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NANOBIOTECHNOLOGY FOR MEDICAL DIAGNOSTICS

NANOBIOTECNOLOGÍA PARA EL DIAGNÓSTICO MÉDICO

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Abstract

Traditional core areas of chemical engineering education are being extended by new expertise in science and engineering at the molecular and nanometer scale. Chemical engineers have been pursuing a dynamic role in the design and development of new generations of diagnostic platforms exploiting different nanomaterials and "*are the forefront of this rapidly developing field, with the potential to propel discoveries from the bench to bedside*" (Ruan *et al*., 2012).

Nanobiotechnology leverages existing expertise from engineering and biology, promotes interdisciplinary discoveries and addresses key elements of next-generation clinical applications.

In the present review we attempt to give an overview of the latest technologies that in our opinion hold great promise as the basis of powerful biodiagnostic tools.

Keywords: bioassays, ELISA, Immuno-PCR, phage, nanofabrication.

Resumen

La áreas tradicionales de la educación en ingeniería química están siendo extendidas por nuevas experiencias en ciencia e ingeniería a escalas molecular y nanométrica. Los ingenieros químicos has estado persiguiendo jugar un rol dinámico en el diseño y desarrollo de nuevas generaciones de plataformas de diagnóstico explotando diferentes nanomateriales y "*son el frente de este campo que se encuentra rápidamente en desarrollo, con el potencial de impulsar descubrimientos que vayan desde el laboratorio hasta el tratamiento de pacientes.*" (Ruan *et al*., 2012).

La nanobiotecnología sirve como palanca en la experiencia que se tiene en ingeniería y biología, promueve descubrimientos interdisciplinarios y atiende elementos clave de la siguiente generación de aplicaciones clínicas.

En la presente revisión tratamos de proporcionar un visión general de las últimas tecnologías que en nuestra opinión constituyen una gran promesa como base para herramientas poderosas de diagnóstico.

Palabras clave: bioensayos, ELISA, Inmuno-PCR, fago, nanofabricación.

1 The state of the art-ELISA and PCR

We begin with a brief discussion of the two most widely-used technologies, which are being advanced by the integration of nanoscale elements and to which new approaches are inevitably compared. For 40 years, the gold standard for detecting protein molecules has been ELISA (enzyme -linked immunosorbent assay) in which a surface-captured analyte is detected by binding an antibody conjugated to a signal-generating enzyme reporter. Enzymes can generate absorbance, fluorescence, chemiluminescence or luminescence from appropriate substrates, and each of these is commonly used with proper instrumentation. Technical innovations such as miniaturization, integration with microfluidics (e.g. GyroLab) and au-

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Table 1 | Detection of protein biomolecules

Tool or technique	Read-out	Illustration	Detection limit*	Molecules per drop (60 µl)	In clinical use
Colorimetry	Visual		150 pM	10^9	Yes
Carbon nanotubes	Electrical		100 pM	10^9	No
Chemi-luminescence	Luminescence		30 pM	10^9	Yes
ELISA	Luminescence		1–10 pM	10^7	Yes
Quantum dots	Fluorescence		500 fM	10^7	No
Silicon nanowires	Electrical		1 fM	10^4	No
Metal nano-particles (bio-barcode)	Scanometric and/or light scattering		30 aM	900	Yes
Immuno-PCR	Fluorescence		20 aM	700	Yes

*Detection limits are best-case examples from the literature and can vary substantially depending on the target and the assay conditions.

Fig. 1. Detection of protein biomolecules; adapted with permission (Giljohann *et al*., 2009).

tomation, along with engineered reporters that carry multiple enzymes (e.g. 10 nm gold nanoparticles (Jia *et al*., 2009)) and development of sensitive substrates have taken the classic assay to a new level, but sensitivity (Figure 1) and narrow linear dynamic range remain still an issue.

Using PCR, detection of nucleic acids achieves remarkable sensitivity, down to a few molecules, by exploiting the natural mechanisms of DNA replication during cell division. PCR, or "polymerase chain reaction", the Nobel-recognized 1983 discovery of Dr. Kary Mullis, is used to amplify a specific region of a given DNA molecule bounded by two complementary DNA primers using a heat-stable DNA-copying polymerase. Heating denatures the double-stranded target into two single strands, each of which is made double-stranded by polymerase extension of the complementary primer, so that the target sequence is doubled in concentration. The reaction is repeated multiple times and leads to exponential amplification of the DNA fragment. After tens of cycles, million-fold amplification of the DNA target region makes detection relatively easy, but at the cost of time and complex temperature-cycling PCR apparatus.

Note that most of the emerging nano-bio-diagnostic methods depend upon molecular recognition, in which a molecule such as an antibody or DNA probe, binds or hybridizes to its target. Biochemistry and physiology depend on molecular recognition in every aspect of their functioning, and the "nano" side of a bio-nano collaboration often is most impressed by the ability of the "bio" side to obtain specific, high-affinity recognition tools, which bind the analyte of interest. The essential following element of a complete diagnostic is the transduction of this recognition and binding into a human-readable output signal, and it is in this transduction step that nanostructured elements usually make their contribution. This review is organized according to signal transduction methods used for detection.

2 Optical readout

Metal nanoparticles (typically gold and silver particles with diameters ranging from 10-150 nm) support surface plasmons (oscillations of the electrons at the nanoparticle surface) that result in extraordinary optical properties that are not exhibited by any other class of material (Saha *et al*., 2012; Weintraub, 2013). By changing their size, shape, and surface coating, the colors of nanoparticles can be tuned across the visible and near-infrared region of the electromagnetic spectrum. Solutions of spherical gold nanoparticles are ruby red in color due to the strong scattering and absorption in the green region of the spectrum. Solutions of silver nanoparticles are yellow due to the plasmon resonance in the blue region of the spectrum (red and green light are unaffected).

Sensors utilizing plasmonic nanoparticles allow for a rather simple detection, even by optical means. Mirkin and co-workers were the first to utilize metal nanoparticles for the plasmonic-based detection of nucleic acids (Mirkin *et al*., 1996; Elghanian *et al*., 1997). The analyte molecules cause the bridging of DNA-functionalized metal nanoparticles (gold or silver) generating aggregates with a concomitant change of solution color from red to blue as a consequence of interacting particle surface plasmons and aggregate scattering properties. More recently, de Rica and Stevens reported a plasmonic ELISA (de la Rica *et al*., 2012) for the ultrasensitive detection of proteins with the naked eye. Their significant observation was that nanoparticles can be generated by the reduction of gold ions in the presence of hydrogen peroxide. However, the concentration of hydrogen

peroxide directly affects the reaction and in the presence of high concentration of hydrogen peroxide, a red colored solution of non-aggregated, spherical gold particles is formed. Then they adapted this process as a signal generation mechanism for ELISA by utilizing the very-active catalase enzyme that when bound by the analyte decreases the concentration of hydrogen peroxide and favors the generation of blue-colored aggregates of nanoparticles. The authors demonstrated the detection of HIV-1 capsid antigen p24 at ag/ml level in serum by visual scoring and thus they opened the road for the adoption of classical ELISA in settings, e.g., in developing countries, that lack sophisticated laboratory instrumentation.

Another approach pioneered by Halas, West, and co-workers is based on the rational design of nanoshells (core-shell spherical particles consisting of a dielectric core with a thin, metallic shell) that attenuate light strongly in the near-infrared region where blood does not. Using nanoshells, they were able to demonstrate an immunoassay performed in whole blood without the need for purification/separation steps (Hirsch *et al*., 2005).

Ultimate sensitivity is at the level of single molecules. Counting single molecules comes with practical challenges but offers distinct advantages over ensemble measurements (Walt 2013). Building on their fiber optic microarray technology, Walt group at Tufts have developed a single-molecule digital ELISA (Rissin *et al*., 2010) where single immunocomplexes captured on beads are detected in arrays of femtoliter size wells using fluorescence imaging. They reported the detection of PSA in serum in femtomolar level using the same reagents as in a classic ELISA. The technology (Single Molecule Array, SiMoA) has been commercialized and a pilot study to quantitatively measure biomarkers of inflammation from patients with Crohn's disease has been reported (Song *et al*., 2011).

Quantum dots (Q dots), semiconductor nanocrystals (2-8 nm), exhibit size-dependent optical and electrical properties (Alivisatos 1996) and show great promise as multiplexable fluorescent reporters in diagnostic assays (reviewed in (Samir *et al*., 2012)).

3 Immuno-PCR

The combination of antibody-like protein molecular recognition with PCR's enormous DNA amplification sensitivity is an intuitively attractive concept which has been visited repeatedly since Sano *et al*. (1992) coined the term Immuno-PCR in 1992. In Immuno-PCR, a chimeric molecule is used consisting of an antibody (which recognizes the target) linked to a sensitively-detectable amplifiable DNA. Immuno-PCR generally achieves a 100-10,000-fold improvement in the detection limit compared to standard ELISAs, but has still failed ubiquitously to establish itself in the analytical laboratory, mainly due to the complicated preparation of immuno-PCR reagents, non-specific binding, and lack of reproducibility (Burbulis *et al*., 2007: Adler *et al*., 2008; Malou *et al*., 2011). The pioneering work of Mirkin *et al*. pushed the limits of protein detection to low femtomolar levels. Ultrasensitive detection is achieved by the introduction of 15 nm gold nanoparticles co-loaded with analyte-specific antibodies and many copies of DNA reporters (extensively reviewed previously (Rosi *et al*., 2005; Giljohann *et al*., 2009)). The DNA reporters are finally detected by hybridization onto a microarray by a conventional flatbed scanner. After significant fine-tuning of the assay format (Bao *et al*., 2006) this technology showed improved dose response and has even become an analytically useful assay (Verigene system; Nanosphere).

Alternative immuno-PCR formats based on nanostructures have been reported. For example, Mason *et al*. (2006) developed a liposome-based PCR detection construct where the DNA reporters were encapsulated inside the lipid bilayer of a 115 nm liposome into which ganglioside receptors (known to bind biological toxins, including cholera toxin) were incorporated and reported sub-attomolar detection sensitivities for cholera toxin in human urine (Mason *et al*., 2006). More recently, "generic" immuno-liposome constructs were reported that can accommodate any biotinylated recognition molecule (e.g. antibodies (He *et al*., 2012)).

4 Phage as reporters

Going beyond traditional phage display library screening, viruses have taken up new roles as building blocks for generation of highly sophisticated structures useful in diverse applications such as drug delivery and diagnostics (Douglas *et al*., 2006). Phage nanoparticles present monodisperse but versatile scaffolds that can accommodate a large number of recognition (antibodies, aptamers, lectins, etc) and reporter (enzymes) elements leading to ultrasensitive, modular, bio-detection reporters

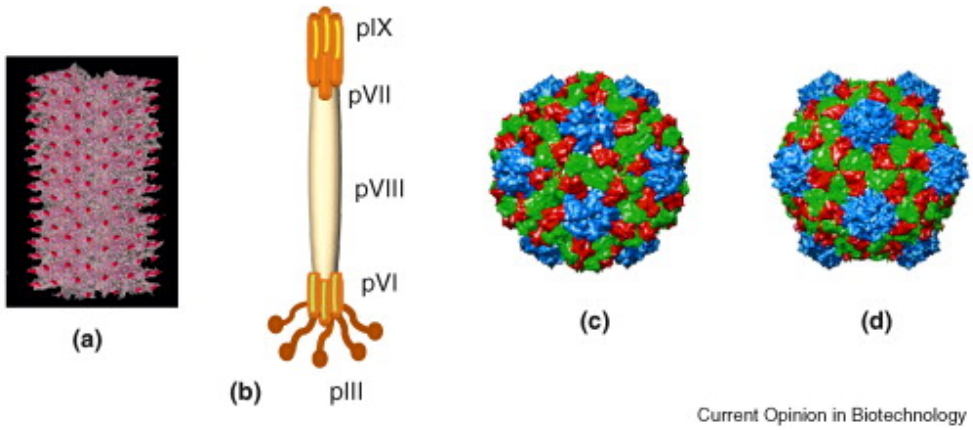

Fig. 2. Viruses used in bionanotechnology. (a) Tobacco mosaic virus, TMV (b) Bacteriophage M13. (c) Cowpea chlorotic mottle virus (d) Cowpea mosaic virus (CPMV); adapted with permission (Soto *et al*., 2010).

(Soto *et al*., 2010) (Figure 2). For example, M13 bacteriophage displaying short peptides that recognize small molecular weight compounds (e.g. 3-phenoxybenzoic acid or brominated diphenyl ether 47) was used as the affinity element in an ELISA and detected by an anti-phage antibody conjugated to horseradish peroxidase enzyme (HRP) (Kim *et al*., 2009, Kim *et al*., 2010). Cowpea mosaic virus (CPMV) decorated with Cy5 fluorescent dye significantly increased assay sensitivity in a microarray-based genotyping of *Vibrio cholera* O139 (Soto *et al*., 2006). A highly sensitive and selective diagnostic assay for troponin I has been reported that utilizes HBV virus nanoparticles. The virus particles display antibody-binding protein A that is used for the oriented immobilization of the anti-analyte antibody as well as a hexahistidine sequence so that the chimeric nanoparticles would have a strong affinity for nickel (Park *et al*., 2009). The analyte-chimeric virus complex is captured on three-dimensional nickel nanostructures, sandwiched by an anti-analyte monoclonal antibody and finally detected by a secondary antibody labeled with quantum dots. The sensitivity was surprisingly boosted to attomolar level which represents six to seven orders of magnitude greater sensitivity than current ELISA assays.

Utilizing PCR-based detection of the phage DNA as the signal generating mechanism also promises ultrasensitive detection of small molecules (Kim *et al*., 2011) and proteins. For example T7 phage modified with antibodies was used for the detection of human HbsAg using real-time PCR as the output (Zhang *et al*., 2013).

5 Mass/size detection

Translating biomolecular recognition into nanomechanical signals offers a label-free approach of detecting the presence of an analyte (Fritz *et al*., 2000, Majumdar 2002). Specific biomolecular reactions confined to one surface of a microfabricated diving-board shaped microcantilever beam induce surface stress and cause the mechanical bending of the cantilever. However, until recently micromechanical cantilevers have been limited to detection of purified targets in high concentrations. The Manalis group at MIT has been developing vacuum-enclosed silicon microcantilivers with embedded microchannels of picoliter volume (suspended microchannel resonators, SMR) whose resonance frequency quantifies the mass of the cantilever (Burg *et al*., 2007, Churana *et al*., 2007). These cantilivers when protected with nonfouling surface coatings enable the sensitive detection of protein molecules in undiluted serum (von Muhlen *et al*., 2010). A commercial instrument that encompasses the suspended microchannel resonators (Archimedes; Affinity Systems) is now available.

Particles can also be detected on smaller size scales by their reduction of the electrical conductance of small orifices. When particles suspended in an electrolyte traverse an aperture they cause a change in resistance or a blockade of the ionic current that is proportional to the particle volume. The idea of resistive pulse sensing has been successfully commercialized as the Coulter counter in the 1950's for characterization of larger colloidal and cellular suspensions, especially blood. Driven by the advances in nanofabrication techniques, making

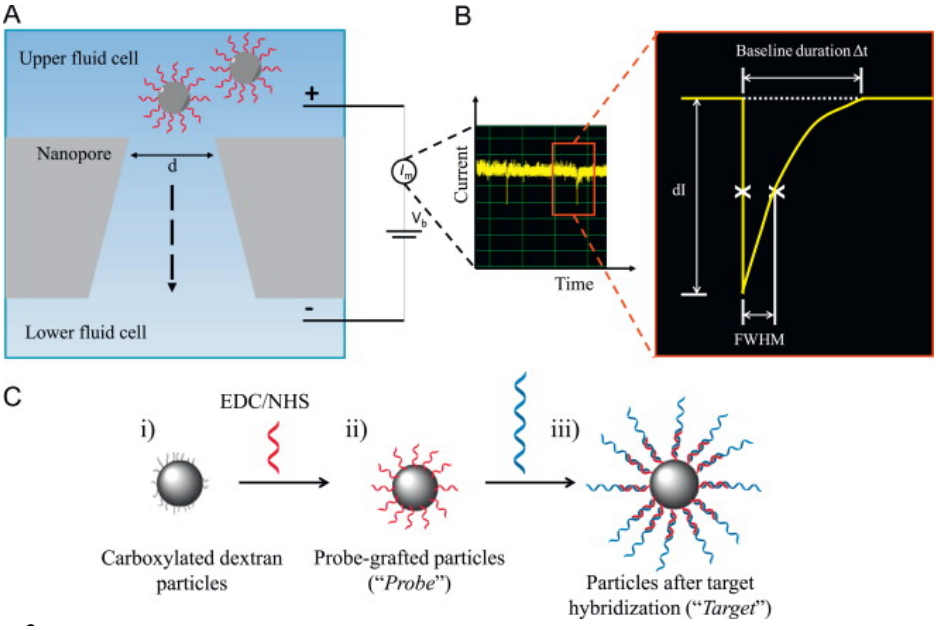

Fig. 3. Nanopore set-up in the IZON qNano instrument, (B) transient current drops created by particles passing through the nanopore, with a scheme indicating calculations for baseline duration, blockade full width at half maximum (FWHM) and magnitude (dI) and (C) DNA hybridization on DNA-functionalized dextran particles; adapted with permission (Booth *et al*., 2013).

artificial nanoscale pores has been an interesting goal (reviewed in (Kozak *et al*., 2011)). However, only recently have dynamically-adjustable (tunable) elastomeric nanopores become available (Garza-Licudine *et al*., 2010, Roberts *et al*., 2010) as part of the qNano particle analyzer (IZON Science Ltd) that allows the characterization of nanoparticles by monitoring the magnitude, duration and frequency of the blockade events as the particles traverse the nanopore (Drescher *et al*., 2012). The magnitude of the generated blockade depends on the particle-to-pore volume ratio and thus for a given aperture the measured change in resistance is proportional to the particle volume; blockade duration correlates with electrophoretic mobility and surface charge. In the qNano, the elastomeric membrane allows for real-time tuning of the pore size by applying a macroscopic stretch to the membrane and enables the real-time tuning of the sensitivity of the measurement. Beyond particle characterization the device appears to be an attractive platform to develop point-of-care diagnostics. Recently detection of DNA hybridization in the qNano has been reported; upon hybridization to complementary DNA, the surface charge of DNA functionalized particles changed (Booth *et al*., 2013) (Figure 3). Platt and coworkers have demonstrated a proof of concept protein detection assay in which analyte-induced particle aggregation is detected by the increased magnitude of the blockade events (Platt *et al*., 2012).

6 Paramagnetic microparticles as labels

We and others have been integrating paramagnetic microparticles, traditionally used in off-line sample capture, cleanup and concentration (van Reenen *et al*., 2013) with optical biosensors for the ultrasensitive detection of proteins and pathogens (Mani *et al*., 2011). We have been developing microfabricated retroreflectors as bio-sensing surfaces. Retroreflectors return light directly to its source and are readily detectable with inexpensive optics. Suspended corner cube retroreflectors (Figure 4), 5 μm in size, consisting of a transparent epoxy core and three surfaces coated with gold are promising ultra-bright labels for use in a rapid, low-labor diagnostic platform (Sherlock *et al*., 2011). On the other hand linear arrays of retroreflectors combined with micron-sized magnetic particles acting as light-blocking labels provide a low-cost platform for multiplex detection of

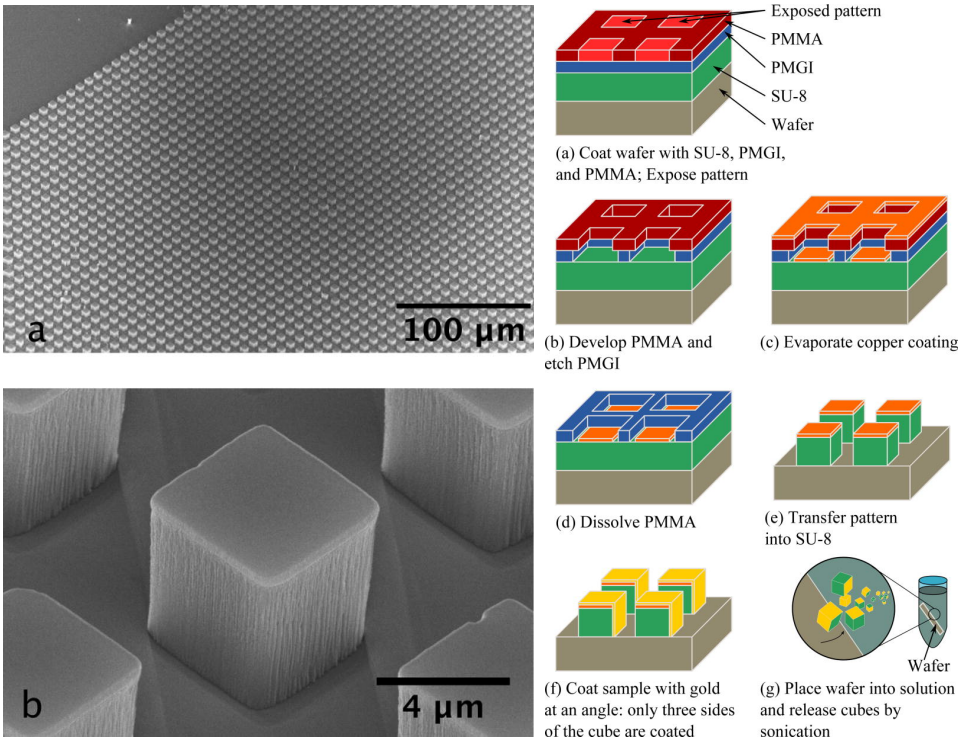

Fig. 4. (Left) Scanning electron micrographs of corner cube retroreflectors. (Right) Schematic of the fabrication sequence for corner cube retroreflectors; pending permission (Sherlock *et al*., 2011)

pathogens (Les reviewed 2013). We have been also investigating the use of an implantable micron-sized retroreflector-based platform and Optical Coherence Tomography (OCT) as a non-invasive and depth-resolved imaging technique for reflectance measurements of micro-retroreflectors in the subcutaneous tissue (Ivers *et al*., 2010).

Diffraction-based biosensors rely on optical scattering and have been explored as sensitive protein detection platforms. Signal enhancement techniques include micro fabricating solid diffraction gratings or by inducing, in the presence of analyte, the assembly of microbeads into diffraction patterns. Lee and co-workers have demonstrated an aptamer-based assay to detect platelet-derived growth actor B on a microprinted gold-coated glass slide where the assembled bead patterns allow visual analysis using a bright-field microscope (Lee *et al*., 2010).

Recently, a microfluidic chip-based magnetic bead surface coverage assay has been reported in which large magnetic beads that have captured analytes from a serum sample 'roll' over a pattern of small beads functionalized with anti-analyte antibodies, to which they can bind selectively, achieving attomolar detection sensitivity using optical microscopy (Tekin *et al*., 2013).

Leveraging existing technology utilized in magnetic data storage hard drives has enabled dramatic progress to be achieved in the magnetic biosensors arena in recent years. Micrometer-sized magnetic particles are promising reporters since even the most complex biological samples lack detectable magnetic background and thus do not interfere with the detection mechanism of the magnetic labels. In 1998, Baselt and co-workers were the first to demonstrate a prototype GMR-based biosensor (Baselt *et al*., 1998). Giant magnetoresistive (GMR) spin-valve sensors detect the presence of magnetic particles by measuring the change of resistance of the conductive layer due to the presence of the magnetic particles. Most recently two research groups have been developing GMR biosensing devices. The Wang group at Stanford has demonstrated multiplex protein detection using 50 nm magnetic nanotags at subpicomolar levels with a dynamic range of more than four orders of magnitude in clinically-relevant serum samples without the need for any washing protocol (Osterfeld *et al*., 2008; Gaster *et*

al., 2009). The J.-P. Wang group at University of Minnesota demonstrated the applicability of sub 13 nm high-moment magnetic nanoparticles in a novel GMR biosensor that achieved detection of as few as 600 molecules (< one zeptomole) of streptavidin (Srinivasan *et al*., 2009) and the possibility to rapidly quantify femtomolar concentrations of a biomarker in human serum in 5 min (Li *et al*., 2010).

7 Conductivity

Inorganic nanostructures exhibit unique tunable electrical properties and are exploited as signal transduction elements for ultrasensitive, rapid, real-time sensing (Rosi *et al*., 2005; Kierny *et al*., 2012).

The tunable conducting properties of semiconductor nanowires allow for label-free electrical detection of analytes through changes in conductance induced by binding events occurring on the nanowire surface and thus provide an attractive diagnostics platform. Since the first report of electrical detection of picomolar concentrations of streptavidin in solution in 2001 (Cui *et al*., 2001) the Lieber group has pioneered bottom-up strategies to fabricate silicon nanowires and they have demonstrated the ultrasensitive detection of various targets including proteins, nucleic acids, and viruses (Patolsky *et al*., 2006). Developments in nanowire sensors for multiplexed detection of biomolecules have been recently reviewed (He *et al*., 2008). The Lieber group has also demonstrated a multiplex assay for three cancer markers with a detection of 0.9 pg/mL in desalted but undiluted serum samples (Zheng *et al*., 2005).

Carbon has been showing great potential in its newly-popular forms, carbon nanotubes (CNTs) and graphene. Nonspecific binding is always an issue with these materials, however. Graphene, a two-dimensional hexagonal network of carbon atoms only one atom thick, has been extensively investigated for various applications due to its prominent structural and electrical properties, and can be used as the basis of extremely powerful biosensor systems and integrated assays with high sensitivity. A number of studies reported the use of graphene in biosensors, and the electrical detection of biomolecules using ultrathin 2D graphene sheets can potentially achieve high sensitivity.

Carbon nanotubes (CNTs) are hollow cylindrical nanostructures composed of single or multiple sheets of graphene containing carbon atoms in a honeycomb arrangement. Single-wall CNTs (SWNT) arrayed vertically are electrically conductive and allow for the construction of high-density sensors with high sensitivity. SWNT arrays combined with multiwall carbon nanotubes decorated with multiple copies of antibodies and enzyme reporters have achieved highly sensitive detection of a cancer biomarker in serum and in tissue lysates (Yu *et al*., 2006). More recently, Cai *et al*. (2010), fabricated arrays of carbon nanotube tips with molecular-imprinted polymer coating for ultrasensive sensing of proteins.

Concluding remarks

Nanoscience, nanotechnology, and advanced fabrication methods have made access to previously-impossible structures almost routine. Chemical engineers are taking advantage of these new tools, integrating them into a wide range of promising new technologies. The future of this synergy looks very promising.

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