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High-sensitivity nanosensors for biomarker detection†

Magdalena Swierczewska[a,b], **Gang Liu**[a,c], **Seulki Lee**[a], and **Xiaoyuan Chen**[a]

Magdalena Swierczewska: Shawn.Chen@nih.gov; Seulki Lee: Seulki.Lee@nih.gov

[a]Laboratory of Molecular Imaging and Nanomedicine (LOMIN), National Institute of Biomedical Imaging and Bioengineering (NIBIB), National Institutes of Health (NIH), 31 Center Dr 1C22, Bethesda, MD 20892, USA. Fax: +1-301-480-1613; Tel: +1-301-451-4246

[b]Department of Biomedical Engineering, Stony Brook University, Bioengineering Building, Stony Brook, NY 11794, USA

[c]Sichuan Key Laboratory of Medical Imaging, North Sichuan Medical College, Nanchong 637007, China

Abstract

High sensitivity nanosensors utilize optical, mechanical, electrical, and magnetic relaxation properties to push detection limits of biomarkers below previously possible concentrations. The unique properties of nanomaterials and nanotechnology are exploited to design biomarker diagnostics. High-sensitivity recognition is achieved by signal and target amplification along with thorough pre-processing of samples. In this *tutorial review*, we introduce the type of detection signals read by nanosensors to detect extremely small concentrations of biomarkers and provide distinctive examples of high-sensitivity sensors. The use of such high-sensitivity nanosensors can offer earlier detection of disease than currently available to patients and create significant improvements in clinical outcomes.

1. Introduction

One of the key challenges in disease control and prevention is early detection. Better clinical outcomes are directly linked with early detection of disease, enabling effective treatment to reduce the suffering and cost to society associated with the disease.[1] However, traditional screening methods such as biopsy, blood detection and clinical imaging are currently not very powerful at very early stages, quite costly and not available to many patients.[2] The use of disease biomarkers is emerging as one of the most promising strategies for our understanding of disease biology and disease management.[2] A biomarker is an indicator of a biological state or condition. It can be a protein, a fragment of a protein, DNA/RNA, or an organic chemical made by abnormal cells. A disease biomarker is a 'molecular signature' of the physiological state of a disease at a specific time and is therefore extremely important for early detection and accurate staging of disease.[2–4] Disease biomarkers also provide information on the underlying mechanism of the initiation of a disease and ultimately offer powerful methods to diagnose and treat the disease at a desired time.

Traditional diagnostic methods, especially for cancer, are based on endoscopy, computed tomography, X-rays, positron emission tomography, mammography and magnetic resonance imaging. However, these methods are neither accessible to large populations nor practical for repeated screenings at early stages of disease. Current models of high throughput

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Correspondence to: Seulki Lee, Seulki.Lee@nih.gov; Xiaoyuan Chen.

screening and detection of a specific biomarker are based on the use of enzyme-linked immunosorbent assay (ELISA), also known as enzyme immunoassay (EIA). This method characteristically involves specific antibodies for a particular biomarker and signals its detection by a chromogenic reporter and substrate. More recently, detection by fluorescence and electrochemiluminescence has also been applied to achieve sensitivities down to picomolar (pM, 10^{-12} M) concentrations.[5] ELISA is a sensitive and well-established method; however, it is time-consuming, technically burdensome, and costly. More detection technologies should focus on biological fluids such as blood and urine for easy to use, low cost, sensitive and quantitative methods of multiple biomarkers, especially towards point-of-care devices in developing countries.[6]

Nanotechnology may be the answer to this need and is already playing an increasingly important role in the improvement of biosensing.[7,8] Nanosensors are devices that sense a force, chemical or biological, where a portion of the sensor operates at the nanoscale. Generally, nanosensors are based on nanoparticles that are conjugated to a targeting ligand where the ligand finds the specific marker of interest, giving the nanosensor specificity, and the nanoparticle acts as the generator or detector of a signal, assigning sensitivity. Nanoparticles offer desirable and unmatched characteristics for detection such as high reactivity, increased electrical conductivity, strength, unique magnetic properties and significant surface area to volume ratio.[9] For example, nanoparticles due to their high surface area to volume ratio can detect a high concentration of markers at extremely limiting amounts of the sample. Additionally, nanosensors offer the use of multi-parametric analysis for real time and direct read outs of detection signals. Furthermore, nanoscale properties are tunable by their shape; therefore, nanotubes, nanowires, thin films, and nanocantilevers give nanosensors versatile and high-sensitivity detection. Such sensitive strategies can also be used to discover novel disease biomarkers. To date, many studies have been conducted on developing high-sensitivity nanosensors for biomarkers (Table 1), which have opened up a new era of early disease diagnosis and better treatment. Detection by nanosensors has reached pico-(p, 10^{-12}), femto- (f, 10^{-15}), atto-(a, 10^{-18}), and even zepto-(z, 10^{-21}) scales.

In this review, we will introduce various novel types of high-sensitivity nanosensors categorized by different signal detection strategies—optical, mechanical, electrical, and magnetic relaxation (Scheme 1). Nanotechnology can provide inimitable detection capabilities for high sensitivity biomarker sensing that was previously not capable. Although many new nanoplatforms are not fully optimized for manufacturing scale up and commercial use, they can provide alternative and irreplaceable diagnostic models.

2. Optical detection

Optical sensing by nanosensors exhibits sensitivity because of the unique interactions between nanomaterials and light waves. The sensitivity, however, is highly dependent on the optical phenomena being detected. For example, fluorescein isothiocyanates (FITC) that interact closely with gold nanoparticles (AuNPs) are highly quenched and no fluorescence signal can be detected; while, this molecule in close proximity with the same nanoparticle can act as a Raman reporter and exhibit enhanced Raman scattering signals. Each type of optical detection employed in high sensitivity nanosensors is introduced.

Surface plasmon resonance (SPR) is a standard method to monitor protein binding interactions in analytical chemistry. It measures the changes in the refractive index of certain types of metal thin films when unlabelled solute molecules bind to the surface. When the surface is excited by electromagnetic radiation, a coherent oscillation of the surface conduction electrons occurs causing resonance that is specific to its environment.[10] Most recent methods allow limits of detection at about 25 ng mL^{-1} and a dynamic range of 2

logs.[11] However, SPR generally has poor resolution due to bulk material interference, suffers from non-specific binding, and is difficult to adjust for high throughput screening.

A unique property of SPR occurs when the light interacts with metal particles that are smaller than the wavelength of light, like metallic nanoparticles. The plasmon oscillates locally around the nanoparticle, known as the localized surface plasmon resonance (LSPR). Works published by Van Duyne and colleagues describe how LSPR can be harnessed for sensing changes in the local dielectric environment.[10,12] El-Sayed and colleagues have greatly characterized the LSPR of noble metal nanoparticles, specifically AuNPs.[13] LSPR sensors are sensitive to the size, shape, and environment of metal nanoparticles during which local refractive index changes.[12] These small refractive index changes lead to changes in the extinction spectra of the nanoparticles. This unique property can be used to detect biomarker molecular binding events. LSPR nanosensors have been developed into a high-throughput, multi-arrayed biochip with limits of detection of 100 pg mL^{-1} (approximately pM detection for the proteins tested)[14] and are becoming commercially available with limits of detection at 1 nM, like the LightPath System™ by Lamdagen Corporation.[15] Both these examples utilize nanostructured self-assembled monolayer (SAM) formation on a substrate, where capture is performed by immobilizing antibodies on the surface. Peak absorption intensity of the LSPR spectra is used to detect various proteins, like immunoglobulins, C-reactive protein, and fibrinogen.[14] Detection is possible by shining white light onto the nanochip in the vertical direction from one optical fiber and the reflected light is collected into the detection fiber and sent for analysis by a UV-vis spectrometer.[14]

However, the development of LSPR nanosensors requires highly uniform nanomaterials to produce a narrow LSPR peak that can shift to a consistent and significant amount. This spectral shift can then be characterized as a detection signal for the culprit that caused the change, like a biomarker. For example, Haes *et al.* utilized specifically shaped Ag nanoparticles to detect amyloid-derived diffusible ligands (ADDL), a biomarker for Alzheimer's disease, from human brain extracts and cerebrospinal fluid.[16] Like ELISA and many nanosensors, a sandwich assay was incorporated to capture the target ligand by a primary antibody and then further labeled with a secondary antibody. First, triangular Ag nanoparticles were synthesized by nanosphere lithography on mica substrates and functionalized with the primary antibody specific for ADDL. After ADDL was captured, a secondary antibody targeted the ligand to enhance the LSPR signal which is measured by ultraviolet-visible extinction spectroscopy. Extinction measurements were collected by a fiber optically coupled spectrometer. Triangular Ag nanoparticles with a perpendicular bisector of 90 nm and height of 25 nm were strategically chosen in order to extend electromagnetic fields 35 nm from the surface, a distance required to detect the ADDL captured in the sandwich assay. This system identified different binding constants of the ADDL to antibody from the brain and cerebrospinal fluid.[16] The control of the dimensions and shape, a unique feature of nanoparticles, allowed for improved optical nanosensors detection. LSPR leads to other phenomena useful for optical nanosensors such as colorimetric detection, unique fluorescence changes and surface-enhanced Raman spectroscopy.[17]

Shifts in LSPR can be detected by absorption spectra and in some cases in a colorimetric manner when the absorption occurs in the visible light region. Au and Ag NPs exhibit oscillation frequencies in the visible region giving Au nanospheres a characteristic red color and Ag nanospheres a yellow color. The color of the particles is affected by the size and shape of the material as well as the dielectric constant of the environment. Any type of anisotropy in the shape, including aggregation of the nanoparticles, can greatly enhance the absorption coefficient, leading to higher detection sensitivity. Mirkin and colleagues firstly demonstrated a detection system for oligonucleotides by controlling AuNPs aggregation

based on the hybridization between target oligonucleotides and nanoparticle–oligonucleotide conjugates.[18] The oligonucleotide of any length is modified with a mercaptoalkyl group at the 5′ terminus, which can conjugate to the Au surface *via* a thiol group. Two of these probes align contiguously when the target oligomer sequence is detected. The target oligonucleotide and two probes hybridize to form an interconnected aggregate of AuNPs. Free AuNPs exhibit a red color but when spacing between the AuNPs decreases, a blue color is formed. Detection is possible visually, when the hybridized particles are transferred to a C18 silica solid support, or spectrally by monitoring the shifts in visible light absorbance. A detection limit of about 10 fmol (10^{-15} mol) of oligonucleotide was achieved.[18]

LSPR affects different cross-sections of optical properties including absorption and scattering. These features have been used in so-called molecular beacons and activatable probes for the detection of specific targets. Metal nanoparticles, due to the strong absorption optical property, can quench fluorescence that is emitted in close proximity to the surface. At larger distances, metallic nanoparticles can enhance that fluorescence due to the high scattering cross-section of the particle. These mechanisms are quite complex and various models are proposed.[19] The unique effect metallic nanoparticles have on fluorophores has been harnessed to detect specific targets, where the fluorophore is released from the surface of the nanoparticles to activate fluorescence after detection.[20] A unique technique proposed by Rotello and co-workers, called nanoparticle “noses”, utilizes the fluorescence effects of AuNPs and fluorescent molecules to sense proteins, cells, and bacteria *in vitro* and *in vivo.*[21] Using six different AuNP-fluorescent polymer probes, Rotello and co-workers devised a rapid detection and differentiation sensor array of seven protein targets (Fig. 1).[21] These probes are formulated by the electrostatic interactions between cationic AuNPs and anionic fluorescent polymers. The six different AuNPs differ by the functionalized surface charge that is responsible for specific interactions with the fluorescent polymer and the target protein. When the polymer closely interacts with the AuNP, fluorescence is quenched. However, when a target disrupts this interaction by competitive binding, the fluorescent polymer is released at a certain rate producing discrete fluorescence patterns. These fluorescent patterns are characteristic of a specific protein and can be used to quantify protein concentration by linear discriminant analysis (LDA).[21]

Optical *in vivo* sensors require wavelengths in the near infrared region (NIR) to reduce signal absorption from blood and tissue. With rationally designed nanoparticles and the use of NIR dyes, *in vivo* fluorescence nanosensors have been designed. AuNPs offer great quenching properties but they suffer from labile surface chemistry, which can be easily reduced *in vivo.* Iron oxide nanoparticles (IONPs), on the other hand, allow for robust surface chemistry that is non-reductive under physiological conditions, but with less efficient fluorescence quenching than gold nanoparticles. To utilize the quenching properties of AuNPs and the surface chemistry of IONPs, Xie *et al.* synthesized a flower-shaped Au–IO nanoparticle, where the IONPs make up three “petals” around the central AuNP (Fig. 2).[20] This nano-flower serves as a substrate *via* high affinity binding between the IONP and dopamine analog for an optical probe that is specifically activated by matrix metalloproteinases (MMPs) within tumors.[20] This conjugate was injected intravenously and located to the tumor site because of the leaky vasculature, called the enhanced permeability and retention (EPR) effect. At the tumor, the probe exhibited high fluorescence signals after sensing MMP, because the MMP cleaved the fluorescent probe away from the quencher.

Another nanoparticle that exhibits properties useful for fluorescence sensing and labeling are quantum dots (QDs). QDs are confined semiconductors that exhibit high quantum yields, broad absorption yet sharp photoluminescence spectra, and large Stokes shifts. Much work has focused on these highly fluorescence nanoparticles,[22] although issues with blinking

signals and toxicity have delayed progress and other optical probes have provided brighter signals.[23] Next generation QDs may show promise as a scaffold for nanosensors. Yet in this review, we focus on unique and high sensitivity nanosensor systems for biomarkers utilizing metallic nanoparticles and other nanotechnologies.

Surface-enhanced Raman scattering (SERS) of single molecules is a major detection signal of interest in nanosensors because metal or core–shell nanoparticles offer large Raman scattering enhancement factors on the order of 10^{14}–10^{15}.[24] This enhancement factor is credited to the LSPR modes at the nanoparticle surface, which can focus the energy to the nanoparticle and increase the density of states at Stokes-shifted wavelengths.[12] Important works by Nie and colleagues have advanced the SERS field in nanotechnology[24] as well as for *in vivo* cancer detection.[23,25] The first demonstration of *in vivo* SERS detection was performed by Stuart *et al.* to measure glucose concentrations in a rat model (Fig. 3).[26] The nanosensors were made by using a Ag film over nanospheres (FON) technique, where a 200 nm thick Ag film was deposited over a 390 nm-diameter nanosphere solution, and further functionalized with decanethiol and mercaptohexanol on the surface to partition glucose and reduce non-specific binding by proteins. This nanosensor assembly, specifically determined by the FON surface thicknesses, allowed detection in the "biological window", where optical signals are not absorbed by blood or tissue. The sensor was then subcutaneously implanted to measure glucose from the interstitial fluid by SERS. The "fingerprint" spectra of glucose are enhanced by the nanosensors and detected by a spectrometer outside the rat. After further optimization, such a sensor could measure glucose concentration or other types of metabolic analytes in diabetic patients in real-time.

Nie and colleagues took a different approach for *in vivo* sensing by developing SERS tags that first target cancer cells and are then detected in a non-contact manner by a Raman spectrometer outside the live animal.[25] Certain types of Raman reporters, which are chromophores with identifiable Raman spectra like malachite green isothiocyanate, crystal violet, Nile blue, can adsorb to PEGylated AuNPs by electrostatic interactions and maintain Raman enhancement factors. Targeting groups, like antibodies, and PEG chains for biocompatibility are bound to the AuNP surfaces *via* thiols and an activated heterofunctional PEG. Combining these elements, Qian *et al.* decorated AuNPs with PEG, antibodies, and small-molecule Raman reporters to non-invasively detect high SERS signals targeted to cancer cells *in vivo*.[25] Other examples have achieved specific biomolecular targeting and detection using SERS nanotags by Au or Ag NPs labeled with fluorescent dyes or surfactants.[25,27,28] In addition, detection limits utilizing target amplification techniques with SERS detection have reached zM (10^{-21} M) concentrations for DNA and RNA[29] and fM concentrations for prostate specific antigen in human serum.[30]

However, the Raman scattering enhancement effect is dependent on the nanoparticle spatial location with respect to the target biomarker as well as among each other. Theoretical work shows that the maximum electromagnetic field enhancement of SERS occurs between the interstitial sites of particles or at locations outside sharp surface protrusions.[31] Chemically, ligands that interact with specific SERS-active surfaces lead to further enhancement of Raman scattering because of charge transfer states. Doering and Nie demonstrate that chemical enhancement of the SERS spectra occurs from Cl^-, Br^-, and I^- ions interacting with a single nanoparticle while citrate, sulfate, and fluoride ions have no effect on single-particle SERS and interestingly thiosulfate ions can quench SERS signals.[31] Additionally, there is still an inherent lack of control of the nanoparticle stability. Hence, a major obstacle in SERS biomarker detection is the large fluctuation in the signal intensities and frequencies under similar conditions, called the "blinking" signal. To overcome this, various shapes and configurations of nanoparticles can be utilized to achieve a more controlled signal. For example, Lim *et al.* engineered SERS-active Au–Ag core–shell nanodumbbells where the

distance between two particles and the Raman dye location are controlled by DNA strands in order to achieve reproducible, non-blinking detection signals of single molecules.[32]

In optical nanosensors, nanoparticles are the signal producers. Sensors are therefore designed to cause changes to the nanoparticle in proportion to the target concentration. However, this requires highly uniform particles that are not affected by their bulk environment and can produce non-blinking and sharp signals. This has not been entirely achieved yet because most nanoparticles are only as stable as the surface stabilizing agents. Biosensors require detection under high salt physiological conditions, which are harsh conditions for most nanoparticles. Although surface chemistry remains a challenge, nanosensor designs have been able to bypass these disadvantages. Optical nanosensors can be optimized, such as taking combinatorial approaches, for a specific role whether it is to measure ultra-low concentrations, detect conformational changes of protein, or utilize *in vivo.* By harnessing the unique optical properties of nanoparticles, nanosensors can produce highly amplified signals for early detection of disease.

3. Mechanical detection

Mechanical detection by nanosensors is based on the ultrasensitive detection of extremely small mechanical forces occurring on the molecular scale. A recently published review provides a thorough overview of mechanical nanosensors.[33] Nanomechanical sensors can measure transport and affinity on the molecular scale as well as forces, displacements and mass changes from subcellular processes. Mass resolution of mechanical devices is proportional to the total mass of the device. So as mechanical sensors decrease to the nanoscale, the mass resolution greatly increases. Detection of zg biomolecules in vacuum[34] and sub-fg sensitivity in fluid[35] has been reported. However, fluid detection, which is an ideal condition for biomolecular targeting, is a major obstacle for mechanical nanosensors because sensitivity is greatly reduced by viscous damping. Suspended microchannel resonators are a unique alternative for biomarker detection in fluid, because particles can be weighed in real time as they flow through the channel (Fig. 4).[35]

The main device in mechanical nanosensors is the microcantilever which can be seen as a “miniature diving board”.[36] When the analyte molecules bind to the immobilized receptors on the surface of a cantilever, the microcantilever undergoes two responses to measure the nm displacement. The resonance frequency of the microcantilever shifts due to mass loading or unloading from molecular interaction. Secondly, the cantilever bends due to surface stress by the adsorption of the molecule. The bending and resonance frequency shifts are measured at high sensitivity using established techniques such as optical beam deflection, piezoresistivity, piezoelectricity and capacitance.[5] Like many nanosensors, detection specificity is low but is usually addressed by selective biochemical reactions like coating the cantilever with self-assembled monolayers, DNA probes, antibodies or peptides.[5] Nanomechanical resonators have reached mass measurements as low as 7 zg.[34]

Braun *et al.* developed a sensor based on arrays of resonating microcantilevers to allow sensing in liquid physiological environments that can measure the interactions between transmembrane protein receptors and their ligands.[37] This array system was tested using a protein receptor of *E. coli.* The protein receptor was crystallized in liposomes, called proteoliposomes, and then immobilized on an Au-coated surface by ink-jet spotting. The sensor was able to measure the mass of the bacterial virus T5 binding with its transmembrane receptor at sub-pM concentrations. The microcantilevers gave specific and time-resolved detection in a micro-array format. The array design improves the sensitivity of the system since all experiments are performed in parallel under identical physiological conditions, reducing false signals to the cantilever by temperature drifts or non-specific

binding. Additionally, this study introduced a universal technique to use proteoliposomes as a target to study receptor–ligand binding, allowing the protein to maintain its biological function. Another array-type technique has been proposed by Waggoner *et al.* to detect prostate specific antigen (PSA) in serum using "trampoline" resonators (Fig. 5).[38] The trampoline resonators improve sensitivity over standard cantilevers because (1) the nanoparticle binding area is larger, increasing the detection limit and (2) the resonance is highly uniform, as compared to the variable frequency response along the length of the cantilevers, reducing the standard deviation of detection. On the resonators, the protein biomarker for prostate cancer is sandwich-captured in fluid phase with a primary antibody and a secondary antibody bound to a 1 fg nanoparticle mass label. Using this unique labeling system and the array of trampoline resonators, PSA was detected at fM concentrations in serum.

For mechanical nanosensors to enter clinical use, assays should be easy to use, conserve reagents, and reduce time. A recent study by Manalis and co-workers proposes a functionalizable surface coating onto silicon oxide suspended microchannel resonators that reduces nonspecific binding.[39] Fluid handling is limited because the polymer coating is directly injected to the silicon resonator and then the antibody is injected to further functionalize the surface. The target is then captured on the immobilized antibodies within the resonator. The adsorbed biomolecule within the microchannel resonator displaces an equivalent volume of solution. This causes an addition of mass, changing the microcantilever resonant frequency in proportion to the bound biomolecules. Using this system, activated leukocyte cell adhesion molecules (ALCAM) were detected in undiluted serum at pM concentrations in about one minute.[39]

Mechanical nanosensors, however, are still limited for clinical settings. The sensitivity and selectivity of nanomechanical sensors are dependent on the nanofabrication of uniform cantilevers and efficient surface coating to improve target binding, respectively. Additionally, efficient mechanical sensing is based on sensitive instrumentation to reduce high noise backgrounds, which can be costly.[40] Inherent environmental limitations hamper biomarker detection since fluid detection has not achieved the high sensitivity measurements as detection in vacuum.

4. Electrical detection

Electrical detection is a rapidly developing field with established, simple and low-cost fabrication techniques. Nanosensors by electrical detection, primarily nano field-effect transistors (FETs), offer simple and direct measurements in real-time as well as portable capabilities.[41] FET-based electrochemical nanosensors utilize nanowires, nanoribbons,[42] and nanotubes[43] to measure the change in resistivity induced by the target molecule binding to the surface. These nanomaterials provide higher sensitivity because the flow of current can occur across the majority of the nano-scale cross-section rather than the planar surface of the sensor.

Silicon nanowires are commonly utilized due to their high sensitivity and ease of chemical modification. Lieber and co-workers in 2001 were the first to demonstrate the use of silicon nanowires for direct, sensitive, and real-time biodetection in aqueous solution (Fig. 6).[44] This proof-of-concept nanowire FET sensor detected protein concentrations as low as 10 pM but further improvements reached fM detection limits.[45–47] Lieber and colleagues continue to be major contributors to the design of direct electrical detection of biomarkers.[44–46,48] Zheng *et al.* developed a multiplexed electrochemical detection system using silicon-nanowire field-effect devices.[46] The nanowires are functionalized with antibodies in a three step process by first introducing terminal aldehyde groups on the oxygen plasma-cleaned

silicon nanowire surface, coupling monoclonal antibodies to the aldehyde groups, and thirdly blocking any unreacted aldehyde groups with amines. The nanowires are functionalized in an array, giving about 200 individual sensors, to detect multiple protein markers at fM concentrations in undiluted serum. When the protein binds to the receptors, only found on a specific nanowire, the change in the conductance across the surface of that nanowire is detected. Arrays of different types of doped silicon nanowires (p- and n-type) were used in order to reduce false-positive signals as well as clearly distinguish protein-binding signals from noise. This was the first demonstration of high sensitivity electrical nanosensors using silicon nanowire arrays.

Carbon nanomaterials are also being introduced into FET nanosensors, specifically carbon nanotubes.[43] Although they currently show weaker detection limits than silicon nanowires, pM sensitivity has been reported using carbon nanotubes for DNA detection.[49] Cai *et al.* developed an array of carbon nanotubes with a molecular imprinted polymer coating on the nanotube tips to recognize proteins with sensitivities below pg mL^{-1} using electrochemical impedance spectroscopy (EIS).[50] Using this nanosensor, human ferritin and human papilloma-virus derived E7 proteins were detected at pg L^{-1} and fg L^{-1}, respectively. A key feature in this design was the electropolymerizing, nonconductive polyphenol nanocoating on the tips of the carbon nanotube arrays. The protein of interest was initially trapped in the polyphenol nanocoating and then removed, leaving an imprint of the protein on the nanotube surface. Because of this confining area, high specificity is achieved. Further, the biosensor can be designed to detect different conformations of proteins, like calcium induced conformational changes in calmodulin. Detection sensitivity is dependent on the impedance, which was found to be the highest at the nanotube tips because there is faster electron transfer along the nanotube tip than the sidewall. The impedance showed concentration dependency from 10 pg L^{-1} to 10 μg L^{-1}. The primary mechanism of signaling is due to the change in permittivity and resistivity in the surface materials in response to protein capture.

A major limitation of electrochemical nanosensors is the inability to sense molecules in physiological solutions, specifically at high salt concentrations.[35,46,51,52] Since electronic detection is based on charge, salt buffers can screen the signal and reduce the nanosensor's sensitivity. For example, nanowire FETs require a salt concentration below 1 mMin order to prevent screening of the electronic signal. One strategy to overcome this salt concentration is to purify the sample of interest upstream of the nanosensors. Stern *et al.* designed a microfluidic purification chip to concentrate the target before electrical detection and demonstrated its use by detecting two cancer antigens from a 10 μL sample of whole blood in under 20 minutes (Fig. 7).[53] This remarkable detection under complex physiological conditions is achieved, not because of the FET device, but by the purification chip that concentrates the sample before being read by the device. The chip captures various types of biomarkers from blood, washes them, and releases the markers into purified buffer for electronic sensing. The markers are released *via* UV irradiation of the photo-cleavable crosslinker between the device and biomarkers of interest. Pre-processing prevents high salt concentrations from interfering with the electronic signal and allows the use of cheaper detectors with clinically relevant samples.

Although electrical sensing can take advantage of fabrication methods developed by the electronics industry, additional design is required to reduce the high salt conditions that biosensors must operate. Nanomaterials can offer high sensitivity because of the increase in current across nanoscale surfaces. Therefore nanowires, nanoribbons, and nanotubes have been utilized in electrical nanosensors for biomarkers. Yet, the high salt concentration of samples can screen signals and reduce sensitivity. This is a remaining challenge in electrical nanosensors. Carbon nanotubes face additional restrictions for electrical detection because of the contamination of metallic nanotubes during fabrication and the complexity of surface

modification.[54] Because electrical signals can be greatly dampened under physiological conditions, electrical and electrochemical nanosensors have shown the most versatility in capture assays that can be translated to different types of nanosensors.

5. Magnetic resonance detection

Magnetic resonance imaging (MRI), one of the most powerful imaging tools in radiology and biomedical sciences, has shown great value in early cancer diagnosis, implant monitoring, and drug discovery. The advantages of MRI include non-invasive imaging, deep tissue penetration, and superior spatial resolution. However, the overriding challenge with MRI for biomedical applications at the cell/molecular level is its relatively low sensitivity.[55] Thus, the introduction of contrast agents, like paramagnetic small molecule agents (gadolinium[56]) and super-paramagnetic iron oxide (SPIO) nanoparticles,[57,58] is essential to detect cancer biomarkers using MRI.

The fundamental principle underlying MRI is that unpaired nuclear spins (hydrogen atoms in water and organic compounds) align themselves when placed in a magnetic field.[55] The contrast agents are able to shorten either longitudinal or transverse proton relaxation time and thus provide higher image contrast. For example, SPIO nanoparticles can shorten transverse relaxation time (T_2) and bring negative contrast, resulting in hypointense images. The controlled clustering or aggregation of a few SPIO nanoparticles can greatly shorten T_2 relaxation time compared to single nanoparticles at the same Fe concentration. Thus, when magnetic nanosensors aggregate through affinity ligands to the biomarkers, a decrease in the T_2 relaxation time of surrounding water molecules is observed, allowing the sensitive and accurate detection of cancer biomarkers (Fig. 8).[59,60] The non-invasive acquisition of information on both temporal changes of labeled-biomarkers and high-resolution anatomy is of great interest in the field of magnetic nanosensor biomarker detection.[61]

When a magnetically responsive nanosensor is used to measure binding kinetics of proteins, detection is enhanced by high spatial and temporal resolution. A model proposed by Gaster *et al.*[62] utilizes giant magnetoresistive (GMR) and magnetic nanoparticles to quantify antibody–antigen binding at 20 zmol of solute sensitivity. GMR biosensors offer extraordinary limit of detection, multiplexing, broad linear dynamic range of measurements and real-time capabilities. These devices operate by changing their electrical resistance during a change in their local magnetic field. As most biosensors, research has focused on applying a sandwich assay to the surface of the GMR nanosensors to quantify the amount of protein. Gaster *et al.* pre-labeled a ligand with a magnetic nanoparticle (MNP) to detect binding kinetics in real time to antigens which are immobilized on the GMR sensor surface.[62] The superparamagnetic NPs are comprised of twelve 10 nm IO cores embedded in a dextran polymer with the coated ligand surrounding the surface. When the MNP labeled ligand binds to the antigen, the magnetic field of the nanoparticle changes and is sensed by the GMR sensor by changes in the electrical resistance. In this way, the kinetics of binding is monitored and the kinetic rate constant can be measured. Gaster *et al.* designed the GMR nanosensor into an array of 72 stripes per sensor to measure multiple types of proteins. The GMR sensor can only detect dipole fields from the magnetic tags within 150 nm of the sensor, therefore only detecting the bound molecules and allowing it to be used in real-time. This high-density technique allows thousands of sensors to be run in parallel with sensitivities of about 10 ng L^{-1} and a dynamic range of 6 logs.

Magnetic nanoparticles such as SPIO nanoparticles are an important class of nanomaterials with interesting properties (biocompatible, physically/chemically stable, and inexpensive to produce), which have been developed into various functional agents for applications in imaging, cell labeling, and drug/gene delivery.[57,63] As the most prominent T_2/T_2^* probes for

MRI, SPIO nanoparticles offer unique magnetic properties that can be exploited for screening biomarkers by magnetic resonance methods. Functional SPIO nanoparticles bound to biological molecules (*e.g.* nucleic acids, peptides, and proteins) have widely been developed for use as nanosensors with the objective of generating or even amplifying measurement signals. For example, Perez *et al.* reported magnetic nanosensors to detect oligonucleotides, proteins, and enzyme activity by MRI with high sensitivity and selectivity (Fig. 9).[60] The SPIO nanoparticles conjugated with oligonucleotides form aggregates by hybridization with addition of complementary oligonucleotides, resulting in a reduction in the T_2 relaxation time. However, no change in T_2 was observed with the addition of scrambled oligonucleotide sequences. Similarly, when the magnetic nanosensor conjugated with green fluorescent protein (GFP) antibody was used to probe the target protein-GFP, the T_2 relaxation time was observed to decrease with time in a dose dependent manner. In each case, there was a decrease in the T_2 relaxation time that could be quantified and related to the biomarkers concentration. In contrast, the reverse SPIO nanosensors have been designed to detect caspase-3 activities. Caspase-3 is a family of intracellular cysteine proteases that is known as a specific mediator of the apoptotic process. The clustered SPIO nanoparticles were prepared using a small peptide, the DEVD (aspartic acid-glutamic acid-valine-aspartic acid), which can be recognized and cleaved by caspase-3. Then, caspase-3 enzyme could disassemble the SPIO aggregates by cleaving DEVD, leading to a corresponding increase in the T_2 relaxation time.

The development of simple and effective techniques based on magnetic nanosensors can be used to delineate cancer cells. El-Boubbou *et al.* developed a magnetic nanosensor bearing carbohydrates to qualitatively and quantitatively profile the carbohydrate-binding characteristics of cancer cells by MRI, which can facilitate both molecular diagnostics and therapeutic tools for cancer.[64] This chip-based micronuclear magnetic resonance system is a powerful biotechnology tool that offers unique advantages in molecular profiling of the cancer cell surface biomarkers (Fig. 10).[59,65] The system consists of microcoils for radio-frequency excitation and nuclear magnetic resonance signal detection, an on-board nuclear magnetic resonance spectrometer, a portable magnet, and microfluidic networks. SPIO nanoparticles were conjugated with antibodies to each target, followed by incubation with cancer cells. The significant differences in T_2 relaxation time could be observed for a variety of cancer cells using this system. This strategy with high sensitivity, specificity and high-throughput shows potential for early cancer diagnosis in the clinic.[65]

When designing nanoparticle-based nanosensors, there are a few important aspects that influence the performance of the sensing system that need to be considered: (1) T_1 and T_2 relaxivity, this is directly related to the sensitivity; (2) colloidal stability, the stability of nanoparticles and their conjugated forms with biomarkers directly affects the efficiency of nanosensors in terms of sensitivity and selectivity; (3) biosafety, problems related to the toxicity of nanosensors should be carefully considered, especially when the final goal is for *in vivo* sample detection.[61]

6. Beyond detection signals

Nanotechnology offers a wide variety of assay read-outs, giving unique sensitivity by signal amplification. Yet there is another amplification technique that can be added to the assay design to achieve highly sensitive nanosensors for biomarkers. The target of interest can be augmented, like in polymerase chain reaction (PCR) where a few copies of nucleic acid sequences are amplified to hundreds of millions of copies. This is usually done upstream of the detection readout. Pre-processing of samples offers: (1) higher sensitivity because non-specific, background signals are greatly reduced and (2) robustness and versatility because ideally the technique could be applied to numerous detection systems. One example is the

use of lab-on-a-chip systems that confine fluid flow within micron sized channels and offer high-throughput pre-processing of clinical samples, like blood, to detect biomarkers.[66]

Mirkin and co-workers developed the bio-barcode assay, which has pushed the limit of detection for DNA to zM concentrations[7] and proteins to aM concentrations (Fig. 11).[67] Utilizing the unique properties of multiple particles, the strategy purifies and amplifies the sample before detection. First a magnetic microparticle targets the biomarker of interest by either a complementary oligonucleotide or a monoclonal antibody. Then AuNPs are introduced to further sandwich the biomarker. These nanoparticles hold hundreds of oligonucleotides, which are referred to as barcodes because the sequence is specific to the biomarker. Next a magnetic field is applied to separate the particles from the solution and the bio-barcodes are released from the particle. The barcode, which occurs at least few hundred times more than the actual target, is then detected. Detection of the bio-barcodes has been accomplished by SERS[29] and light scattering.[68] Complementary to the bio-barcode assay is scanometric detection that utilizes LSPR properties of nanoparticles for detection.[68] The use of the bio-barcode assay with scanometric detection was able to detect the first amyloid-derived diffusible ligands (ADDL), a biomarker for Alzheimer's disease, in cerebrospinal fluid of 30 individuals.[69] Previously, no system was able to measure the fM concentration of ADDL in the cerebrospinal fluid.

Additionally, selectivity of targets is still a limiting factor towards highly sensitive nanosensors for biomarker detection. Nanosensors select biomarkers, oligonucleotides and proteins, mainly by base-pairing or antibody binding, respectively. DNA targets are easy to select in solution because of the strong interactions of Waston–Crick base pairing, where the unique sequence binds to its complementary code. Protein targets, on the other hand, are captured by antibodies, which do not include base pairing recognition. Antibodies have shown nonspecific binding with other proteins, making antibody–protein detection difficult in heterogeneous clinical samples. Aptamers are emerging as rivals to antibody–protein selectivity.[22] Systematic evolution of ligands by an exponential enrichment (SELEX) process can isolate specific nucleotide sequences, aptamers, for protein biomarkers of interest. Less cross-reactivity and higher selectivity are reported than antibody recognition.[70]

7. Conclusions

Highly sensitive nanosensors provide unique signal detection and amplification strategies to push the limits of detection to zM concentrations. Highly sensitive sensing can detect prognostic and predictive biomarker levels earlier in disease stages, distinguish between favorable and unfavorable outcomes of tumors, and guide further disease treatment. It also can be used to detect recurrence of the disease much earlier on after treatment. Unique strategies have been introduced using various nanomaterials in this tutorial review. Yet, the sensitivity, specificity, and predictive value of nanosensors have to be determined using clinical samples before they can be used in patients. Furthermore, nanosensors need to meet the following requirements to provide appropriate diagnostic capability:

1. Early detection readings
2. Specificity
3. Simplicity, low cost
4. Minimally invasive/non-invasive procedure
5. Site-specific detection (targeted organ/tissue specific)
6. Observer-independent

The synthesis of extremely reproducible particles is necessary to achieve reproducible detection signals. For optical detection, the generated signal is directly linked to the stability of the nanoparticles. If the particles are not synthesized in the same way each time, signals will vary and the assay will be limited in reproducibility and sensitivity. Mechanical and electrical detection has overcome some of the reproducibility concerns by testing a control sample on the same test strip. Yet this can make comparing within the same patient, like following biomarker concentration during a treatment regime, a challenge as samples may not always be available to test alongside each other. It also adds cost to the system. Current nanosensor sensitivities are only achievable under highly optimized conditions in a lab. Mass production is greatly limited because little to no nanosensors have been scaled up and manufacturing is costly. Although a long way to go, nanosensors have the potential to be used as point-of-care devices due to their size and sensitivity. However, point of care devices should be limited to easy to use systems, like colorimetric detection. Other assays and detection types may require technicians to run the equipment or expensive signal receivers, like in magnetic resonance methods, which will reduce its point of care uses due to high health care costs. Therefore, there is a need to involve manufacturing skill into the design of nanosensors in order to produce simple systems that are robust in settings outside of the lab and towards point-of-care devices.

Nanotechnology offers exclusive solutions and promises towards diagnostic applications. The high-sensitivity detection signals introduced in this review can only be achieved by the unique interactions that nanomaterials have with their environment. By harnessing this power, nanosensors can offer high sensitivity detection to a large population of people for routine screening. Additional functionalities can be assigned to them to go beyond diagnostic applications and towards therapeutic agents when used *in vivo*; so far, optical and magnetic resonance detection has been used to detect *in vivo* biomarkers. Targeted nanosensors can serve as imaging, diagnostic and therapeutic agents, so called theranostic systems. However, the interplay between these elements must be carefully investigated to ensure a synergistic benefit.

Fundamental questions of how nanoparticles react to different environments, like *in vivo*, still remain. Stringent toxicity studies are required to address the full cycle a nanoparticle takes *in vivo* from uptake and metabolism to clearance. The nanofield requires a highly interdisciplinary setting in order to meet its vast capabilities. Beyond robust synthesis nanosystems, there is a need to introduce numerous skills into the field. Scientists, engineers and clinicians must operate in unison to collect the clinical needs of a biosensor, design a nanosensor that utilizes its full potential towards robust signal amplification, and develop an efficient manufacturing scheme.

Acknowledgments

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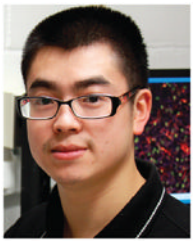
Biographies

Magdalena Swierczewska is a Graduate Partnerships Program (GPP) student between the Biomedical Engineering Department at Stony Brook University and Dr Xiaoyuan Chen's LOMIN of the NIBIB, NIH. Her previous work includes the development and characterization of novel nanomaterials for biomedical imaging and tissue engineering. Using her background in nanotechnology and material science, Maggie is focused on the development of intracellular biomarker nanoprobes towards her PhD thesis.



Gang Liu received his PhD in Biomedical and Bioengineering from Sichuan University in China, under the supervision of Professor Hua Ai. In 2009, he joined Dr Xiaoyuan Chen's LOMIN of the NIBIB, NIH, as a postdoctoral researcher. His current research interests focus on the development of theranostic nanomedicine carrying both chemotherapeutics, gene therapeutics, and imaging tags.

Seulki Lee is a Group Leader of Theranostic Nanomedicine Section in the LOMIN at NIBIB, NIH. He received his PhD from the Department of Materials Science and Engineering at Gwangju Institute of Science and Technology (GIST) in Korea. He focused his training on nanomedicine and molecular imaging at the Molecular Imaging Program at Stanford (MIPS) under the supervision of Dr Xiaoyuan Chen. In 2009, he joined Dr Chen's new LOMIN at the NIBIB, NIH, as a group leader. With a background in nanomedicine and molecular imaging, his research aims to develop smart nanoplatforms for future diagnosis and therapy of various diseases with the emphasis on theranostics.

Xiaoyuan Chen received his PhD in chemistry from the University of Idaho in 1999. He joined the University of Southern California as an Assistant Professor of Radiology in 2002. He then moved to Stanford University in 2004 and was promoted to Associate Professor in 2008. In the summer of 2009, he joined the Intramural Research Program of the NIBIB as a tenured Senior Investigator and Chief of the LOMIN. Dr Chen has published over 300 papers and numerous books and book chapters. He sits on the editorial board of 12 peer-reviewed journals and the editor-in-chief of Theranostics. He is interested in developing molecular imaging toolbox for better understanding of biology, early diagnosis of disease, monitoring therapy response, and guiding drug discovery/development. His lab also puts special emphasis on high-sensitivity nanosensors for biomarker detection and theranostic nanomedicine.

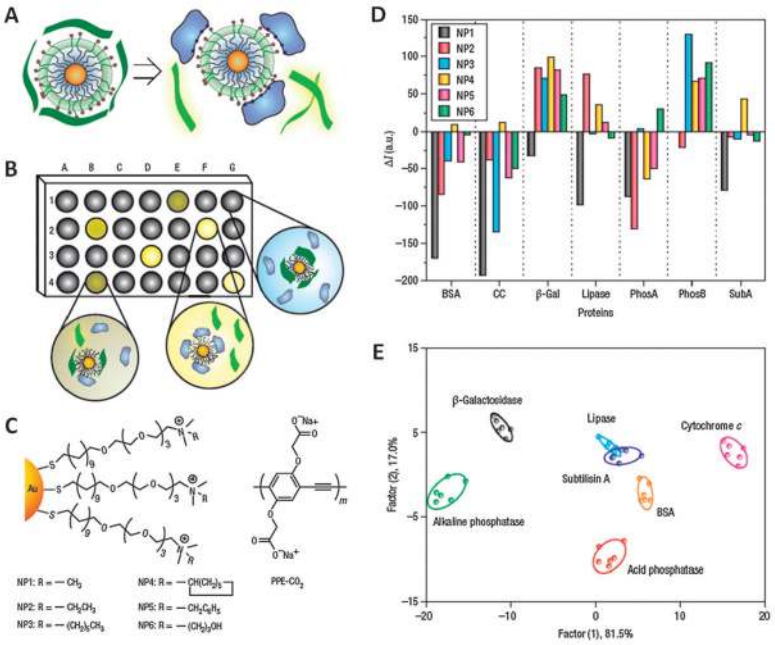


Fig. 1.

Chemical "nose" sensor. (A) Schematic of the fluorescent polymer interaction with AuNPs. When the polymer interacts with the nanoparticle surface, fluorescence is quenched (left). When the polymer is displaced by a protein target, the fluorescence is restored (right). (B) Protein sensor array made up of chemical noses. A fluorescence pattern is generated based on the specific interaction between the particle and fluorophore. Each well contains different nanoparticle–polymer conjugates. (C) Chemical structure of six different cationic AuNPs that interact with the anionic fluorescent polymer ($m \approx 12$) used to sense protein analytes. (D) Fluorescence response patterns of the chemical nose array. (E) Canonical score plot calculated from LDA used to identify seven proteins. Image adapted with permission from ref. 21.

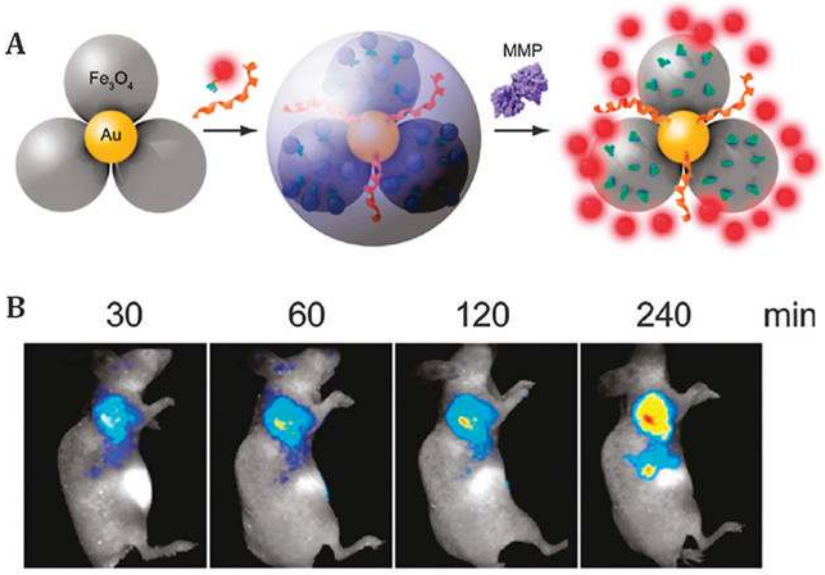


Fig. 2.

Fluorescence activatable nanosensors. (A) Schematic illustration showing the mechanism of flower-like Au–Fe_3O_4 nanoparticles. First the AuNPs and surrounding Fe_3O_4 nanoparticles are synthesized to form a flower like structure. Next, a dye labeled matrix metalloproteinase (MMP) substrate is attached to the probe. When the MMP substrate is intact, the dye is quenched because of the close interaction with the AuNP. When MMP is present, the dye is separated from the AuNP regaining its fluorescence and the probe is activated. (B) *In vivo* near-IR fluorescence imaging after injection of flower like activatable probes into a mouse model. The probe shows high fluorescence signals at the tumor site where MMP concentrations are high. Modified with permission from ref. 20.

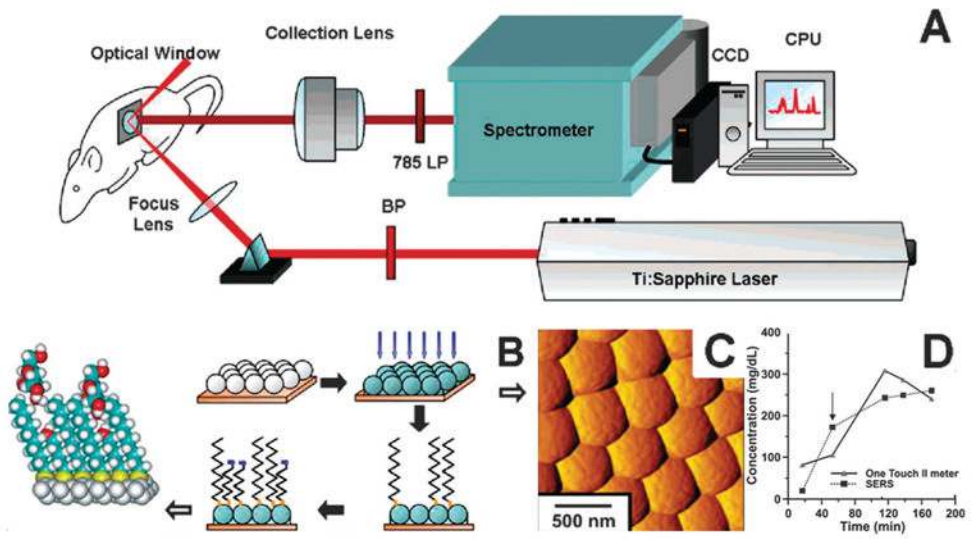


Fig. 3.

In vivo SERS detection of glucose in a mouse model. Schematic image of (A) instrumental set-up for SERS detection of an implantable glucose sensor and (B) Ag film on nanoparticles (AgFON) sensor, showing glucose capture across the surface. (C) Morphology of the sensor as seen by atomic force microscopy. (D) Time course of in vivo glucose measurements. Glucose was infused at 60 minutes (arrow) and measurements were performed by a commercial glucose meter (triangle) and SERS nanosensor (square). Modified with permission from ref. 26.

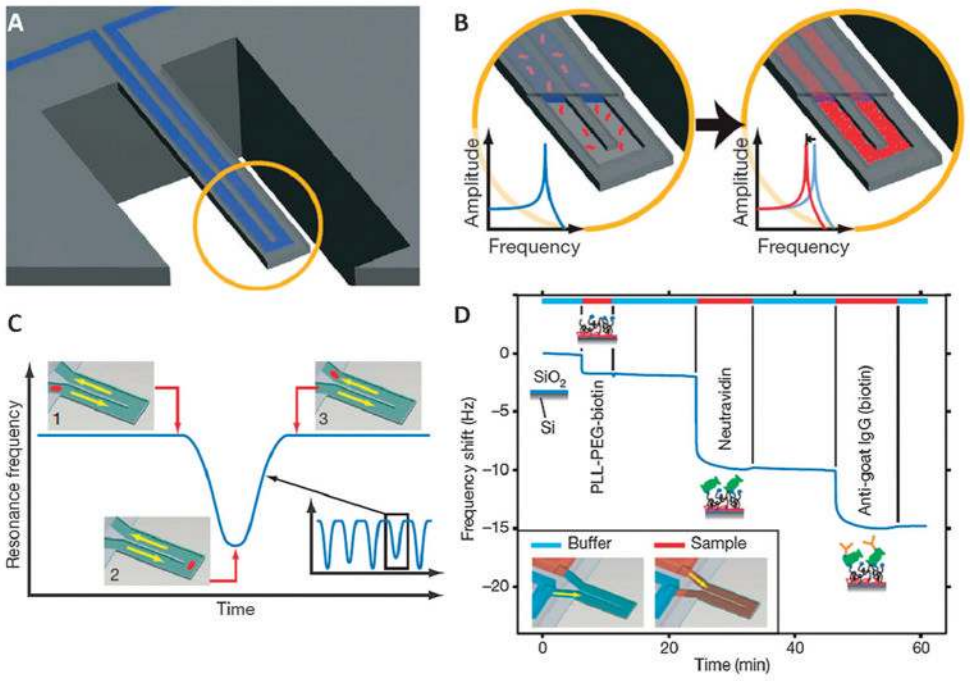


Fig. 4.

Suspended microchannel resonator. (A) Schematic drawing of a suspended microchannel. This microchannel allows continuous fluid flow through the channel while still achieving sub-femtogram mass resolution by reading the signal from the cantilever under high vacuum. This type of system overcomes the challenge of fluid detection by mechanical nanosensors. Two types of detection by this microchannel is possible: (B) bound molecules by a sandwich assay accumulate in the channel and increase the mass (right) while non-accumulated molecules continue to flow through the channel (left). The frequency therefore shifts due to the change in mass. (C) Non-bound, free-flowing particles within the channel can also by monitored in real-time by peak frequency measurements, as seen in the frequency *vs.* time graph. (D) Representative frequency shifts over time as antibodies are coated on the suspended microchannel resonator. First a biotin linker is adsorbed on the silicon dioxide surface, then Neutravidin (streptavidin analog) is coated on the channels, and finally biotinylated antibodies bind to the Neutravidin. Between each injection (red bars), rinse cycles were incorporated (blue) where no change in frequency shift is observed. Modified with permission from ref. 35.

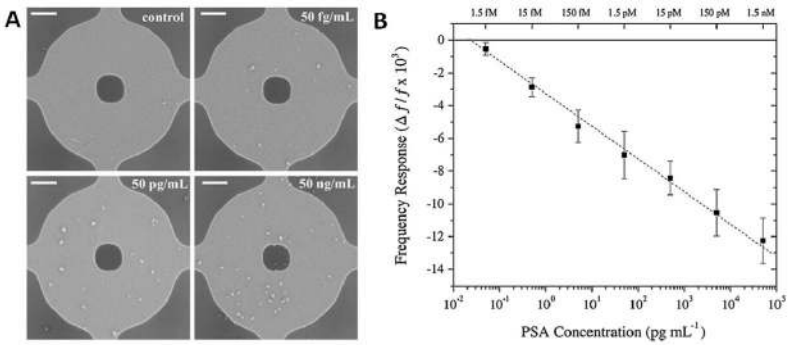


Fig. 5.

Electromechanical detection of protein. (A) Representative scanning electron micrographs of trampoline resonators with varying concentrations of prostate specific antigen (PSA) with nanoparticle labels. Scale bar 1 mm. (B) Frequency response based on the PSA concentration showing sensitivity to the fM. Modified with permission from ref. 38.

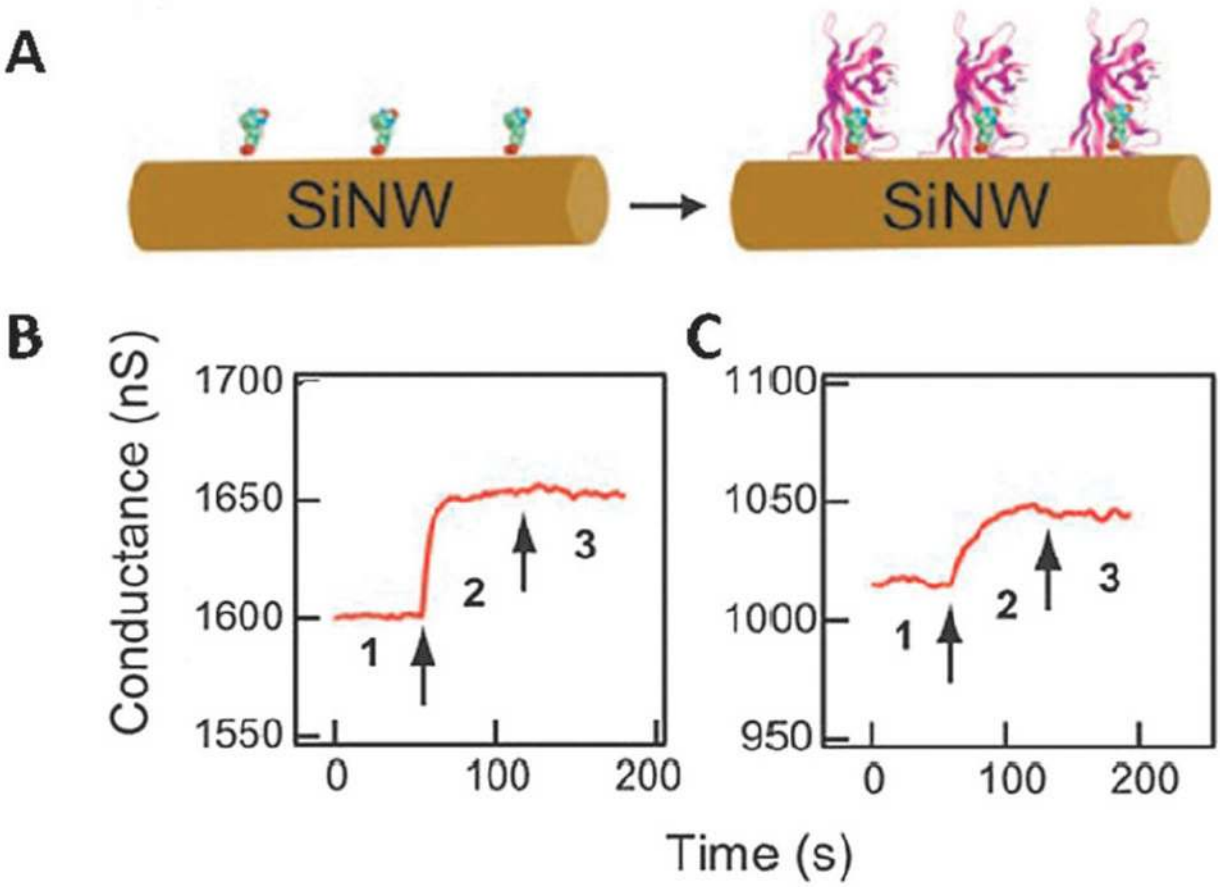


Fig. 6.

First demonstration of real-time detection of protein using silicon nanowires (SiNW). (A) Schematic figure showing protein binding (right) onto biotin-decorated SiNW (left). (B,C) Conductance *versus* time graph where the nanowire is originally in buffer solution (region 1) and then (B) 250 nM or (C) 25 pM of streptavidin binds to the nanowire (region 2) and finally the nanowire is in pure buffer (region 3). The arrows indicate where solutions were changed. Modified with permission from ref. 44.

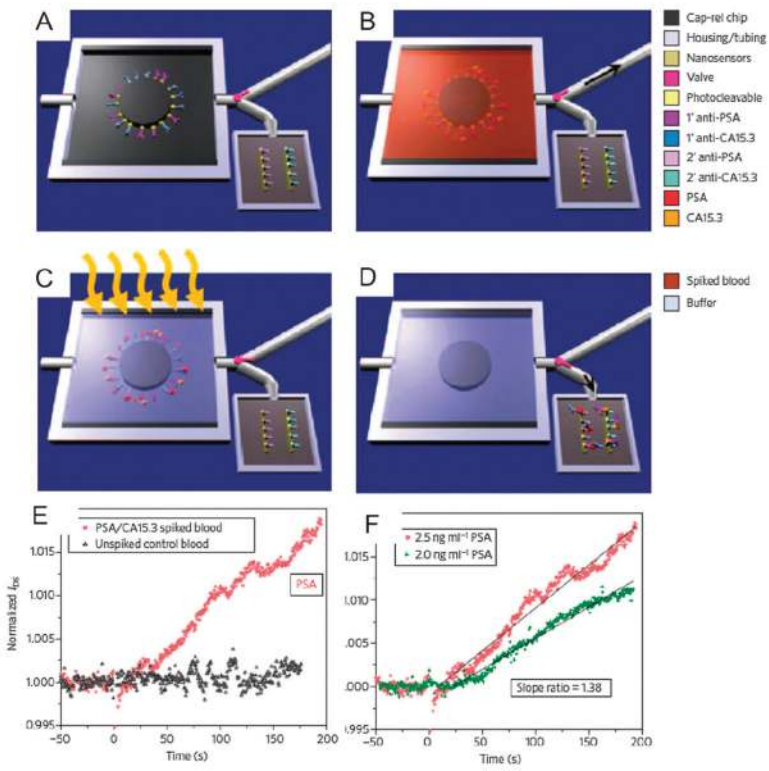


Fig. 7.

Electrical detection using a unique pre-processing method. (A) Primary antibodies to numerous biomarkers are bound to the sensor *via* a photocleavable crosslinker. (B)Whole blood is injected into the chip (black arrow) and biomarkers bind to the device. (C) The probe is washed and then UV irradiation (orange waves) is applied to cleave the linker between the captured biomarker and sensor. (D) Finally, the antibody–antigen complexes are washed out of the sensor for detection. (E) Response to anti-prostate specific antigen (PSA) purified from a blood sample, initially containing 2.5 ng mL^{-1} PSA compared with no protein. (F) Normalized response to different concentrations of PSA. Modified with permission from ref. 53.

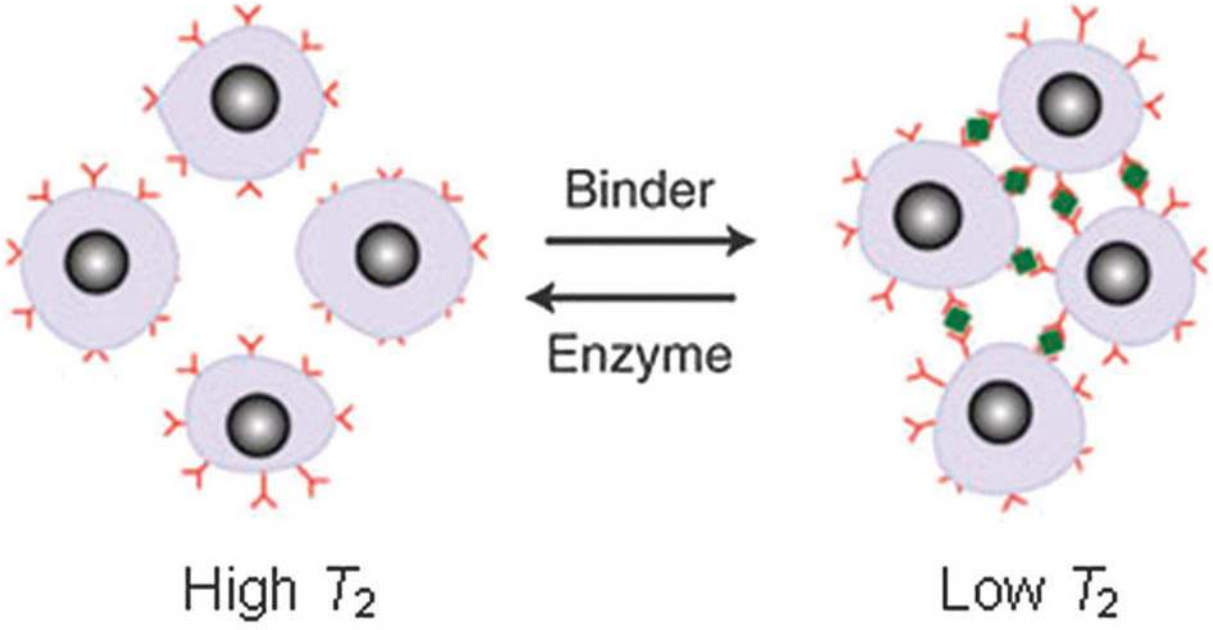


Fig. 8.

Principle of a magnetic relaxation switches assay using magnetic nanosensors. When monodisperse magnetic nanoparticles conjugated with a binder (*i.e.* protein, antibody or complementary oligonucleotide sequences), the spin–spin relaxation time (T_2) of neighboring water protons decreased as the self-assembled clusters become more efficient at dephasing nuclear spins of many surrounding water protons. However, when these nanoclusters are treated with a cleaving agent (*i.e.* enzyme), the nanoparticles become dispersed, switching the T_2 of the solution back to the lower values. These qualities render the developed magnetic nanoparticles as magnetic relaxation switches capable of screening biomarkers by magnetic resonance methods. Modified with permission from ref. 59.

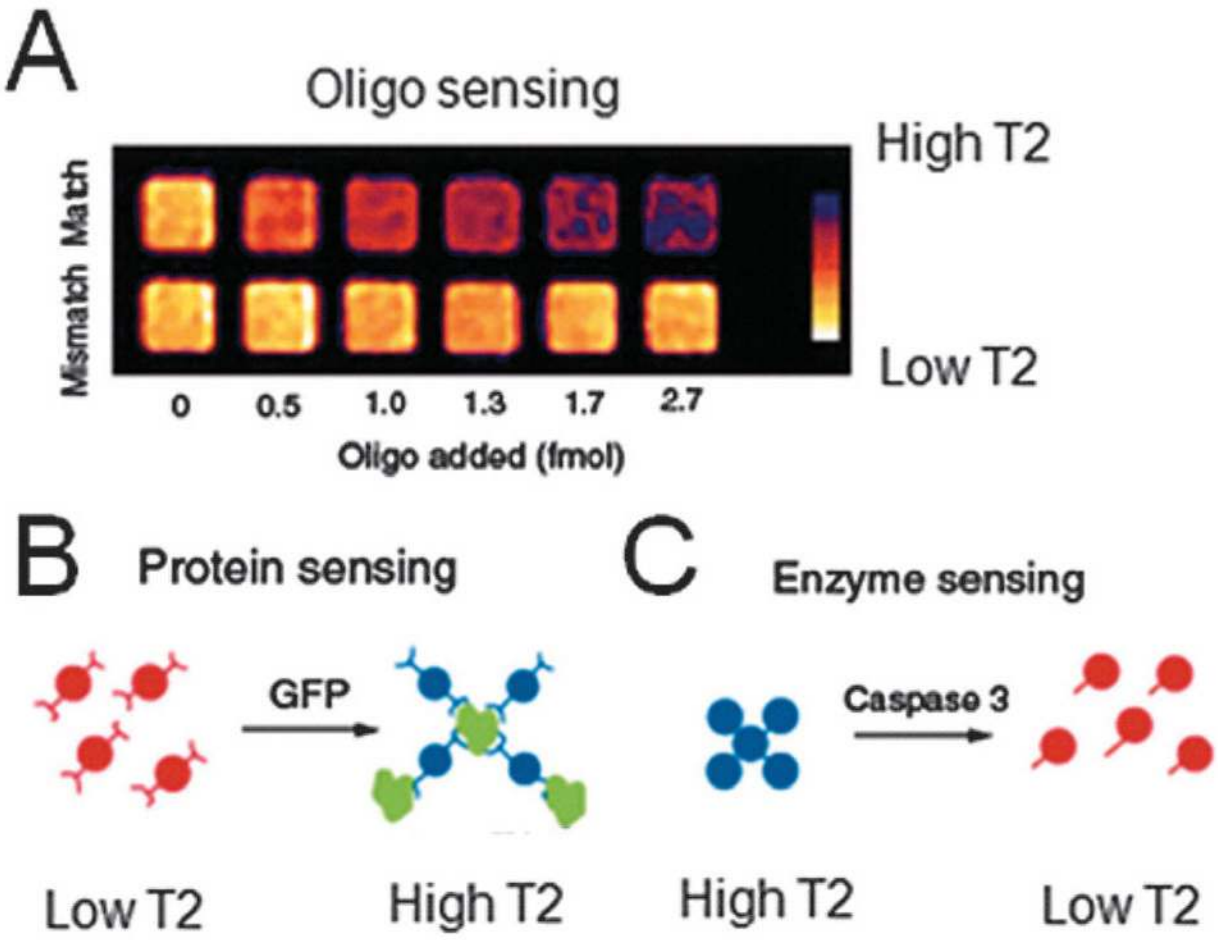


Fig. 9.

Detection of oligonucleotides, proteins, and enzyme activity using magnetic nanosensors. (A) T_2 relaxation time decreased with complementary oligonucleotides when SPIO nanoparticles conjugated with oligonucleotides. (B) T_2 relaxation time decreased with targeted protein-GFP when SPIO nanoparticles conjugated with GFP antibody. (C) T_2 relaxation time increased with the addition of caspase-3 enzyme when SPIO clusters linked with DEVD, a substrate sequence of caspase-3. Modified with permission from ref. 60.

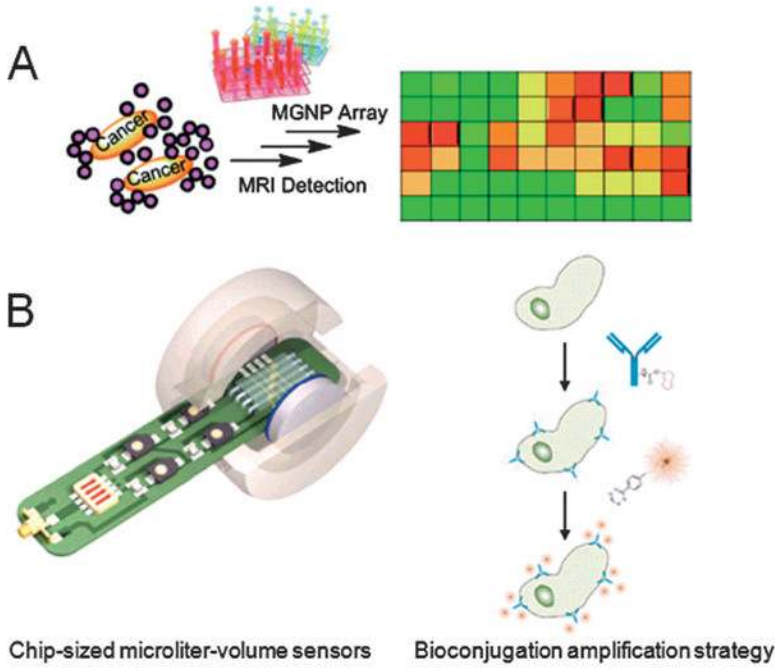


Fig. 10.

Cancer cell detection and profiling using magnetic nanosensors. (A) Magnetic nanosensor bearing carbohydrates was used to profile the carbohydrate-binding characteristics of cancer cells by magnetic resonance imaging. Modified with permission from ref. 64. (B) Cancer biomarkers detection based on chip-sized microlitre-volume sensors and SPIO-targeting strategies. Modified with permission from ref. 65.

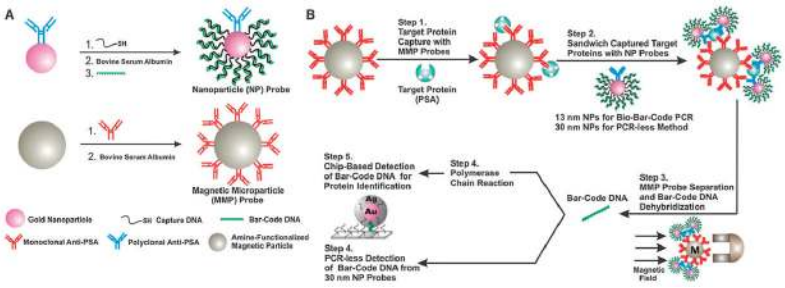
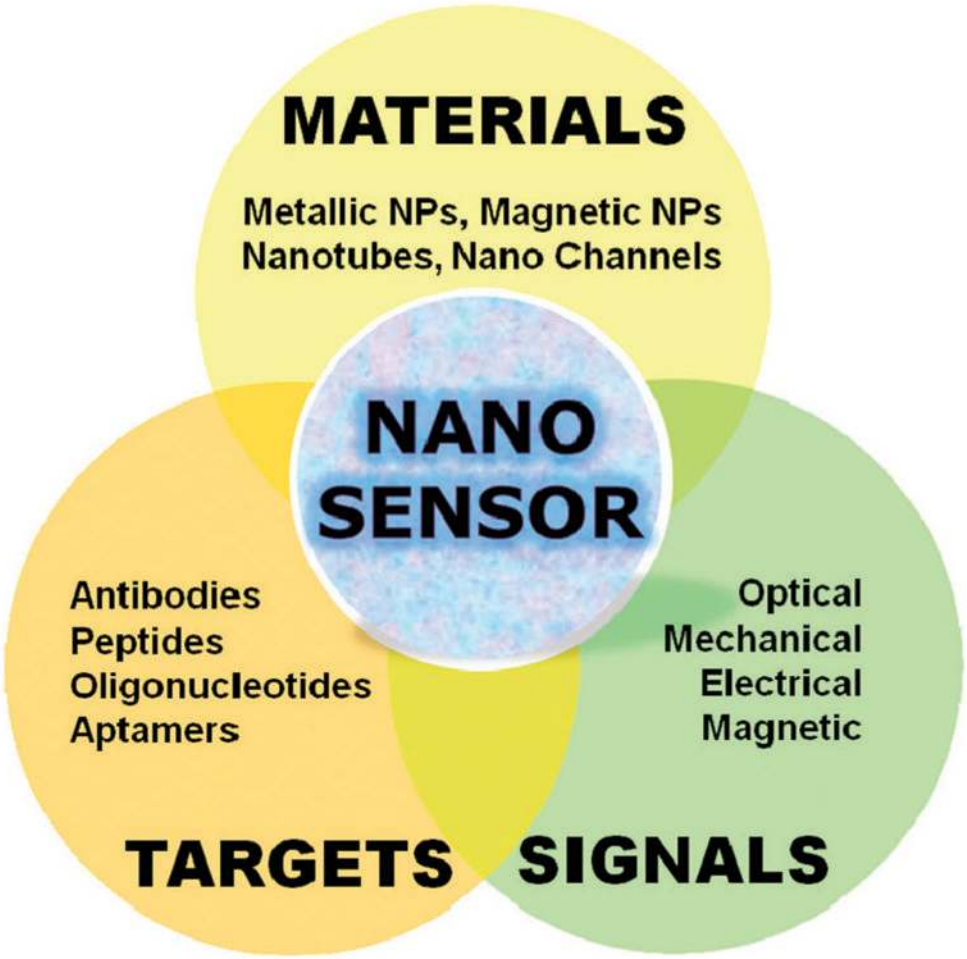


Fig. 11.

The bio-barcode assay technique. (A) The initial probe development of AuNPs. (B) The method of detection for an example protein–prostate specific antigen. Magnetic probes are functionalized with monoclonal antibodies for the protein and mixed with the protein (Step 1). The probes are then separated from the buffer and concentrated on the walls of the tube. The magnetic probes are resuspended in buffer where the secondary probe is introduced. The secondary probe is a AuNP functionalized with polyclonal antibodies and barcode DNA strands. This probe sandwiches the protein target (Step 2). The hybrid particles are separated by magnet again and the barcode DNA is dehybridized (Step 3). The isolated barcode DNA can then be amplified by PCR (Step 4, top) and the probes undergo scanometric DNA detection (Step 5). Modified with permission from ref. 67.



Scheme 1.
Three components necessary for nanosensors.

Table 1

Nanosensor detection limits for biomarkers

Detection method	Nanotechnology	Biomarker detection	Prefix metric	Pros	Cons	Ref.
Optical						
LSPR	Metal NPs, silica NPs enhanced with Au	pM	pico-10^{-12}	Flexible detection	Highly uniform particles	10–17
Colorimetric	AuNPs, AgNPs	nM	nano-10^{-9}	Easy to read signal	High probe concentration	18
Fluorescence	AuNPs-dye, quantum dots	pM		*In vivo* detection	Bleaching or blinking signal	19–23
SERS	AuNPs-dye enhanced with Ag, Au–Ag core–shell nanodumbbells	zM	zepto-10^{-21}	*In vivo* detection	Blinking signal	12, 23–32
Mechanical	Microcantilevers, suspended microchannel resonators	fM	femto-10^{-15}	Low sampling volumes	Sensitivity affected by viscous fluid	5, 33–40
Electrical	Silicon nanowires and nanoribbons, carbon nanotubes. graphene sheets	fM		Fast analysis time	Sensitivity affected by salt concentrations	35, 41–54
Magnetic Resonance	Superparamagnetic iron oxide nanoparticles	zmol		*In vivo* detection	Intricate signal detection system	55–65

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