Opinion TRENDS in Biotechnology Vol.23 No.4 April 2005

Nanobiotechnology: the promise and reality of new approaches to molecular recognition

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Nanobiotechnology is the convergence of engineering and molecular biology that is leading to a new class of multifunctional devices and systems for biological and chemical analysis with better sensitivity and specificity and a higher rate of recognition. Nano-objects with important analytical applications include nanotubes, nanochannels, nanoparticles, nanopores and nanocapacitors. Here, we take a critical look at the subset of recent developments in this area relevant to molecular recognition. Potential benefits of using nano-objects (nanotubes, quantum dots, nanorods and nanoprisms) and nanodevices (nanocapacitors, nanopores and nanocantilevers) leading to an expanded range of label multiplexing are described along with potential applications in future diagnostics. We also speculate on further pathways in nanotechnology development and the emergence of order in this somewhat chaotic, yet promising, new field.

Introduction

Nanobiotechnology is a recently coined term describing the convergence of the two existing but distant worlds of engineering and molecular biology. Engineers have been working for the past three decades on shrinking dimensions of fabricated structures to enable faster and higher-density electronic chips, which have reached feature sizes as small as 20 nm using deep UV-lithography. In parallel, molecular biologists have been operating for many years in the domain of molecular and cellular dimensions ranging from several nanometers (DNA molecules, viruses) to several micrometers (cells) [1]. It is believed that a combination of these disciplines will result in a new class of multifunctional devices and systems for biological and chemical analysis characterized by better sensitivity and specificity and higher rates of recognition compared with current solutions [2].

This article assesses recent advances and illustrates emerging applications of nanobiotechnology in separation, sequencing and detection by describing progress in the development of 'nano-objects' such as carbon nanotubes and quantum dots, and highlighting their use in molecular recognition. Finally, it outlines the future directions of these new developments and speculates on the realistic potential for their applications in bioanalysis.

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Molecular detection

Innovation in molecular detection has several objectives. Most notably, it is currently focused on: (i) moving towards highly multiplexed molecular recognition using re-configurable arrays, and thus departing from arrays with fixed recognition sites printed on solid surfaces [3]; and (ii) developing new methods for registering and quantitating a specific binding event through electrochemical or electronic measurements, preferably without using a label [4]. Nanotools such as nanochannels, nanopores, quantum dots (QDs), nanotubes, nanowires and nanocapacitors are becoming of great importance in the technological solutions for these ambitious objectives [5].

Multiplex molecular tagging: quantum dots, nanorods and nanoprisms

Multiplex tagging of unknown molecules (different DNA fragments or proteins) in a sample and their subsequent tag-by-tag recognition in a flow system provides an appealing alternative to monitoring a binding event and single color detection 'by location' in planar, fixed arrays. Highly multiplex tags have been developed based on QDs [6] (Figure 1), metallic [7] (Figure 2) and glass [8] (Figure 3) barcodes, among others. Tagging molecules with QDs has several advantages over standard fluorophore tags [9,10]. The absorption spectra of QDs (e.g. colloidal inorganic semiconductor nanocrystals consisting of a CdSe core and a ZnS shell) are very broad, extending from the ultraviolet to a cutoff wavelength in the visible spectrum. The position of this cut-off is dictated by dot size (larger size results in longer wavelengths) and by the composition of the core. Emission is confined to a narrow band (typically 20–40 nm full width at half maximum intensity) likewise centered at a wavelength characteristic of the particle size. QDs can be excited with a single wavelength to produce multiple colors with minimal photobleaching. The differentiation of emission color, intensity and spectral width can lead to several thousand unique signatures. Nie's group [6], in their

 0167-7799/$ - see front matter doi:10.1016/j.tibtech.2005.02.007

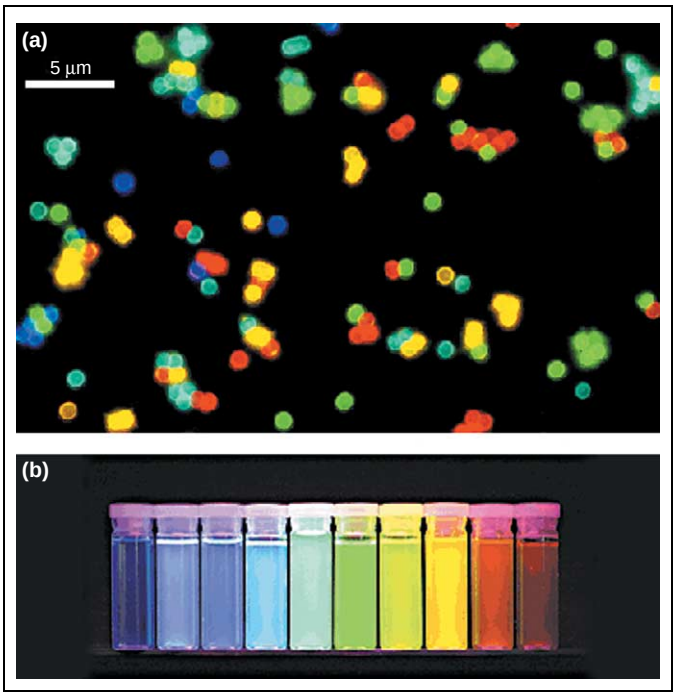

Figure 1. Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules **(a)** Fluorescence micrograph of a mixture of CdSe/ZnS QD-tagged beads emitting single-color signals at 484, 508, 547, 575, and 611 nm. The beads were spread and immobilized on a polylysine-coated glass slide, which caused a slight clustering effect. **(b)** Ten distinguishable emission colors of ZnS-capped CdSe QDs excited with a near-UV lamp. From left to right (blue to red), the emission maxima are located at 443, 473, 481, 500, 518, 543, 565, 587, 610, and 655 nm. Reproduced with permission from [45] http://www.nature.com/.

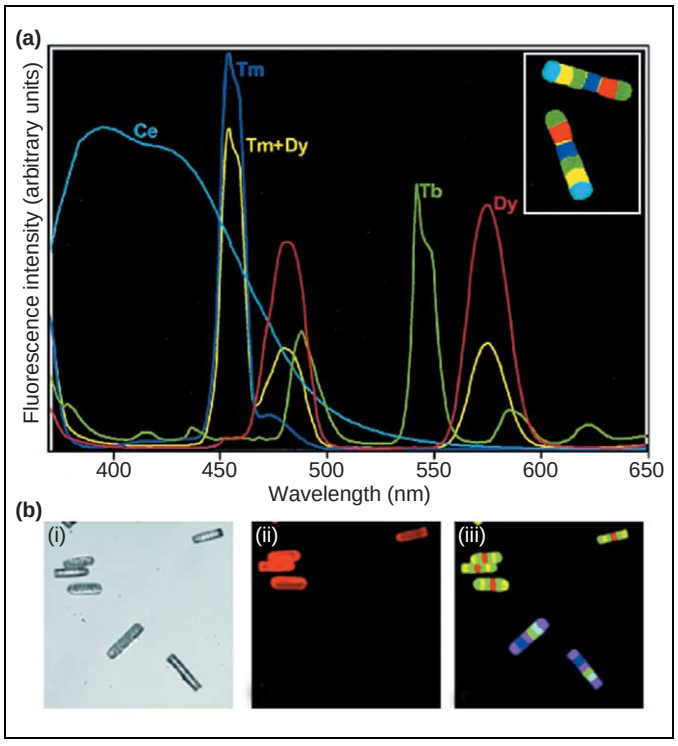

Figure 3. Micro barcodes. **(a)** False-color image of two 100 Å ~20 μm barcodes (inset) and corresponding fluorescence spectrum barcode elements. The same color scheme is used for the spectra and the image [e.g. the yellow band in the barcode corresponds to the yellow [combination Tm + Dy (*Thulium* + *Dysprosium*)] line spectrum]. **(b)** Fluorescent false-color images of two different barcode particles A and B (seen in panel iii) used in a DNA hybridization assay using Cy3-labeled DNA. (i) White light image; (ii) Cy3 channel image; (iii) rare earth image obtained by using a 420 nm long pass filter. Results from Cy3 channel (panel ii) show that only one of the two barcode elements (e.g. yellow-green-red-green-yellow) participates in hybridization to Cy3-labeled DNA (compare images in panels ii and iii). Reproduced with permission from [39] © 2002 National Academy of Sciences, U.S.A.

development of complex material microbeads built with embedded QDs, claim theoretical multiplex capacity of one million. The QD signatures can be recognized optically and Wang showed recently that they can also be used for coding in electrochemical detection schemes [11].

QDs in core or shell format are rendered water-soluble by providing a layer of functionalized silica or linkers, such as mercaptoacetic acid, dihydrolipoic acid, or modified polyacrylic acid, for conjugation to macromolecules and ligands. Successful application of bioconjugated QDs towards labeling cells and macromolecular components of cells has been demonstrated [12,13], and early technical difficulties, such as reproducibility in manufacturing, quenching in solution and adsorption and toxicity when used in living cells, have been overcome. There are other questions that must be addressed concerning QDs,

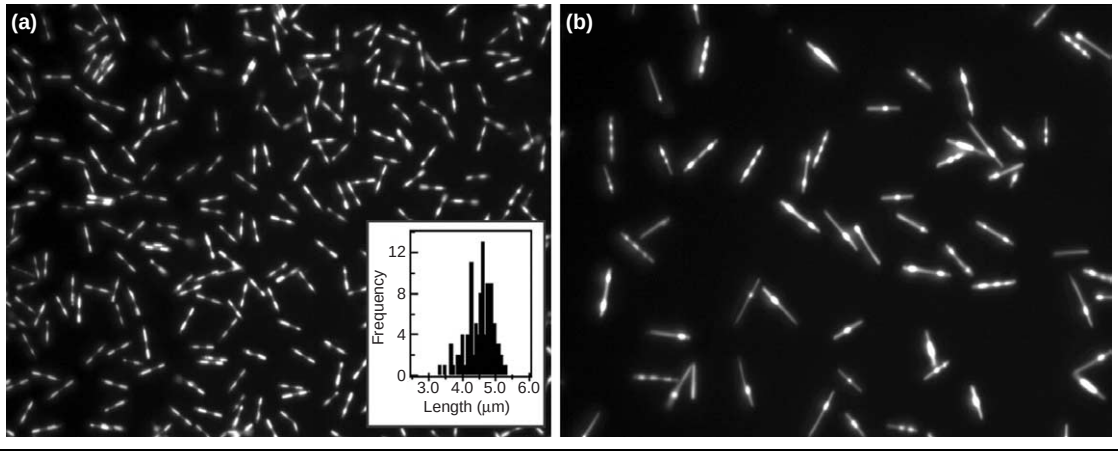

Figure 2. Metallic barcodes. **(a)** A population of particles of 'flavor' 0110011, where 0 denotes Au and 1 denotes Ag segments. The inset shows a histogram of particle lengths for 106 particles in this image. Particle length was 4.5±0.4 μm, or a ±9% variation in overall particle length. Images were acquired with a Zeiss Axiovert 100 microscope with a Plan APO 100Å ~1.4 NA objective. Illumination was provided by 175-W Xe lamp and LiquidLight Guide (Sutter Instruments, Novato, California; www.sutter.com) filtered with a HQ 405-nm, 20-nm bandpass filter (Chroma Inc., Brattleboro, Vermon; www.chroma.com). Illumination was split with a 50/50 BS (Chroma). **(b)** Mixture of nine barcode batches with different striping patterns. Particle 'flavors' in increasing binary order: 0000000, 0000001, 0000010, 0000100, 0001000, 0001010, 0011100, 0100010 and 0101010. Reproduced with permission from from Nicewarner-Pena, S.R. *et al.* (2001) Submicrometer metallic barcodes. *Science* 294, 137–141. www.sciencemag.org.

including their access to targets in confined cellular compartments or multi-component molecular complexes, their use in fluorescence lifetime or fluorescence resonance energy transfer measurements of molecular association and conformation, and in multiphoton techniques.

The good news is that these promising reagents are finally maturing and becoming available to the general scientific community from commercial sources, a process that should be accelerated by the introduction of alternative QD cores and shells. We anticipate that the vision of high-throughput screening, highly multiplexed bioassays and other applications of QDs will soon also become a reality.

As quantum dots are becoming established, yet another nanotechnological development is emerging as a possible challenger to this type of label. Multi-metal nanorods with barcoded stripes that can be recognized using reflectivity measurements have been demonstrated recently [7]. The nanorods are produced using a lithographic process whereby different metal stripes (barcodes) are electrodeposited into pores in an Al_2O_3 (aluminium oxide) membrane. A subsequent read-out of these barcodes is performed using an optical microscope. The nanorod tagging can be performed in parallel with optical tagging because the two detection methods do not interfere with each other and thus further increase the scale of the multiplexing capability.

Another intriguing prospect of differentiation in labeling is due to the variation of nanoparticle shape. An early example in this arena has been nanoprisms (100 nm edges) fabricated in silver [14] (Figure 4). Nanoparticles with this shape interact with light differently when compared with spherical particles and as a consequence appear differently colored [15]. This difference provides the basis for multiplexed assays in which the nanoparticle labels are all made from exactly the same material but rely on differences in shape to achieve unique optical signals.

Improved detection sensitivity: the use of nanotubes and nanotube matrices

Potential for improved sensitivity in recognizing a DNA hybridization event might come from the use of nano-size electrodes based on carbon nanotubes (CNTs) (Figure 5). Li *et al.* [16] developed DNA microarrays containing sensing pads constructed from assembled multi-walled carbon nanotubes (MW-CNTs) grown with controlled density and built into a matrix within a silicon nitride template. The upper (open) ends of the tubes act as nanoelectrodes and are functionalized with ssDNA probes. Target DNA that hybridizes to the ssDNA probe on the ends of the electrically conductive CNTs is detected using an electrochemical method that relies on guanine oxidation. The authors demonstrate ultra-high detection sensitivity in the range of a few attomoles (amol) and relate it to the anticipated reduction of signal to noise (S:N) ratio with the reduction of the electrode size (i.e. CNT size). To maintain an appreciable level of signal while maintaining this improved S:N value, groups of CNTs are deposited within each sensing pad.

Wang and Musameh [17] used composite electrodes containing CNTs dispersed in a teflon matrix in electrochemical detection experiments. This composite material combines advantages of CNTs and bulk composite electrodes, and is characterized by accelerated electron transfer

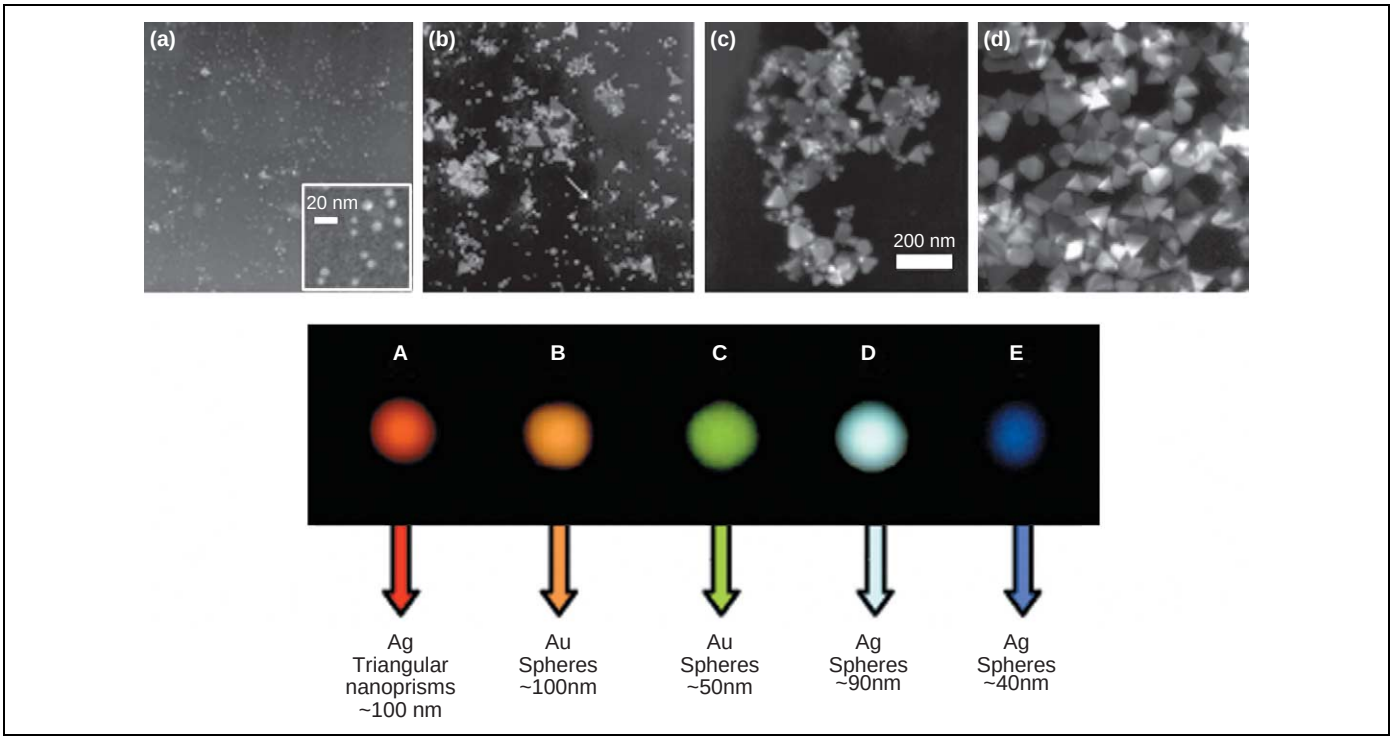

Figure 4. Photo-induced conversion of silver nanospheres to nanoprisms. Transmission electron microscopy images (reverse print, upper panel) mapping the morphology changes before irradiation **(a)** and after 40 **(b)**, 55 **(c)** and 70 **(d)** hours of irradiation. Rayleigh light-scattering of particles deposited on a microscope glass slide illustrating the different colors of nanoparticles of different shapes and sizes (lower panels). The slide is used as a planar waveguide, which is illuminated with a tungsten source. The image was taken with a digital camera. Reproduced from Jin *et al.* (2001) Photoinduced conversion of silver nanospheres to nanoprisms. *Science* 294, 1901–1913. www.sciencemag.org.

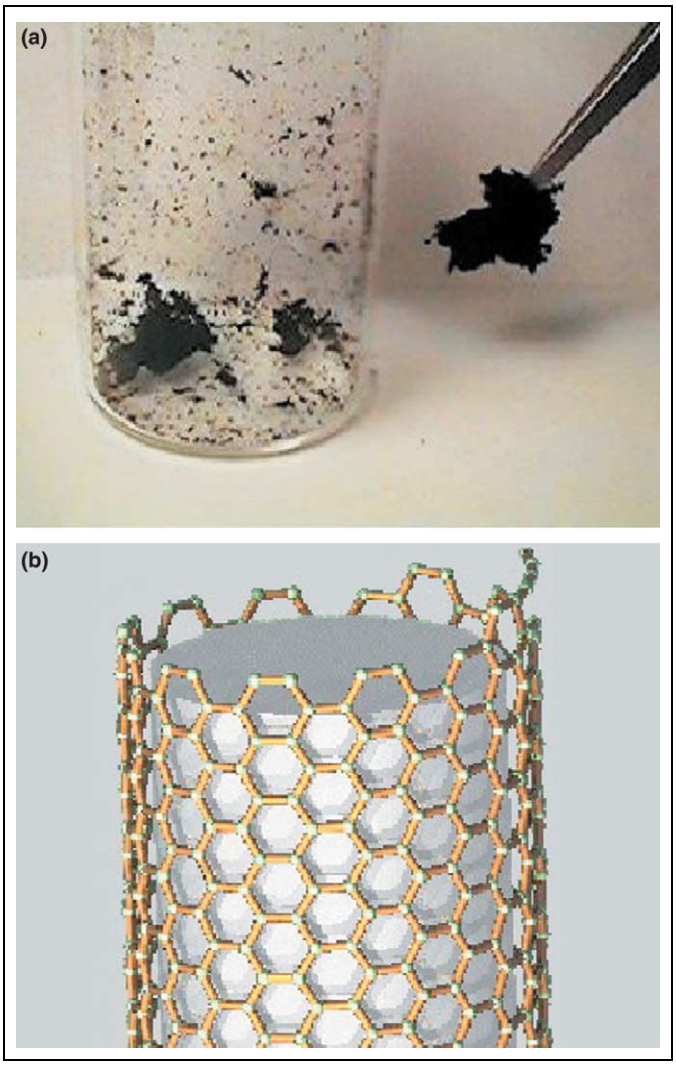

Figure 5. Carbon nanotubes. **(a)** A mass of carbon nanotubes, tiny tubes 10 000 times thinner than a human hair. **(b)** Rolled up sheets of carbon hexagons.

and minimized surface fouling as demonstrated in amperometric biosensing of glucose and ethanol [17].

CNTs can also be used as narrow conduits for flow-based assays. Ito [18] developed a Coulter counter chip containing a membrane with a single MW-CNT channel. The membrane was prepared from an epoxy section containing a MW-CNT channel mounted on a polydimethylsiloxane (PDMS) support structure allowing for evaluation of size and surface charge of carboxy-terminated polystyrene nanoparticles. The measurement was applied to particles with sizes in the range of 60–100 nm and was characterized by a high S:N ratio.

CNTs also have been used as tips in atomic force microscopy (AFM) for high resolution imaging of DNA fragments on a surface. Lieber's group designed special oligonucleotide probes which, under appropriate hybridization conditions, bind only to fully complementary ssDNA fragments. However, binding does not occur when a single-base mismatch is present [19]. Subsequently, they used single wall CNTs as AFM tips to enable high-resolution, multiplex detection of the different labels.

One of the challenges for nanotubes is simply just keeping abreast of the developments in this fast-moving field. The variation in the structure and properties of nanotubes is extensive and includes single and multi-wall varieties. What started from the isolated discovery of carbon nanotubes [20–22] has evolved into a full-fledged field, with nanotubes fabricated from a range of materials including boron nitride (BN), gallium nitride (GaN), boron carbide (BC) and organic polymers. The scope of nanotubes is further enhanced by functionalization and attachment of different types of molecules and filling of the nanotube with other molecules (e.g. loading CNTs with enzyme labels and using the filled CNT as a label for an antibody) [23]. The different types of nanotubes will offer great potential for nanobiotechnology tools as has already demonstrated by the impressive variety of current applications [24].

Nanodiagnostics

The ability to analyze simultaneously multiple nucleic acid sequences for variation in a rapid and accurate fashion is pivotal in all areas of diagnostics. Several studies suggest that nanoparticle-based technology could enable sensitive detection of sequence variation in DNA [25–27]. Early studies demonstrated array-based DNA discrimination by allele-specific oligonucleotide hybridization using gold nanoparticle reporters containing bound oligonucleotides for hybridization to complementary targets [25]. Detection was achieved by silver enhancement resulting in deposition of silver on the surface of the nanoparticles, which enabled scanometric detection to define the location of the gold nanoparticles on an array [26]. Attachment of a dye close to the surface of the nanoparticles via linkage to the oligonucleotide also enabled detection after silver enhancement by surface-enhanced Raman spectroscopy (SERS) [27]. Attachment of different dyes having distinctly different Raman spectra was also demonstrated, showing feasibility for genotyping of two different alleles at the same array register. Further work is needed to realize the full potential of this approach for multiplex analysis of genetic variation in genomes of high sequence complexity.

Comparable protein-based target detection (attomolar to femtomolar levels) was also achieved [28–30]. A nanoparticle oligonucleotide bio-barcode assay was used to detect free prostate-specific antigen (PSA) using magnetic microparticles coated with PSA monoclonal antibody. The PSA is captured by the magnetic microparticle and reacted with gold nanoparticles coated with a polyclonal PSA antibody and barcode DNA hybrids. The PSA-sandwiched complexes are magnetically captured and the barcode DNA is released. The barcode DNA is then detected on arrays after annealing to bound complements and detection using complementary oligonucleotide bound to gold nanoparticles [31]. An intermediate PCR amplification of the released barcode DNA before the array detection increased the PSA detection limit from 30 attomolar to 3 attomolar [31]. These detection limits are lowered by six orders of magnitude than the current conventional immunoassays for PSA but the assay is multi-step and lengthy and this level of sensitivity is not needed clinically as yet. However, this assay illustrates the potential of this type of assay

strategy, and it could become important as new and less abundant protein targets are identified in on-going proteomic studies.

Label-less detection: nanocapacitors, nanopores, nanochannels and nanomechanics

Most molecular recognition techniques rely on a binding event and subsequent interrogation of the optical, electrochemical or magnetic [32–35] tag carried by the molecule involved in binding. An attractive and highly desirable alternative to this strategy would eliminate the tagging step, and instead, would rely on detection of the change of an inherent property of the analyte or the molecular aggregates formed upon binding.

Lee's group developed nano-gap capacitors (50 nm electrode spacing) that are fabricated using silicon nanolithography [35]. The ssDNA probe is immobilized on the electrode surface. The dielectric properties of the ssDNA probe and dsDNA formed on hybridization with the target are different and can be measured through capacitance measurements in these nano-gap capacitors. An immediate prospect is large two-dimensional arrays of capacitors that could be used to provide capacitive, label-free simultaneous measurement of nucleic acid targets in a sample.

Nanomechanical deflections on micromachined silicon cantilevers have also been used to recognize the occurrence of molecular events such as DNA hybridization and protein binding [5,36]. In the case of DNA hybridization, ssDNA is immobilized on the surface of a cantilever and subsequently target ssDNA is introduced. The cantilever acts as a miniature 'balance' and deflects proportionally to the amount of hybridized target. The deflection is measured accurately using optical detection methods [4,37]. Extension of this method to multiplex recognition and prevention of non-specific binding is still a challenge – however, the method is a clever demonstration of making use of the power of sophisticated microfabrication and assay methods.

Nanopore devices have been built to interrogate on-bead, label-less immunoassays in flow systems. Sohn's group [38] used a Coulter counting principle to determine particle size based on the 'electronic signature' of the particle as it flowed through a microfabricated PDMS pore. Antibody binding to antigen immobilized on the particle surface contributes to the diameter change of the complex, and thus can be detected using these nano-pore chips [39].

Sequencing of ssDNA fragments has been proposed based on electrophoretic transport of DNA chains through a single pore fabricated in a silicon nitride membrane [40] or through an α-hemolysis pore in lipid bilayers [41]. The pore dimensions were, in both cases, <10 nm in diameter. Measurements of the cross-pore current indicated that individual polynucleotides transported through the pore could provide a unique 'signature' and that this could eventually lead to low-cost, rapid and direct methods for DNA sequence analysis. Nanochannels that would stretch out the DNA molecule and simplify the sequencing are also in the investigational stage [42]. Estimates for the sequencing rate that could be achieved using nanopores range from a conservative 1000 bases per second to an optimistic 10 000 bases per second, which greatly exceeds the current rate of $\sim$30 000 bases per day using conventional sequencers. A remaining challenge for nanopore technology is to refine the technique to distinguish single nucleotides as opposed to only distinguishing signatures on the basis of sequence differences.

Current status

Nanobiotechnology is still at its early stages of development; however, the development is multi-directional and fast-paced. Universities are forming nanotechnology centers and the number of papers and patent applications in the area is rising quickly. The nanotechnology 'tool-box' is quickly being filled with nanotools, but realistically, some of these newly developed tools might not have viable applications and could end-up on the 'technology shelf' in the future. The flurry of new nano-based sensors, for example, looks at first glance to be appealing, but in many cases, the techniques for preparing these sensors are complex; the sensor performance might not be superior to existing methods relying on micro-approaches (as opposed to nano-approaches). Nevertheless, there are definite benefits emerging from these developments. Nanobiotechnology is interdisciplinary and brings together life scientists and engineers. This, in turn, fuels further growth of ideas, which would not occur without these interdisciplinary interactions. Finally, many bets have been placed on the future importance of nanobiotechnology and nanobiotech start-ups, which constitute nearly 50% of the venture capital invested in nanotechnology [43].

Future trends

Nanobiotechnology is here to stay! We expect that the current multi-directional and chaotic developments will gradually become more ordered and develop sharp focus as applications mature to produce useful and validated technologies. It is still not entirely clear whether nanobiotechnology will be the basis of the next technological revolution and, if this is indeed the case, what is a realistic timescale for this potentially industry-transforming event. There is little doubt that there is great optimism among scientists, politicians and policy makers who anticipate significant job creation associated with the growth of this new field (www.foresight.org/Nanomedicine/NanoMedArticles.html). Nanobiotechnology will certainly provide opportunities for developing new materials and methods that will enhance our ability to develop faster, more reliable and more sensitive analytical systems. A gradual, rather than explosive incorporation of these new discoveries into molecular recognition is predicted. The progression of fabrication techniques driven by the semiconductor industry will allow realization of smaller and smaller structures, challenging researchers to provide new applications for those structures, which reach beyond electronic devices, for which they were initially made. QDs and gold nanoparticles are just two examples of this transfer of technology into molecular detection applications.

Finally, future applications of nanobiotechnology include development of *in vivo* sensors. Nano-sized devices are envisaged that could be ingested or injected into the body, where they could act as reporters of *in vivo* concentrations of key analytes [44]. These devices would

have a capability for sensing and transmitting data to an external data capture system. The constant vigilance of these devices would provide a real-time, 24/7 scrutiny of the state of a person's health. The regulatory issues that will have to be addressed for such devices are as yet unknown; however, the basic technology that would underlie their development can already be discerned.

Acknowledgements

This work was supported in part by grants from the NIH (NCI R33-CA83220 – P.F., S.S. and NCI RO1-CA 78848–04 – L.J.K.) as well as from the US Department of Energy (LDRD – P.G.).

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