolysin [81]. Similarly, the activity of exonuclease I bound to α-hemolysin has been monitored by nanopores [82]. The kinetics of these reactions in pilot studies is not sufficient for effective sequencing but demonstrates the future possibilities if they could be optimized. In addition to detecting the electronic signal directly from nucleotides/nucleobases, there have also been efforts to detect signals optically after converting the naturally occurring sequence to fluorescent emitters [83]. However, methods described thus far introduce far more complexity into sample preparation than is required by other approaches so are not as appealing as the simpler nanopore readouts.

While biological nanopores offer advantages in terms of being easily manipulated with respect to changing the charge and inclusion of complex functional groups in or near the pore, there are only a small number of suitable pore proteins and they provide a limited set of scaffolds with which to work. Also, construction of highly parallel arrays does not have the economies of scale that solid-state

nanopores offer. Solid-state nanopores have the advantage of being much thinner and thus can generate signals arising from a single base more easily. Indeed, graphene offers the thinnest possible nanopore, the thickness of a single carbon atom. Furthermore, nanopores can be constructed with a wide variety of widths and this can be readily changed using well-established techniques [84]. For the most part, solid-state nanopores are made from silicon derivatives. Very long DNAs (97 kb) have been reported to be translocated through silicon at a speed greater than 10 kb/ms [85] and it is likely that much longer DNAs could be used. Even at 97 kb, these DNAs far surpass the lengths that can be interrogated with current sequencing technologies and thus would immediately provide a substantial benefit for genomic analysis if information beyond simply the length could be obtained.

As with biological nanopores, the speed of translocation with current solid-state nanopores is too fast for sufficient signal to be generated for each base to be effectively read. As a result, modifications of the pores and translocation conditions have been carried out to slow the rate of translocation [86]. Capture rate can be adjusted by varying salt concentrations [87] or by altering the manner in which the pores are made [88, 89]. Pores can be chemically modified so that the charge slows translocation speed. Because silicon nanopores can be made wider, ssDNA and dsDNA as well as protein-bound DNA can be successfully translocated through pores. When bound to recA, dsDNA moves through the pores much more slowly and generates a much higher blockade current [90].

While varying translocation conditions can slow the rate of transit through the nanopore that creates new issues with the positioning of the DNA with respect to the nanopore constriction and assuring that the DNA is read sequentially [91]. However, if DNA is translocated through a nanopore at a speed necessary to ensure predictable positioning, it is going too fast to generate sufficient signal. As a result, a variety of methods have been tested in order to provide the optimal mix of speed and sensitivity [92, 93]. Additionally, hybrid pores modified with DNA [94] or protein [95] can also be used. Another approach employs oligonucleotide probes of known sequence to tag regions of interest in DNA [96] and generate position-specific changes in blocking current. By using pools of probes, the entire sequence can be generated *de novo*. This method has the advantage of using groups of nucleotides with each signal and spreading that signal out over a longer physical distance for enhanced signal to noise and ease of detection.

Graphene nanopores also have the subject of much recent interest due to the absolute minimum thickness of their constrictions. Thus far, translocation of DNA has been observed through pores experimentally [97–99] along with theoretical predictions of how sequence signals might be possible [100]. While offering tremendous potential, graphene nanopores are now in their infancy in terms of characterization and their very simplicity will make them difficult to modify as can be accomplished with protein and silicon-based nanopores. Thus, the varying flavors of nanopore sequencing provide the best hope for the next quantum leap in sequencing capacity and capabilities but many issues need to be overcome before they are a commercial reality.

11.5 The Future

The power of amplification-based next generation sequencing has enabled countless new approaches to a host of previously inaccessible biological questions. This treasure trove of new applications and knowledge has obscured the fact that there are severe limitations and artifacts present in this powerful but not omniscient technology. The tremendous variety of single-molecule approaches holds the potential for filling many of those gaps and expanding the reach of massively parallel sequencing ever further. An important example, sequence reads of unprecedented length achieved with orders of magnitude faster speed to results could be generated, all at lower cost than current technologies. Different single-molecule approaches existing today, or in development, offer the potential for simplifying and lowering sample requirements, improved quantitation for gene expression, protein binding, and epigenetics. Thus, we can look to the next 10 year when the promise of the $1000 genome will be realized – and with this new milestone new opportunity for unraveling the complexity of human disease.

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