

Nanotechnology

Moving From Microarrays Toward Nanoarrays

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Summary

Microarrays are important tools for high-throughput analysis of biomolecules. The use of microarrays for parallel screening of nucleic acid and protein profiles has become an industry standard. A few limitations of microarrays are the requirement for relatively large sample volumes and elongated incubation time, as well as the limit of detection. In addition, traditional microarrays make use of bulky instrumentation for the detection, and sample amplification and labeling are quite laborious, which increase analysis cost and delays the time for obtaining results. These problems limit microarray techniques from point-of-care and field applications. One strategy for overcoming these problems is to develop nanoarrays, particularly electronics-based nanoarrays. With further miniaturization, higher sensitivity, and simplified sample preparation, nanoarrays could potentially be employed for biomolecular analysis in personal healthcare and monitoring of trace pathogens. In this chapter, it is intended to introduce the concept and advantage of nanotechnology and then describe current methods and protocols for novel nanoarrays in three aspects: (1) label-free nucleic acids analysis using nanoarrays, (2) nanoarrays for protein detection by conventional optical fluorescence microscopy as well as by novel label-free methods such as atomic force microscopy, and (3) nanoarray for enzymatic-based assay. These nanoarrays will have significant applications in drug discovery, medical diagnosis, genetic testing, environmental monitoring, and food safety inspection.

Key Words: Atomic force microscopy (AFM); carbon nanotube (CNT); CCD camera; chemical vapor deposition (CVD); CNT nanoelectrode array; electrochemical; enzymatic assay; multiwalled carbon nanotube (MWCNT); nanoarray; nanochip array; nanoelectrode; nanolithography; nanotechnology; nucleic acid analysis; plasma-enhanced chemical vapor deposition (PECVD); protein detection; scanning probe microscopy; self-assembly; single-walled carbon nanotube.

1. Introduction

Microarrays are important tools for high-throughput analysis of biomolecules. The use of microarrays for parallel screening of nucleic acid and protein profiles has recently become an industry standard for drug discovery and biomarker identification. But it requires relatively large sample volumes and elongated incubation time because of the large spot size. This also limits the sensitivity of detection. In addition, traditional microarrays utilize bulky instruments for detection and require extensive efforts in sample amplification and labeling, which increases the cost and time for getting the results. These drawbacks are the biggest impediment to move the nucleic acid- and protein-based detection to point-of-care and field applications. Nanoarrays brought by recent development in nanotechnologies have potential to overcome these limitations. Two types of nanoarrays are discussed in this chapter, i.e., carbon nanotube (CNT) nanoelectrode array and nanochip array (*1–10*). CNT nanoelectrode arrays are produced by reducing the electrode size to <100 nm using carbon nanotubes. This type of array has been demonstrated for ultrasensitive nucleic acid analysis by label-free electrochemical (EC) detection (*1–3*). Nanochip arrays, on the other hand, are fabricated by reducing the spot size of the traditional microarray to the nanometer scale using nanolithography techniques such as dip pen nanolithography (*4–8*). Nanochip arrays have been demonstrated for ultrasensitive protein detection in small volume by conventional optical fluorescence microscopy and also using novel label-free methods such as atomic force microscopy, eliminating the need for specialized optical readout instrumentation (*9,10*). Both types of arrays have also been applied to enzymatic assays (*11,12*). These characteristics suggest that nanoarrays will be advantageous over microarrays for many applications in biomedicine and biotechnology.

1.1. What is Nanotechnology?

The concept of nanoscience arose in a lecture by the physicist Feynman in 1959 (*13*), and the remarkable possibilities of nanotechnology in molecular manufacturing were discussed by Drexler in 1986 (*14*). After Iijima discovered the cylindrical structure of carbon nanotube in 1991 (*15*), nanoscience and nanotechnology have undergone great advances recently.

Nanotechnology is the creation of useful materials, devices, and systems through the manipulation of matter on this miniscule scale. The emerging field of nanotechnology involves scientists from many different disciplines, including physicists, chemists, engineers, and biologists. Nanotechnology is in a broad sense the science and engineering concerned with the design, synthesis, characterization, and application of materials and devices that have a functional organization in at least one dimension on the nanometer scale, ranging from a

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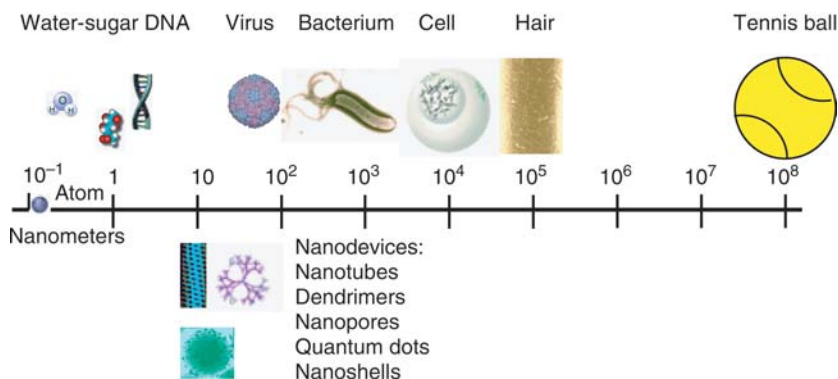


Fig. 1. Size of nanomaterials, molecules and live organisms.

few to about 100 nm. A nanometer is a billionth of a meter as illustrated in **Fig. 1**. It is difficult to imagine anything so small, but think of something only 1/80,000 the width of a human hair. Ten hydrogen atoms could be laid side-by-side in a single nanometer. A nanometer is roughly the size of a molecule itself (e.g., the width of duplex DNA is about 2.5 nm, whereas, the diameter of a sodium atom is about 0.2 nm) (16–18). There are many interesting nanodevices being developed for both diagnostics and therapeutic applications. For example, quantum dots have been demonstrated for cancer diagnostics, CNT and nanowire-based field-effect-transistor for biosensors, and dendrimers for drug delivery (1,19–22). The development of nanoarrays is focused in this chapter.

1.2. Why Nanoarray?

Nanomaterials offer significant advantages over conventional methods in biodetection regarding sensitivity, specificity, speed, portability, throughput, and cost because of their (1) small size (1–100 nm) and correspondingly large surface-to-volume ratio; (2) chemically tailorable physical properties, which directly relate to size, composition, and shape; (3) unusual target-binding properties; and (4) overall structural robustness (20). **Figure 2** summarizes the three types of arrays. Conventional microarray spot sizes are typically 200 μm in diameter (see **Fig. 2A**). In nanoelectrode array platform, individually addressed ultramicrospot are about $20 \times 20 \mu\text{m}^2$, consist of tens or hundreds of nanoelectrodes. Each nanoelectrode has a diameter of 50–100 nm, which are laid in insulating materials such as SiO_2 with only the end being exposed. The average spacing between neighboring nanoelectrodes is over 1500 nm to avoid the overlap of the hemispherical diffusion layer around each individual

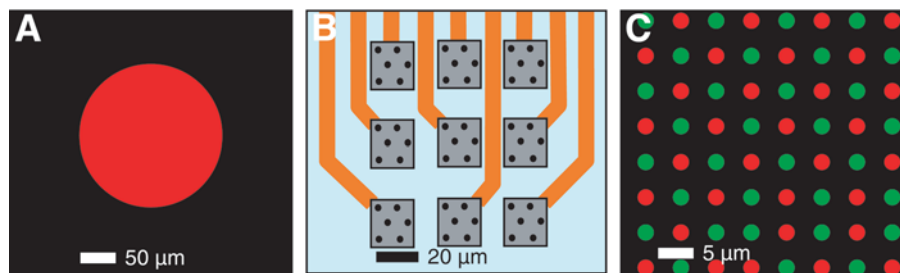


Fig. 2. Three types of array (A) microarray, (B) CNT nanoelectrode array, and (C) nanochip array.

one (Fig. 2B). In nanochip arrays spot sizes are typically $0.25\text{--}1\ \mu\text{m}^2$ with pitch $>2\ \mu\text{m}$ (see Fig. 2C).

1.3. CNT Nanoelectrode Array

CNT technology is ideal as a nanoelectrode array platform. A CNT is a cylindrical form of carbon, configurationally equivalent to a two-dimensional graphene sheet rolled into a tube (23,24). A CNT might consist of a single wall, known as a single-walled carbon nanotube, or multiple concentric cylindrical grapheme walls, known as multiwalled carbon nanotube (MWCNT). The outside diameter of a MWCNT varies from a few nanometers to about 200 nm and the length varies from a few microns to hundreds of microns. The physical dimension of MWCNTs is ideal for fabricating nanoelectrodes. MWCNT shows highly conductive metallic properties. The open end is an ideal electrode similar to graphite edge plane, while the sidewall is very inert similar to graphite basal plane. The difference in electron transfer rate at these two sites differs by five to six orders of magnitude (25). This makes MWCNT an ideal nanoelectrode, which can pick up the signal at the tip and transfer it to the measuring unit connected at the other end with minimum interference by the surrounding environment. With proper EC etching or acid treatment, the open end of MWCNTs contains --COOH and --OH groups, which can be used for attaching biomolecules through the formation of amide bonds (26).

The sensitivity of an electrode is mainly determined by its *signal-to-noise ratio*. The noise is the background current mainly from the capacitive charging/discharging current at the electrode/electrolyte interface and thus proportional to the surface area (A) of the inlaid electrode, i.e.,

$$i_n \propto C_d^0 A \quad (1)$$

where C_d^0 is the specific capacitance at the interface. In most common voltammetry measurements, the magnitude of the peak current of the redox

signal is the sum of two terms: a linear diffusion as described in the Cottrell equation, and a nonlinear radial diffusion (27)

$$i_{l,peak} = nFAC_0 * \sqrt{\frac{D_0}{\pi t}} + nFAC_0 * \left(\frac{D_0}{r}\right) \quad (2)$$

where n is the number of electrons involved in the reaction with one electroactive species, F is the Faraday constant, C_0 is the concentration of electroactive species, D_0 is the diffusion coefficient, t is time, and r is the electrode radius. As the radius is decreased below 25 μm , the second term increases and dominates the signal, $i_{l,peak}$, which is typically referred to as ultramicroelectrodes (UME) (28). In this regime, the signal-to-noise ratio follows the equation

$$i_{l,peak}/i_n \propto nFC_0D_0/r \quad (3)$$

Clearly, if the electrode size is reduced from 200 μm to 200 nm, *the signal-to-noise will be improved by 1000 times*. At the same time, the electrode also *responds 1000 times faster*, similar to the fact that smaller transistor features afford faster computer speed.

It is clear that the performance of an electrode with respect to the temporal and spatial resolution scales inversely with the electrode radius. Therefore, the sensitivity can be dramatically improved by reducing the size of the electrodes to nanoscale. Even a single redox molecule can be detected using a Pt-Ir electrode with 15 nm diameter (29). With the diameter approaching the size of the target molecules, nanoelectrodes can interrogate biomolecules much more efficiently than conventional electrodes. For practical applications, hundreds of nanoelectrodes are desired to be attached to each microelectrode as a local nanoelectrode array in order to increase the statistical reliability. From our previous work (2), each individual electrode will behave as an independent nanoelectrode as long as the average spacing between the neighbors is approx $>1 \mu\text{m}$. The total signal summed over all nanoelectrodes on the same local microspot will increase the signal amplitude so that it will not be interfered by instrumental and environmental noise. The EC behavior of such low-density nanoelectrode arrays or ensembles is similar to a single nanoelectrode, which is fundamentally different from the high-density nanoelectrode ensemble fabricated with a templated method by Menon (30). The diffusion layer of each nanoelectrode in a high-density array/ensemble will overlap giving similar behavior as a solid macroelectrode.

The nanoarray format also has a second advantage in improving the efficiency of target capture and kinetics of the target/probe hybridization. Peterson has demonstrated that 100% of probes can be hybridized following Langmuir-like

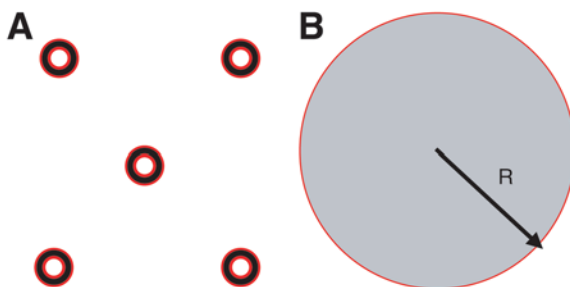


Fig. 3. Schematic of the relative amount of the probes at the edge (in red) vs those at the high density area (in black). (A) At the end of an inlaid MWCNT nanoelectrode array and (B) at a microspot of a conventional DNA microarray.

kinetics at a low probe density (2×10^{12} probes/cm²) and only about 10% probes are hybridized with a high probe density (12×10^{12} probes/cm²) with much slower kinetics (31,32). Better mismatch discrimination is obtained by reducing the probe density. This observation can be as a result of the steric hindrance from the neighboring molecules. In nanoelectrode array platform, the efficiency and kinetics for the hybridization of target molecules faces the same problem, particularly if long single-stranded DNA or mRNA are the target molecules (33,34). Because of the small size and geometry of each nanoelectrode, a large portion of capture probes is well-exposed at the edge and can be easily accessed by the target molecules. The hollow structure of MWCNTs forms an additional inner circular edge. Approximately, as high as 27% of the probes are in the 2 nm wide edge at the end of an inlaid MWCNT nanoelectrode with the outer radius of $R_{\text{out}} = 25$ nm and inner radius of $R_{\text{in}} = 10$ nm (Fig. 3). In contrast, the percentage of probes at the edge is negligible in conventional DNA microarrays. Therefore, the nanoelectrode platform would naturally be more efficient for hybridization and mismatch discrimination.

For electronic nano-chip technology, a key advantage is that a large local electrical field can be applied right at the tip of nanoelectrodes on each microspot. Because DNA and RNA molecules are negatively charged, the electric field might enhance or retard hybridization and denature surface immobilized DNA duplexes. Discrimination between fully matched and mismatched hybrids can be achieved by programming each microsites with a specific electrode potential (35–37). The electrostatic force generated by the local electric field coupled with global temperature control provides much better stringency control over the whole chip. The electrode potential can be further programmed into biphasic pulses, which is synchronized with the fluid flow to accelerate the process for the right target to hybridize onto the electrode.

1.4. Nanochip Array

Nanochip arrays are obtained by printing nanometer size spots on surface using dip pen nanolithography technology (4–8). The surface area of a single nanoarray spot is about several thousand times less than that of conventional microarray, with estimated volumes of approx 30 attoliters/spot. Therefore, the amount of analyte in one microarray spot (100 μm in diameter) is adequate for fabrication of many thousands of nanoarray spots. This suggests that nanoarrays are compatible with picoliter volumes of analyte, which is consistent with the volumes of a single cell (9–10). The small volume requirement of nanoarrays enables sensitive detection for less analyte such as protein extracts generated from just a few cells of interest harvested by laser capture microdissection from a cancer biopsy.

According to Einstein's square root law of Brownian motion (38), $\langle |r|^2 \rangle = 6 D t$, the distance of molecular diffusion, r , will be proportional to the square root of the time, t , and therefore, a modest reduction in volume will yield a significant decrease in diffusion time. A reduction in array size statistically ensures that every analyte molecule will have an opportunity to sample the entire capture surface in a reasonable amount of time. A traditional microarray utilizing tens or hundreds of microliters of sample spread out across several centimeters of capture domains results in the effective wasting of the majority of the sample because it will never be in close proximity to a relevant capture domain unless additional energy is added to enhance the movement of the analyte (e.g., agitation, mixing, or electromotive force). In typical static 2-h incubation, each microarray spot theoretically samples not more than a 1.7-mm radius half-sphere. In contrast, a multiplexed nanoarray in a tiny microfluidic chamber measuring $100 \times 100 \mu\text{m}^2$ could expose each capture domain to every analyte molecule in a matter of minutes. The small size of array with small volume of sample enables fast hybridization and efficient target capture (6,9,10).

Because of the small size, nanoarrays are more accessible optically than microarrays as the entire nanoarray field can be read in a single optical microscopy image with conventional optical fluorescence microscopy or by novel label-free methods such as atomic force microscopy, eliminating the need for specialized optical readout instrumentation (6,9,10).

2. CNT Nanoelectrode Array for Nucleic Acid Analysis

CNTs belong to a family of materials consisting of seamless graphitic cylinders with extremely high aspect ratios (39–45). The typical diameter varies from about one nanometer to hundreds of nanometers and the length spans from tens of nanometers to hundreds of microns or even centimeters.

As a result of the intriguing nanometer-scale structures and unique properties, CNTs have quickly attracted intensive attention in the past few years in many fields such as nanoelectronic devices (46–51), composite materials (52), field-emission devices (53,54), atomic force microscope probes (55–58), and hydrogen/lithium ion storage (59,60). Many studies reported potential for ultrahigh sensitivity sensors as well (51,61–64). The extremely high surface-to-volume ratio of CNTs is ideal for efficient adsorption. The one-dimensional quantum wire nature makes their electronic properties very sensitive to gas or chemical adsorption. Both of these factors are essential for high-sensitivity sensors.

In the past few years, CNT sensors have been demonstrated in many applications involving gas molecules, liquid-phase chemicals, to biomolecules with improved performance compared with conventional sensors utilizing micro- or macromaterials and thin films (65). This section summarizes the recent progress in the fabrication of CNT nanoelectrode for rapid, ultrasensitive, label-less nucleic acid analysis using nanotechnologies.

2.1. The Fabrication of CNT Nanoelectrode Array

CNTs are critical components of the sensing devices, which are integrated either directly or indirectly during the fabrication routes. A variety of methods, ranging from advanced micro- or nanolithography techniques to handmade processes, have been demonstrated by various researchers to build functional devices. Generally, CNTs could be the sensing elements whose properties are changed on occurrence of the biological events or by the transducers that transfer the changed signal to the measuring units. The sensing device could use a single CNT or an ensemble of such materials.

An innovative bottom-up approach based on vertically aligned MWCNTs was developed to fabricate CNT nanoelectrode arrays (2,65–67). This technique is compatible with semiconductor processes and can be employed to mass produce nanoelectrode array chips. The processing flow is illustrated in **Fig. 4** and the details are described next in six steps:

1. A microelectrode array with individual addressing lines is fabricated on a Si chip using standard ultraviolet (UV)-lithography patterning techniques.
2. A thin layer (~10–30 nm) of catalyst metal such as Ni is deposited at the microelectrode site to define the location for MWCNT growth.
3. Arrays of vertical aligned MWCNTs are grown at the catalyst covered sites with plasma-enhanced chemical vapor deposition (PECVD) (68–70).
4. A SiO₂ layer is deposited by tetraethoxysilane chemical vapor deposition (CVD) to encapsulate all MWCNTs as well as the metal lines.
5. A chemical mechanical polishing or etching step is then applied to remove excess SiO₂ and expose only the very ends of MWCNTs.

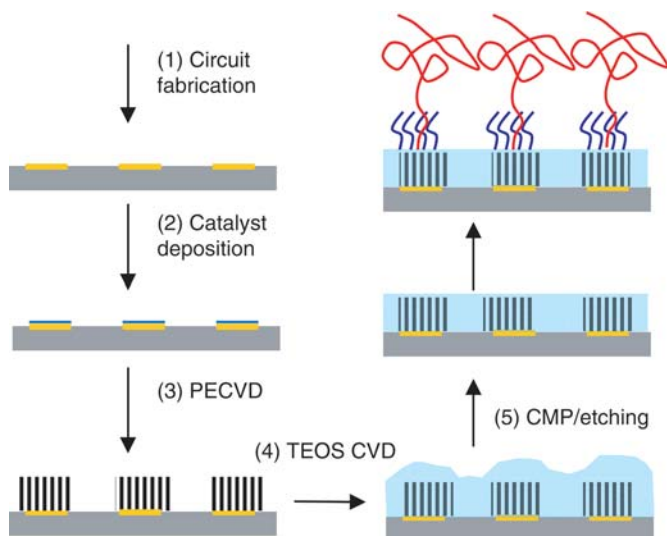


Fig. 4. Schematic of the processing flow for fabricating MWCNT nanoelectrode array Nano-chips for DNA/RNA analysis. MWCNT nanoelectrode arrays are connected to individually addressed microelectrodes on a Si chip in an array-in-array format.

6. Such nanoelectrode arrays are then functionalized with specific oligonucleotide probes by forming amide bonds. Further hybridization and washing steps are carried out using the similar protocols for conventional DNA microarrays.

As a first step for demonstrating the feasibility of multiplex electronic Nanochip, we designed a 3×3 array and successfully fabricated well-defined MWCNT nanoelectrode arrays using the procedure described in **Fig. 4**. **Figure 5A,B** shows scanning probe microscopy images of a 3×3 array of individually addressed electrodes on a Si (100) wafer covered with 500 nm thermal oxide. The electrodes and contact lines are 200 nm thick Cr patterned with UV-lithography. Each electrode can be varied from 2×2 to $200 \times 200 \mu\text{m}^2$, consisting of a vertically aligned MWCNT array grown by PECVD from 10–30 nm thick Ni catalyst films. **Figure 5C,D** shows MWNT arrays grown on 2 μm and 200 nm diameter Ni spots defined by UV- and e-beam lithography, respectively. The spacing and spot size can be precisely controlled. The diameter of the MWCNTs is uniform over the whole chip and can be controlled between 30 and 100 nm by PECVD conditions. The number of CNTs at each spot can be varied by changing the thickness of the Ni film. Single nanotubes can be grown at each catalyst spot if their size is reduced to <100 nm. The tetraethoxysilane CVD process produces a conformal SiO_2 film, which encapsulates each nanotube and the substrate surface, resulting in a mechanically stable and well-insulated matrix so that aggressive polishing or etching processes can be applied.

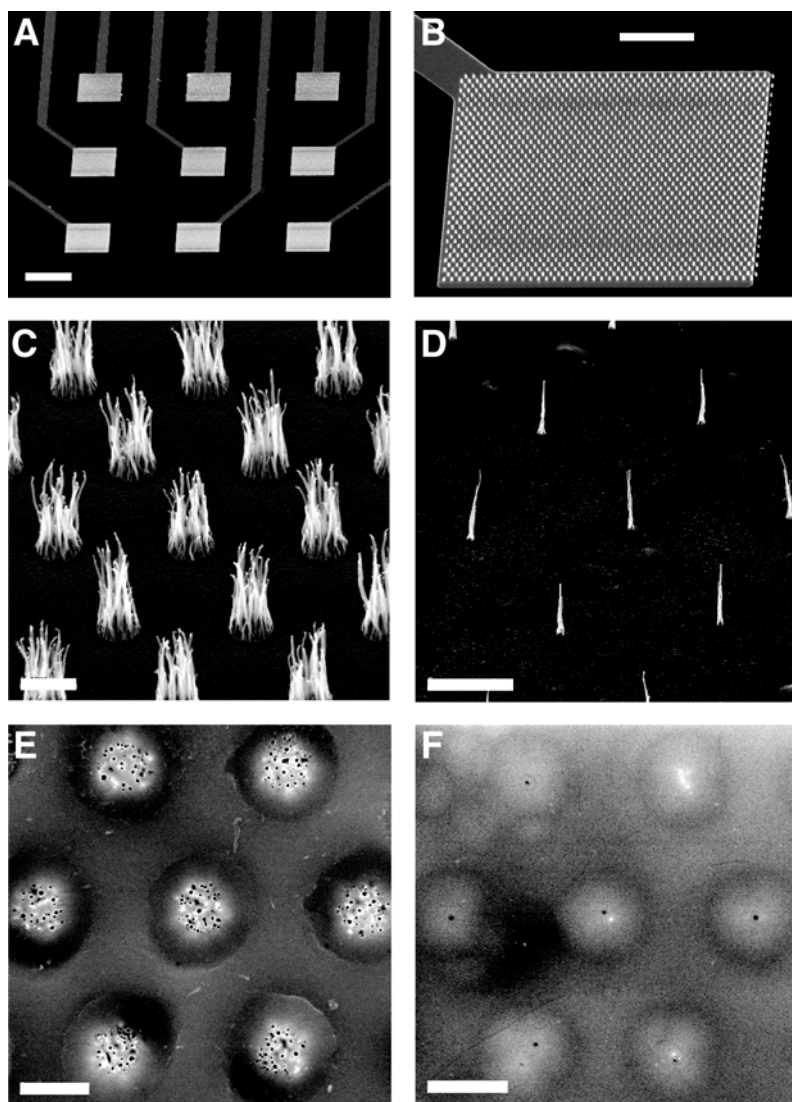


Fig. 5. Scanning probe microscopy images of (A) a 3×3 electrode array, (B) an array of MWNT bundles on one of the electrode pads, (C) and (D) array of MWNTs at UV-lithography and e-beam patterned Ni spots, respectively, (E) and (F) the surface of polished MWNT array electrodes grown on $2 \mu\text{m}$ and 200 nm spots, respectively. (A)–(D) are 45° perspective views and (E–F) are top views. The scale bars are 200, 50, 2, 5, 2, and $2 \mu\text{m}$, respectively.

Figure 4E,F shows the top-view of MWCNT nanoelectrode arrays inlaid in SiO_2 after polishing from the UV and e-beam patterned samples, respectively. Clearly, CNTs retain their integrity and are separated from each other. The CNTs in as polished samples normally protrude over the SiO_2 matrix by about 30–50 nm because of their high mechanical resilience. An EC etching method is employed to shorten CNTs and level the tips approximately in the surface plane of the SiO_2 matrix. The MWCNTs grown by our PECVD technique present a bamboo-like structure with a series of closed shells along the tube, which seal off the hollow channel, leaving only the very end accessible by electrolytes.

2.2. Functionalization of Oligonucleotide Probes

A common feature for biological sensing is the requirement of immobilization of biomolecules with specific functionalities to the sensing device. These biomolecules serve as probes to specifically either bind particular species in the testing sample or catalyze the reaction of the analyte. Such an event produces a change in chemical or physical properties that can be converted into a measurable signal by the transducer. The specific recognition of the target molecules is the essential feature for biological sensing. The common probe and target (analyte) recognition mechanisms include: (1) antibody/antigen interactions, (2) nucleic acid hybridizations, (3) enzymatic reactions, and (4) cellular interactions. Depending on the device and its sensing mechanism, different functionalization methods have to be adapted.

The oxides at the CNT ends can mostly be converted to carboxylic groups using EC etching. A ferrocene (Fc) derivative, $\text{Fc}(\text{CH}_2)_2\text{NH}_2$, can be selectively functionalized at the tube ends through amide bonds facilitated by the coupling reagents dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) (71). Similar carbodiimide chemistry can be applied to functionalize primary amine terminated oligonucleotides using water-soluble coupling reagents 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride and N-hydroxysulfo-succinimide as shown in **Fig. 6**. A probe [Cy3] 5'-CTI-IATTTTCICAIITCCT-3' [AmC7-Q] and a target [Cy5] 5'-AGGACCTGC-GAAATCCAGGGGGGGGGGG-3' are used, which are related to the wild-type gene of *BRCA1* Arg1443 stop (72). Fluorescence images of Cy3 and Cy5 labels are taken with a laser scanner after each step of functionalization, hybridization, and washing to confirm the attachment. The Cy3 and Cy5 images after hybridization and washing obtained from a functionalized spot of 3 mm in diameter on a piece of MWCNT nanoelectrode array chip with a size of $\sim 1 \times 1 \text{ cm}^2$. Clearly, the hybridization only occurs at the spot with probe

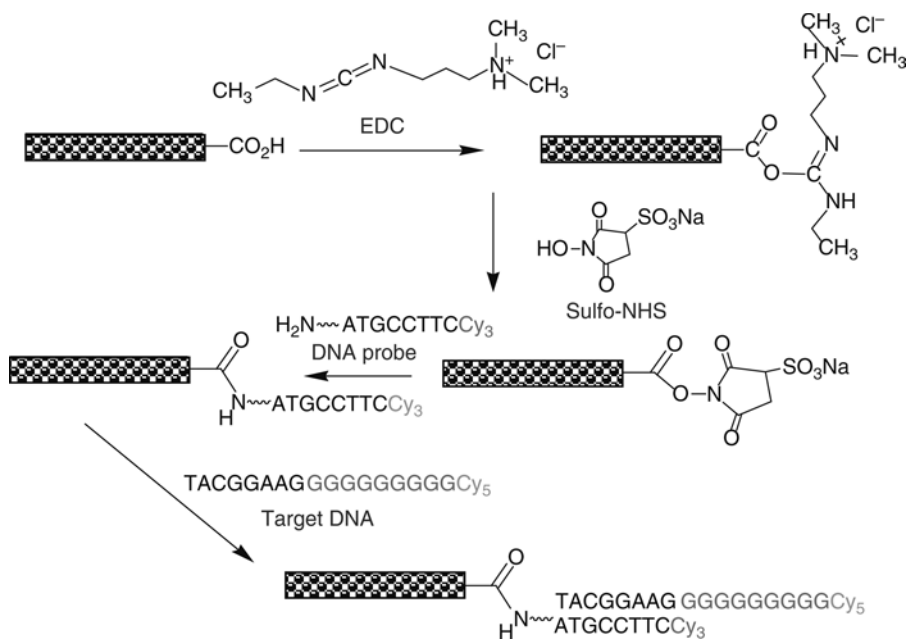


Fig. 6. The schematic of functionalization of oligonucleotide probe to the end of a CNT through the formation of amide bonds.

molecules, indicating that the target binds to the surface only through specific hybridization with the probe. Nonspecific binding was removed by washing procedures.

2.3. Nanoelectrode Array-Based EC Detection

It has been reported that the guanine bases in DNA can be oxidized at about 1.05 V (73). This oxidation reaction can be used for DNA detection (74). EC detection of an oligonucleotide target was first demonstrated as shown in **Fig. 7B**. A 10 bp polyG tag was attached to the sequence fully matched with the probe, which serves as the signal moiety. The guanine bases in the probe are replaced with inosine to ensure no guanine contribution from the probe. To further amplify the guanine oxidation signal, a mediator $\text{Ru}(\text{bpy})_3^{2+}$ was introduced, which can transfer electrons from the guanine bases dangling near the electrode surface as schematically illustrated in **Fig. 7**. Combining the advantages of nanoelectrode array, guanine oxidation, mediator amplification, and AC voltammetry altogether, extremely high sensitivity of EC detection has been achieved. The net AC voltammetry data is presented in **Fig. 8A**. If scaling down with a MWCNT array on a $20 \times 20 \mu\text{m}^2$ micro-contact, we estimated that $<10^6$ oligonucleotide target attached to this spot

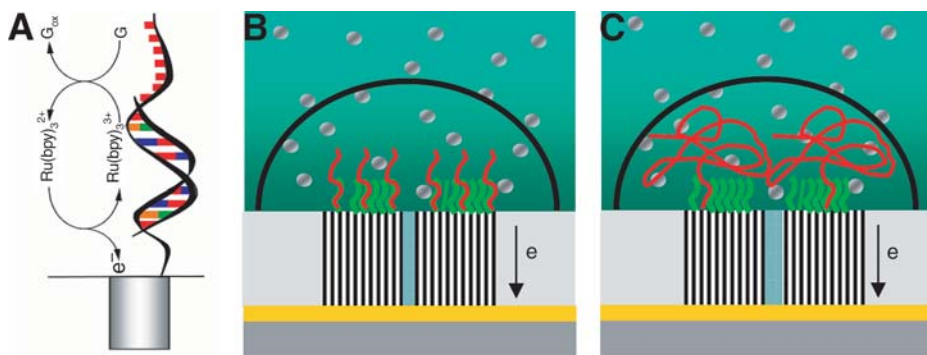


Fig. 7. The schematic of (A) the mechanism of $\text{Ru}(\text{bpy})_3^{2+}$ mediator amplified guanine oxidation, (B) the detection of oligonucleotide targets, and (C) PCR amplicon targets.

could be easily detected. It needs to be noted that this number is the maximum estimation, whereas the real number of target DNA that were detected could be orders of magnitude smaller.

Direct EC detection of *PCR amplicons* using CNT nanoelectrode arrays has also been demonstrated (3). In this case, the abundant inherent guanine bases are directly used as the signal moieties without employing a signal tag labeling process as fluorescent techniques do. The same *BRCA1* gene probe was employed. But the target was changed to about 300 bases normal allele within *BRCA1* gene containing 5'-AGGACCTGCGAAATCCAG-3', which is complementary to the specific oligonucleotide probe. Genomic DNA from a healthy donor was used in PCR amplification. Considering the physical size (radius of gyration of ~ 6 nm for 300 base ssDNA), it is expected that not more than ~ 70 targets can hybridized with the probes on each MWNT of ~ 100 nm diameter. The nonspecific binding is removed by stringent washing with 2X SSC/0.1% SDS, 1X SSC, and 0.1% SSC at 40°C for 15 min, respectively. The major part of the target molecules likely dangles near but might not be necessarily be in direct contact with the electrode surface (Fig. 8C). $\text{Ru}(\text{bpy})_3^{2+}$ mediators can efficiently transport electrons from the guanine bases to MWCNT nanoelectrodes to provide an amplified guanine oxidation signal as long as target DNA molecules are within the three-dimensional diffusion layer (typically a hemisphere with a radius of ~ 300 nm, i.e., $\sim 6 R_{\text{ave}}$, where R_{ave} is the average nanoelectrode radius). Because the approx 300 base long PCR amplicon statistically contains about 75 inherent guanine bases, about the same level of net EC signal was obtained (as shown in Fig. 8B) compared with the 10-mer polyG tagged oligonucleotide target even though the number of PCR amplicon target are much less. The sensitivity for detecting <1000 target molecules can be achieved at a $20 \times 20 \mu\text{m}^2$ microcontact. In contrast, target samples that underwent the

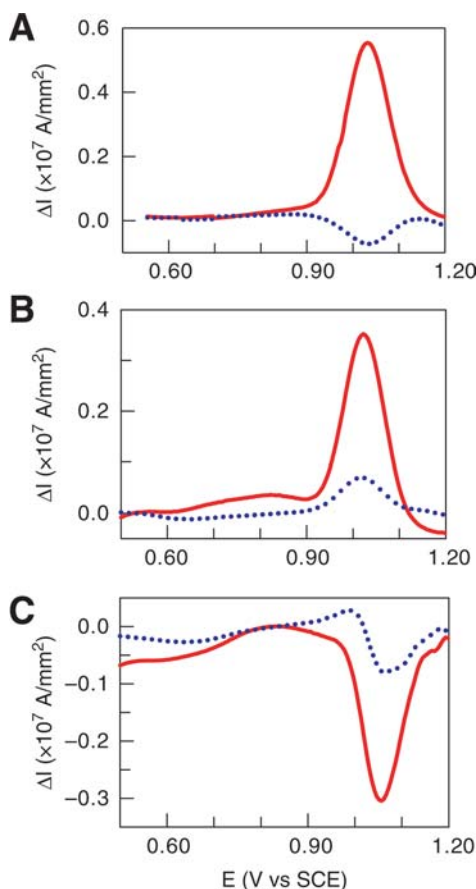


Fig. 8. The net AC voltammetry signal in 5 mM $\text{Ru}(\text{bpy})_3^{2+}$ and 0.20 M NaOAc (pH 5.2) with an AC sinusoidal wave of 10 Hz and 25 mV amplitude on top of a staircase DC ramp after incubation in (A) specific polyG tagged oligonucleotide target, (B) specific PCR amplicon, and (C) random PCR amplicon. The horizontal dashed lines indicate the maximum background noise.

same incubation condition, except were unrelated about 400 bases PCR amplicon, give net negative EC signal (as shown in **Fig. 8C**). The normalized net EC signal from a clean MWCNT nanoelectrode array without probe oligonucleotide has shown a reproducible background noise as marked by the dashed lines in **Fig. 8**. The hybridization of specific targets (whether oligonucleotides [**Fig. 8A**] or PCR amplicons [**Fig. 8B**]) generates a distinct positive signal over the noise, while the absence of hybridization gives a distinct negative signal (**Fig. 8C**). Such high reliability has not been reported by using other nanotechnology-based DNA sensor yet.

One big advantage of the CNT nanoelectrode array platform is that it can be integrated with electrical field stringency control and microfluidics to improve the accuracy and hybridization speed. Controlled electric fields are known to be able to regulate the transport and concentration of biomolecules (75), and control the hybridization and denaturation of DNA molecules (35,76). Such electric methods can be readily applied to MWCNT nanoelectrode array to speed up the hybridization. Ideally specific electric field can be applied at each individual microspot to gain specific stringency control for each probe. This makes the screening of single-nucleotide polymorphism (SNP) much more precisely (77,78), particularly in highly multiplexed analysis, which is critical for nucleic acid analysis.

3. Nanochip Array for Protein Detection

The advantages of ultra miniaturization and multiplexing enable one to use smaller sample volumes and achieve greater sensitivity and quicker analysis owing to the small size of entire array and the individual features that comprise the array. There is significant development in use of nanolithography for patterning surfaces with proteins on the submicrometer length scale to create protein nanoarrays. Each 1 μ spot in these array-based assays covers less than 1/1000th of surface area of a conventional microarray spot while still maintaining enough antibodies to provide a useful dynamic range (9–10,15). Nanoarray-based protein assay exceeds the limit of detection of conventional enzyme-linked immunosorbent assay-based immunoassays by >1000-fold (10). This could lead to the discovery of other useful diagnostic biomarkers that currently escape detection. This section summarizes the practical methods and protocols for protein nanoarrays, especially for the surface modification and antibody immobilization that are the key factors in determining the overall success of a nanoarray.

3.1. Nanoarrayer

The basic principle of the NanoArrayer is mechanically mediated direct deposition of materials on surfaces with high spatial precision, surface sensing capabilities and environmental control. Briefly, a microfabricated deposition tool is loaded by immersing the distal end in a drop of the protein solution. The surface to be patterned is positioned on a piezoelectric inchworm-driven XY stage with 20 nm resolution over 25 mm of travel (Burleigh exfo, New York). Manipulating the local humidity at the interface between the deposition tool and surface with gentle bursts of wet or dry air allows precise control of the molecular transfer rate. All stage movement and patterning parameters are controlled with a custom software package called NanoWareTM (Phoenix, AZ) (5,6,9,10).

3.2. Surface Modification

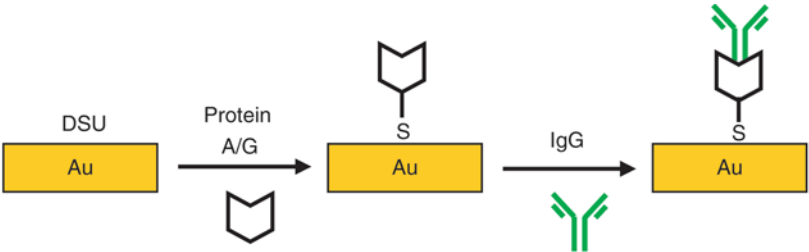
The use of gold-based substrates for monitoring protein interactions has a relatively long history. Instead of using polycrystalline gold surfaces, thin gold films (10–200 nm) can easily be deposited on solid supports similar to glass or silicon wafers by evaporation or sputtering in an ultrahigh vacuum (79). Because the adhesion of gold on glass or silicon is poor, precoating of a thin layer of Cr or Ti (1–10 nm) is generally required ahead of gold deposition.

Since the work of Allara (80,81) and Whitesides (82,83) in the mid-1980s, the spontaneous adsorption of organosulfur compounds onto gold has been a widely used method for the preparation of self-assembled monolayers (SAMs). If the alkyl chain is of sufficient length, the resulting molecular architecture is very stable and orients itself nearly perpendicular to the surface (84,85). The close proximity of the adsorbed molecules causes van der Waals forces to play an important role in intermolecular stabilization. The thickness of these monolayers is typically in the nanometer range in the vertical direction and as a result, they can be termed nanoassemblies. Unlike glass- or polymer-based arrays, SAMs on gold also have the added advantage of the surface properties being efficiently characterized by optical, mechanical, and EC analytical tools.

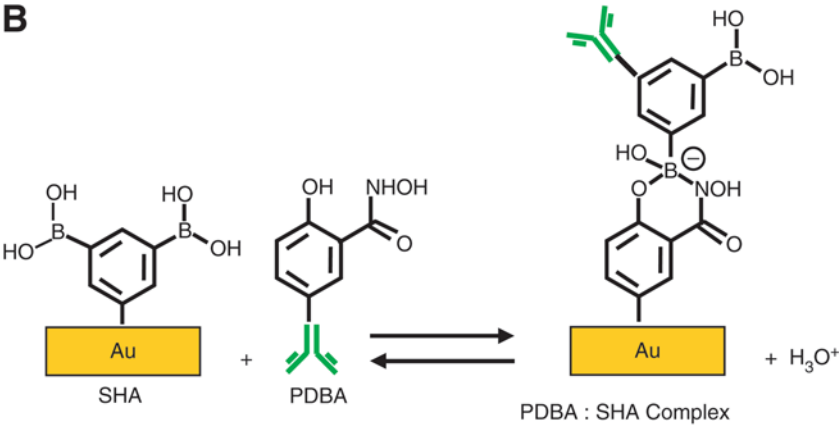
A major limitation to the use of SAMs as an immobilization matrix is their low temperature stability resulting from the weak bonding (interactions) between sulfur and gold. Although this is not a concern in the case of protein arrays (where experiments are conducted at low temperatures), such nanoarrays are found to be unsuitable for DNA applications where highly stringent washing and temperature conditions are employed.

Efficient immobilization of biomolecules is a key factor in determining the overall success of a nanoarray or, for that matter, any high-throughput screening tool. If the immobilized probes are not correctly oriented on the microarray surface or are denatured, it can dramatically affect the downstream biomolecular interaction events. **Figure 9** summarizes three types of SAMs for antibody immobilization. Protein A/G surface is very useful for properly orienting antibodies and boosting specific net target binding, because protein A/G is the mixture of protein A and Protein G that specifically bind immunoglobulin (Ig)G Fc and leave IgG F(ab)₂ open for antigen capture as shown in **Fig 9A**. Protein A and protein G have different binding affinity to IgG Fc of different species. The combination of protein A and protein G work well for all types of IgG (antibody). Salicylhydroxamic acid (SHA) binds IgG functionalized with 1,3-phenyldiboronic acid (PDBA) while exhibiting very low nonspecific binding to target protein without a PDBA functional group as shown in **Fig. 9B**. Once formed, SHA–PDBA complex is stable to a wide variety of conditions. PDBA–IgG conjugates can be released from these SHA-affinity surfaces in a

A



B



C

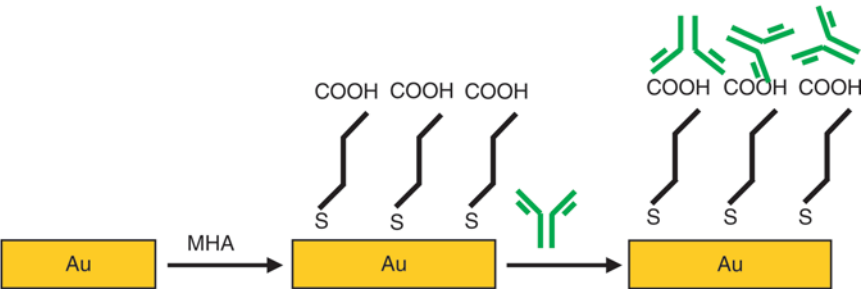


Fig. 9. Schematic of surface modification and functionalization of (A) Protein A/G chip, (B) SHA-SAM chip, (C) MHA chip.

controlled manner that allow separation of IgG captured protein for the further analysis. The 16-mercaptohexadecanoic acid (MHA) is deprotonated at pH 7.4, and therefore the nanofeatures are negatively charged as shown in **Fig. 9C**. Antibody will adhere to negatively charged surface features through electrostatic interactions, thus retaining its biological activity toward its targets. MHA-based chips have robust two-dimensional structure and less steps for antibody immobilization. This antibody immobilization is simple, but not directional

(5,6,9,10,86–89). The following descriptions summarize the three common surface modification and functionalization protocols.

3.2.1. Protein A/G Chips

1. Sputtered gold chips are immersed in alkanethiolate solutions (0.5 mM dithiobis-succinimidyl undecanoate (DSU) [89] in 1,4-dioxane) to allow SAM formation overnight.
2. Wash the chips in dioxane and dry the chips with 100% ethanol.
3. The chips are blown dry with argon and allowed to react with 0.1 mg/mL protein A/G (Pierce) in phosphate buffered saline (PBS) for 30 min at 25°C.
4. Unreacted succinimide groups are blocked with 10 mM Tris-HCl, pH 7.5, 1 M glycine for 30 min at 25°C.
5. Without washing, chips are transferred into StabilGuard (SurModics, Eden Prairie, MN) protein stabilizing and blocking agent and incubated for 1 h at 25°C.
6. Chips are then rinsed briefly with a few drops of double-distilled water, dried with argon, and stored in desiccators at 4°C before use.

3.2.2. SHA SAM Chips

1. Fresh gold-coated silicon wafers are incubated in 5 mM SHA alkanethiolate SAM reagents and ethanol as directed by the manufacturer's protocol (Versalinx™, Prolinx, Bothell, WA) (87).
2. Chips are sonicated and washed successively in ethanol and double-distilled water before use.

3.2.3. MHA Chips

1. Pattern MHA into dot features on a gold thin film.
2. To minimize nonspecific binding of proteins on the inactive portions of the array, the areas surrounding the MHA patterned features are passivated with PEG-alkythiol (11-mercaptopundecyl-tri[ethylene glycol]) by placing a droplet of a 1 mM ethanolic solution of the surfactant on the patterned area for 2 h.
3. Rinse the chips with ethanol and, then, Nanopure water.

3.3. Antibody Immobilization and Hybridization

Proper antibody immobilization and background blocking are critical for the highly specific and sensitive detection of target proteins. Here summarizes the common antibody immobilization and hybridization protocol for these three chips.

1. The capture antibody are immobilized by arrayed deposition of IgG onto a protein A/G chip, PDBA-labeled IgG on SHA SAM chip, or immersing MHA chip in a solution containing IgG at 0.2–1 mg/mL in 10 mM PBS for 1 h.
2. The chips are stored overnight in a humid environment for 1 h at 25°C or overnight at 4°C to facilitate binding of antibody to activated surface.

3. Wash chips with 500 μL of PBS + 0.1% Tween-80 (PBST) for 5 min and double-distilled water for 5 min to remove salts and detergent.
4. Add prehybridization buffer containing 5 mg/mL bovine IgG (Sigma St. Louis, MO) in PBST to block remaining binding sites for 30 min at 25°C.
5. Incubate antigen in PBST + 4% IgG-free, protease-free BSA with agitation for 30–45 min at 25°C.
6. For atomic force microscopy (AFM) imaging, stop here with three successive washes for 3 min each in 500 μL of PBST. Otherwise, continue next steps for optical image acquisition with CCD camera.
7. The chips are then immersed in 500 μL of PBST + 1.5 $\mu\text{g/mL}$ Cy3-labeled detection antibody for 30–45 min at 25°C.
8. Alternatively, biotinylated detection antibody is added to the reaction at 0.25 $\mu\text{g/mL}$ and incubated with agitation for 30–45 min at 25°C.
9. The chips are then transferred into 500 μL of Cy3-streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1.5 $\mu\text{g/mL}$ in PBST and incubated with agitation for 30–45 min at 25°C.
10. Three successive washes are carried out for 3 min each in 500 μL of PBST before image acquisition.

3.4. Detection

Nanoarrays that utilized fluorescent reporting molecules are visualized on optical microscope equipped with a Cy3 filter and a Texas Red filter from Chroma Technology (Rockingham, VT). Images are captured using a scientific grade CCD camera (Cohu, Inc., San Diego, CA). Immediately before visualization, chips are briefly rinsed with a few drops of double-distilled water and inverted onto a drop of *n*-propyl gallate/glycerol antifade solution (5% *n*-propylgallate, 70% glycerol, 25% 0.5 *M* Tris-HCl, pH 9.0, adjusted to a final pH 7.4). Fluorescent images were analyzed for spot size, intensity, and coefficient of variance with the Array Pro Analyzer software package (Media Cybernetics, Carlsbad, CA).

AFM images are recorded in tapping mode with a Dimension 3100 AFM from Digital Instruments (Santa Barbara, CA). The chips are washed 5 min in PBS + 1% Tween-80, 5 min in double-distilled water, and dried before final AFM imaging. To amplify the signal associated with antigen binding to the array, gold nanoparticle probes, which are heavily functionalized with detection antibodies to the antigen, are reacted with the nanoarray by soaking the array in a solution containing detection antibody coated gold nanoparticles (20 nM, 10 nM in 10 mM PBS) for 1 h.

4. Nanoarray for Enzymatic Assays

Enzymatic assays are extremely useful for clinical diagnostics, environmental monitoring, and drug discovery. The development of nanoarray for enzymatic

assay significantly reduces the amount of biological and chemical reagents and samples and shortens the throughput time and improves the detection sensitivity.

Both CNT nanoelectrode arrays and nanochip arrays have been applied for these assays (*11,12*). CNT nanoelectrode array-based EC transducers offer substantial improvements in the performance of amperometric enzyme electrodes. The greatly enhanced EC reactivity for numerous of hydrogen peroxide and NADH at CNT-modified electrodes make these nanomaterials extremely attractive for numerous oxidase- and dehydrogenase-based amperometric biosensors. There are over 200 dehydrogenases and 100 oxidases. Many of these enzymes catalyze specifically the reactions of clinically important analytes (e.g., glucose, lactate, cholesterol, amino acids, urate, pyruvate, glutamate, alcohol, hydroxybutyrate) to generate the electrochemically detectable products NADH and hydrogen peroxide. Similar sensitivity and stability improvements have been illustrated for EC biosensors based on other enzymes, including tyrosinase, peroxidase, organophosphorous hydrolase, or alkaline phosphatase (*11*).

Nanochip array-based enzymatic assays for alcohol dehydrogenase, pyruvate kinase, and enolase were compared with conventional assays monitored in 96-well microtiter plates. All miniaturized reactions could be performed in maximum volumes of 6.3–8 nL and were read out with a conventional fluorescence microsystem equipped a scientific grade CCD camera. Enzymatic-based assays using nanoarrays are a promising low-volume, low-reagents, and low sample-consuming alternative to current methodology (*12*).

5. Conclusion

This chapter summarized recent progress in the development of CNT nanoelectrode array for nucleic acid analysis and nanochip array for protein detection with improved miniaturization, higher sensitivity and rapid analysis. The CNT nanoelectrode array for nucleic acid analysis platform can be adapted for protein detection and enzymatic assay. Nanochip array for protein detection platform can also be adapted for nucleic acid analysis and enzymatic assay. Single copy detection within minutes for these novel devices is expected by integrating microfluidics and electric field control of probe–target hybridization. CNT nanoelectrode array-based EC sensors are more amenable to miniaturization. In addition, the continuous response of an electrode system allows for computer control, which makes the EC detection simpler, more reliable and less expensive than other analytical techniques.

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