Colloidal nanoparticles as advanced biological sensors

Philip D. Howes, Rona Chandrawati, Molly M. Stevens*

Department of Materials, Department of Bioengineering, and Institute of Biomedical Engineering, Imperial College London, Exhibition Road, London SW7 2AZ, UK.

*Corresponding author. E-mail: m.stevens@imperial.ac.uk

Abstract

Colloidal nanoparticle biosensors have received intense scientific attention and offer promising applications in both research and medicine. We review the state of the art in nanoparticle development, surface chemistry, and biosensing mechanisms, discussing how a range of technologies are contributing toward commercial and clinical translation. Recent examples of success include the ultrasensitive detection of cancer biomarkers in human serum and in vivo sensing of methyl mercury. We identify five key materials challenges, including the development of robust mass-scale nanoparticle synthesis methods, and five broader challenges, including the use of simulations and bioinformatics-driven experimental approaches for predictive modeling of biosensor performance. The resultant generation of nanoparticle biosensors will form the basis of high-performance analytical assays, effective multiplexed intracellular sensors, and sophisticated in vivo probes.

Evolution has given rise to organisms of staggering complexity. Our now extensive knowledge of biological systems pales in comparison to the remaining mysteries. Unraveling these requires tools that probe the molecular machinery of life and provide detailed feedback on complex networks of subtle interactions. Such tools are cornerstones of biomedical research and practice, and improvements in these lead directly to a better understanding of fundamental biology, monitoring of health, and diagnosis of disease. Colloidal nanoparticle biosensors are a class of biological probe that will not only yield improved biological sensing but also provide a step change in our ability to probe the biomolecular realm. Nanoparticles can act as high-performance sensors because nanomaterials exhibit unique and useful behaviors not present in their bulk form: for example, bright tunable fluorescence from semiconductor nanoparticles and localized surface plasmon resonance (LSPR) phenomena in metallic nanoparticles. These particles exhibit intense responses to incident light (or other stimuli), and the ability to modulate this response by interaction with target analytes makes them excellent biosensor signal transducers. Outputs can be quantitative or qualitative depending on the functionality of

the sensor, with both in vitro and in vivo applications. Nanoparticle biosensors can dynamically interact with and respond to their environment, which can be a considerable advantage compared to passive labeling techniques with nanoparticles, dyes, and stains. Another key advantage is that washing steps are often not required, as they are with passive labeling, making these biosensors suitable for simple, rapid analytical quantification assays, detecting analytes in cells, and sensing and tracking analytes in vivo in real time.

Fluorescent quantum dots (QDs) and plasmonic gold nanoparticles (AuNPs) are commonly used in nanoparticle biosensors. QDs are fluorescent inorganic semiconductor nanoparticles, typically 2 to 10 nm in diameter, that possess high fluorescence brightness and photostability (*1*, *2*). Their broad absorption and sharp emission spectra are tunable by particle size, allowing multiplexing where a mixed population of QDs can be excited with a single excitation source. Energy transfer between QDs and proximal donors or acceptors can be extremely efficient, and they can be functionalized with large numbers and varieties of functional molecules, factors that are vital for QD biosensors (*3*). Plasmonic nanoparticles exhibit unique optical properties due to LSPR (*4*). AuNPs receive particular attention because of their stability and ease of synthesis. Spatial confinement of surface plasmons in plasmonic nanoparticles yields a pronounced extinction peak in the visible spectrum, and intense surface fields polarize the local volume around the nanoparticles. External agents entering this volume (e.g., biomolecules, ions, dyes, other nanoparticles) interact with the field, leading to various effects, including LSPR peak shift, surface-enhanced Raman scattering (SERS), and quenching or enhancement of fluorophores. This yields an optical signal that can be modulated by the presence of an analyte, forming the basis of a biosensor (see Box 1 for typical nanoparticle biosensor architectures).

Analytical techniques such as enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and fluorescence in situ hybridization (FISH) are the workhorses of biomolecular research and diagnostic laboratories. However, they are labor intensive, requiring various combinations of washing, heat cycling, and incubations. Probing the molecular content and function of cells is currently reliant on organic fluorescent dyes; however, these offer poor photostability, broad absorbance and emission, and small Stokes shifts that make prolonged or multiplexed analysis very difficult. For in vivo sensing and imaging, near-infrared fluorescence can yield extremely high resolution real-time imaging and tracing of analytes but is limited by the poor spectral qualities of current dyes. All of these applications will benefit from robust and highly sensitive nanoparticle biosensors. The introduction process could follow two paths: Nanoparticle biosensors could form the basis of competing technologies to replace existing approaches: for example, an ultrasensitive nanoparticle biosensor assay to quantify proteins in physiological samples could replace ELISA; or, they could become advanced components that enhance the performance of existing technologies: for example, directly replacing the fluorescent dyes used in PCR, FISH, cellular fluorescence microscopy, and in vivo sensing.

Nanoparticle biosensors have achieved a high level of development, but there are still hurdles that must be overcome. The main challenges include reproducible synthesis of high-quality nanoparticles and maximizing performance in physiological conditions. Deploying these sensors in unfiltered and unpurified bodily fluids such as blood, urine, or saliva is challenging owing to the effects of interferent molecules, ionic concentrations, and changes

in pH; therefore, great care must be taken to engineer sensors to withstand such conditions. Overcoming these challenges requires careful consideration of nanoparticle cores, surfaces, and biosensing mechanisms.

Nanoparticle cores

Nanoparticles for biosensing must be chemically robust to withstand complex conditions; show minimal perturbation of the probed system (e.g., low or no toxicity); and produce intense but switchable responses to incident light, yielding a strong signal change upon analyte interaction. For fluorescent biosensors, the optical properties of QDs are an excellent basis for signaling (*5*). However, the heavy-metal content of standard-composition QDs (typically CdSe and ZnS) means that toxicity might prohibit wide-scale use, particularly in clinical applications where disposal costs of toxic heavy metals could be prohibitive. Toxicological concerns have driven development of heavy metal–free alternatives with materials like silicon (*6*), carbon (*7*), ternary I-III-VI alloys of Cu, Sn, Zn, Ag, In, and S (*8*), and conjugated polymers (*9*, *10*), and biosensing has been demonstrated with all of these. The challenge is to replace heavy metals while maintaining excellent properties, and as yet none of the alternatives have surpassed Cd-QDs in terms of optical quality and biofunctionalization. However, research activity is intense, and new formulations are constantly emerging. It is likely that some will eventually supersede Cd-QDs and have a substantial impact on the translation of nanoparticle biosensors.

Fluorescent nanoparticles absorb high-energy light and emit at a lower energy. Luminescent upconverting nanoparticles (UCNPs), a recently emerged class of nanoparticle, do the opposite, harvesting low-energy light by multiphoton absorption and upconverting to higher-energy emission. This property is excellent for biosensing, as UCNPs can operate at red–infrared wavelengths, where autofluorescence is avoided and greater penetration in biological matrices occurs (*11*). Current work is striving to improve the luminescence brightness and surface chemistry of these nanoparticles, and although application in biosensing is only a recent advance, it is becoming the next thrust of the field.

Unique phenomena emerge as the shape and size of noble-metal nanoparticles vary. Below ~2 nm, noble-metal nanoparticles (including Au, Ag, and Pt) exhibit fluorescence tunable across the ultraviolet, visible, and near-infrared (NIR) wavelengths (*12*). Their biocompatibility, strong fluorescence, long emissive lifetimes, and excellent photostability make them attractive for nanoparticle biosensing (*13*). A major advantage of noble-metal nanoparticles over QDs is their small size, which is below the 5.5-nm renal clearance limit (*14*). Above 2 nm, noble-metal nanoparticles exhibit LSPR, which gives rise to superquenching, fluorescence enhancement, and LSPR field perturbation phenomena, which are all used for biosensing (*15*). The production of precisely engineered metal alloys or core-shell architectures allows fine tuning of plasmonic properties (*16*, *17*). Common alloying elements include Cu, Pd, and Pt, and new synthesis routes are in active development (*18*). Furthermore, changing the shape of noble-metal nanoparticles shifts the LSPR peak position, allowing tuning for specific applications.

Beyond core composition, the way in which nanoparticles are synthesized also has a tremendous influence on their character. Nanoparticle properties are extremely sensitive to

size, shape, crystal structure and associated defects, dopants, surface morphology and charge, and density of capping ligands. Although this sensitivity allows control and tuning of particle characteristics, it also makes syntheses very difficult to reproduce. Solution-phase synthesis, where precursors react in solutions containing coordinating ligands, yields high-quality nanocrystals with optimal physical and chemical characteristics and allows control over nanoparticle structure and morphology. Reaction parameters such as stirring rate, vessel morphology, and precursor injection position, which are often thought to be of minor importance in traditional synthetic chemistry, are critical in nanoparticle synthesis. Reproducible synthesis is therefore a major hurdle. Analyzing, understanding, and optimizing nanoparticle synthesis procedures is important both in the development of novel nanoparticle formulations and in moving toward robust nanoparticles for commercialization and advanced applications of nanoparticle biosensors. Complete control of reaction parameters and conditions can be achieved with automated robotic systems, such as the WANDA system at the Lawrence Berkeley National Laboratory (Fig. 1A) (*19*). Batch-to-batch variability can be avoided with continuous-flow systems, and incorporation of "in-line" analytical platforms (e.g., absorbance and fluorescence spectroscopy) that feed information back to reaction controllers allows real-time tuning and optimization of products, which is impossible with batch synthesis (*20*). Also noteworthy are microfluidic synthesis platforms, which have reduced reaction volumes with increased uniformity in the chemical and thermal environment and allow precise control over reaction conditions (Fig. 1B) (*20*, *21*). Microfluidic systems will likely have a key role in optimization and discovery of nanoparticle compositions and synthesis procedures. Given the extremely low reaction volumes, rapid throughput, and real-time tuning of product properties in continuous-flow formats, these systems can yield a large amount of information for relatively low inputs of reagents, time, and energy (*22*).

Nanoparticle synthesis methodologies have progressed substantially in the last two decades, but there is still much to learn about the exact nature of nanoparticle formation and growth. Examples include elucidating the importance of nonclassical (aggregation, coalescence) growth mechanisms in which particle-particle interactions play an important role (*23*, *24*) and the effects of capping ligand concentration on growth trajectory (*25*–*27*). Unraveling these mysteries will influence development of sophisticated syntheses that yield reproducible results. A number of techniques have emerged to probe the processes involved in nanoparticle growth (Fig. 2). In situ x-ray irradiation from synchrotron sources can be used to follow various parameters during growth, including particle morphology, tomography, crystalline structure, size distribution, and particle assembly, and allows spectroscopic measurement of chemical and electronic configurations, all in real time (Fig. 2A) (*28*–*31*). Liquid cell transmission electron microscopy (TEM) allows real-time visualization of morphological, structural, and chemical changes of colloids (Fig. 2B) (*23*). A small volume of reaction solution is trapped between two electron-permeable membranes, such as graphene (*32*, *33*), and energy delivered by the beam initiates particle nucleation. Techniques such as electron energy loss spectroscopy (EELS) and energy-dispersive spectroscopy (EDS) allow atomic-resolution imaging and chemical identification with liquid cells. Computer simulation is emerging as an excellent complement to these experimental techniques, allowing modeling and analysis of specific components and processes of nanoparticle nucleation and growth (Fig. 2C) (*34*). For example, density functional theory (DFT) has been used in combination with three-dimensional electron microscopy to study the growth of Ag shells on Au nanoparticles, revealing that growth on the (100)

facets is preferred regardless of the morphology and crystallinity of the Au core (*35*). DFT has also been used to study the effect of alkanethiol capping ligands of different lengths on the shape evolution of AuNPs during colloidal growth, showing that ligand concentration on particular faces drives variations in morphology (*26*). Furthermore, capping ligands themselves can be explicitly modeled with molecular dynamics (MD) simulations: for example, to reveal that cetyltrimethylammonium bromide (CTAB) forms a layer of distorted cylindrical micelles on Au nanorod surfaces and that channels among these micelles allow $AuCl_2^-$ ions direct access to the surface during growth (*27*). These tools are changing our understanding of various processes, including particle nucleation, coalescence, shape control mechanisms, and surfactant effects (*36*).

Nanoparticle surfaces

Nanoparticles require a surface covering to provide a barrier between the core and its environment. This is generally a layer of capping molecules that bind directly to the surface and ideally stops particles aggregating, disperses them in water at a range of pH values, resists nonspecific adsorption of surrounding molecules, and provides a conjugation point for functional biomolecules (Fig. 3). The interface between a nanoparticle core and a biological environment is a key area and must be engineered carefully to optimize nanoparticle biosensor performance (*37*).

The many biomolecules (enzymes, lipids, etc.) and ions in physiological fluids such as blood, urine, and saliva provide a hostile environment for nanoparticles; therefore, capping ligands must provide effective protection. For nanoparticle biosensors, capping layer thickness is key. The biosensing mechanisms that modulate nanoparticle signals in response to analyte interaction are usually governed by distance-dependent interactions across the capping layer, where thinner capping layers result in stronger signal modulation. These interactions include resonance energy transfer (RET), where energy is transferred across space (typically up to 10 nm) by nonradiative dipole-dipole coupling, and LSPR field perturbation, where molecules entering the LSPR field alter the refractive index of the sensing volume surrounding a plasmonic nanoparticle (typically up to 30 nm from the surface). This puts a constraint on the capping layer; it must be both compact and highly protective. Thinner capping layers are generally less protective against particle aggregation, nonspecific adsorption, and surface degradation; hence, there is an inherent conflict between decreasing capping thickness and increasing protection.

The most successful surface-capping approaches to date have made use of custom molecules with modular combinations of low–molecular weight components that convey a range of beneficial properties to the nanoparticle. A notable breakthrough is that of modular zwitterionic ligands that yield nanoparticles with exceptional stability and functionality (*38*–*41*). Through electrostatic interactions and hydrogen bonding, zwitterionic ligands can bind large numbers of water molecules that act to restrict nonspecific interactions with surrounding biomolecules (*42*, *43*). This is important, as nanoparticles show a propensity to adsorb proteins and other biomolecules, creating a “corona” of material that, if too thick or too strongly bound, will block access of analytes to surface-bound biosensing mechanisms. The zwitterionic groups (e.g., sulfobetaines,

carboxybetaines) contain a diversity of charges with net zero charge, which allows stability over extended pH and ionic concentration ranges, an important attribute given the diverse characteristics of different physiological fluids. Ligands can be designed with multiple surface-binding groups (e.g., thiols, which form dative covalent bonds with many metals) to ensure strong and lasting binding: for example, bidentate dihydrolipoic acid (DHLA)–based (*44*) and tridentate tris(mercaptomethyl) ligands (*45*). Short ethylene glycol segments can also be included to control nonspecific adsorption (*46*). Zwitterionic ligands yield exceptionally stable nanoparticles and are the state of the art for compactness and fouling resistance, particularly when combined with novel phase transfer routes like photoligation (*47*).

Polymeric capping layers offer several advantages, including many surface-binding groups per molecule and increased control over functional group number and position, but historically it was difficult to obtain compact layers owing to their relative bulk. However, improved control over polymerization and surface coordination has yielded excellent polymer capping agents (*48*). Examples include hydrophilic polymers grafted with short thiolated alkyl chains (*49*) and reversible addition fragmentation chain transfer (RAFT)–mediated polymerization of mixed-functionality monomers (*50*). Given that thiol oxidation is problematic and seemingly unavoidable, research is starting to avoid their use; thus, inclusion of groups such as imidazoles as coordinating moieties is noteworthy (*51*).

The exact nature of the nanoparticle–environment interactions is yet to be elucidated, and there are several phenomena that have a notable effect upon the functionality of nanoparticle biosensors (*52*). Charged nanoparticles attract large numbers of ions that alter the local environment, creating ionic and pH gradients. These can affect the biosensing mechanisms (e.g., protein–antibody binding or DNA–DNA hybridization) and the conformation of component biomolecules. Nanoparticles are similar in size and shape to many proteins, and their interactions are mediated by the same forces, such as van der Waals interactions, dipolar attractions, electrostatic attraction and repulsion, and hydrogen bonding. They can therefore undergo dynamic interactions with each other that can alter properties of both nanoparticle (e.g., inducing phase transformations, restructuring, and dissolving the nanoparticle surface) and protein (e.g., denaturation, altering enzyme activity). Precise understanding of these emerging phenomena is important to inform the design and development of nanoparticle biosensors, and such studies are currently a key part of nanoscience (*53*).

Biosensing mechanisms

Modulation of nanoparticle signals by interaction with target analytes is governed by the architecture of surface-bound biosensing mechanisms. The key elements are (i) the way constituent biomolecules are conjugated to the nanoparticle, (ii) the signaling modes employed, and (iii) the analyte-receptor mechanism.

Bioconjugation is used for constructing biomolecular sensing mechanisms (Fig. 3B) (*3*). Biomolecular recognition elements are immobilized on particle surfaces, and their interaction with analytes yields a physical or chemical response that modulates the particle-derived signal (Fig. 4, A to G). Traditional covalent approaches often require multiple

washing and purification steps, hydrophobic reaction intermediates, and multiple reagent additions that often destabilize colloidal nanoparticles. Unintended cross-linking between nanoparticles, and between biomolecules, is also a common problem, and such techniques allow little control over the number of conjugated species.

Ideal bioconjugation procedures should be simple, high yield, and nondamaging to nanoparticles and preferably should require no intermediate reagents. The binding of biotinylated biomolecules to streptavidin-coated nanoparticles achieves all of these characteristics; however, the relative bulk of streptavidin (52.8 kD, ~6 nm) severely restricts RET efficiency. Oligohistidines are excellent alternative bioconjugation vectors, as they can bind directly to particle surfaces, sitting between surface ligands. They have been used on both QDs and AuNPs (*54*). A short sequence of histidine amino acids can be included in a recombinant protein (*54*), in a peptide sequence (*55*), or as part of an oligonucleotide construct (*56*), and its high affinity for metal nanoparticle surfaces allows attachment with excellent control over number and orientation of attached species. The many emerging highly site- or sequence-selective conjugation techniques, such as click reactions (*57*, *58*), strain-promoted cycloadditions (*59*), and enzyme-mediated ligations (*60*, *61*), are excellent prospects for nanoparticle bioconjugation, as they offer rapid, efficient, and strong binding. Synthetic biomolecules, such as peptides and DNA oligomers, can easily be functionalized with the required groups. Proteins can have appropriate functional groups inserted into their peptide sequence by the recombinant inclusion of non-natural amino acids. This yields proteins that can be conjugated to nanoparticles with high control over both number and orientation of attached species (*62*). Such precise control over position and selective reactivity of conjugating groups is an important step toward a sophisticated “plug-and-play” approach to nanoparticle bioconjugation (*3*).

Signaling modes are key for nanoparticle biosensors. Fluorescence-based tools are used extensively in biological sciences—for example, in analyte quantification assays and cellular imaging—and they offer high resolution and excellent sensitivity. Sensing mechanisms of fluorescent nanoparticle biosensors generally involve charge or energy transfer to modulate fluorescence intensity, lifetime, or spectral profile. Charge transfer acts by perturbing excited states in the particle core to either enhance or quench emission. RET processes include Förster resonance energy transfer (FRET) and bioluminescence or chemiluminescence (BRET and CRET) and can involve a variety of materials, including organic dyes, lanthanide complexes, fluorescent proteins, graphene, chemi- and bioluminescent enzymes, and inorganic materials like AuNPs, which may function as acceptors or donors and can be arranged in relays (*63*). The use of long-lifetime luminescent nanoparticles and RET agents allows for time-gated detection, which will be an important area of development for commercial nanoparticle biosensors (*64*). Minimizing donor–acceptor separation is paramount, and nanoparticle biosensing components must be carefully chosen and constructed to achieve maximum sensitivity.

Plasmonic nanoparticles act as biosensor signal transducers in three main ways. First, the LSPR peak can shift in response to the action of an analyte, usually matter entering or exiting the LSPR field. Such matter may be analytes binding directly to the particle (*65*), plasmonic nanoparticles aggregating or disaggregating (*66*), or selective deposition of a metallic layer on the nanoparticle surface (*67*). Second, enhancement of Raman scattering

intensity due to the binding of secondary agents to plasmonic nanoparticles allows sensitive detection: for example, by binding analytes directly to nanoparticles (direct intrinsic SERS) (*68*), modulation of SERS due to perturbation of a receptor (indirect intrinsic SERS) (*69*), or selectively adding or removing Raman-active dyes (extrinsic SERS) (*70*). Third, plasmonic nanoparticles can enhance or quench nearby (<10 nm) fluorophores through interactions of their excited electrons with the LSPR field (*71*), which allows for combining advanced plasmonic and fluorescent nanoparticle constructs.

Analyte–receptor interaction events activate the biosensing mechanisms that modulate nanoparticle properties. Examples include the following:

1) Enzyme–substrate: Enzymes are essential in maintaining physiological homeostasis, and aberrant expression or activities act as biomarkers for various diseases. Systems to measure enzyme activity and abundance are vital in both research and clinical practice. Enzyme substrates, such as proteins and peptides, can act as active elements in biosensing mechanisms (*55*). For example, a RET-active agent tethered to a fluorescent nanoparticle by a short protease-selective peptide sequence can be removed by protease cleavage, modulating nanoparticle fluorescence (Fig. 4A) (*72*, *73*). Enzymes can also alter the aggregation state of plasmonic nanoparticles: for example, by protease cleavage of peptide-bound aggregates of AuNPs (*74*), kinase-induced aggregation of peptide/antibody-coated AuNPs (*66*), or transglutaminase-induced covalent cross-linking of peptide-coated AuNPs via the formation of isopeptide bonds (*75*).

2) Antigen–antibody: Antibodies are used in protein biosensors for their high specificity and form the basis of ELISA. The advent of controlled antibody fragmentation and single-domain antibodies is yielding compact immunosandwich complexes that allow for low limits of detection (LOD) in nanoparticle biosensing applications (*76*). Nanoparticle FRET biosensors have been constructed with such fragments (Fig. 4C) (*77*). For plasmonic nanoparticles, proteins binding to antibody-functionalized surfaces can induce a measurable LSPR (*65*), or a secondary antibody can be used to introduce an additional agent for signal amplification (*67*). Furthermore, studying the SERS spectra of receptor or bridging molecules, such as antibodies, upon binding an analyte allows plasmonic nanoparticles to act as effective label-free biosensors (Fig. 4G) (*69*).

3) Nucleic acid interactions: Nucleic acids can perform complex structural and mechanistic roles in engineered systems, and they are excellent for constructing biosensing mechanisms. For nucleic acid targets, molecular beacons are common and have been used in nanoparticle-based configurations (*78*). Nucleic acid displacement can selectively remove donors or acceptors from a double-stranded complex on a nanoparticle surface, which is the basis of nanoflares (Fig. 4E) (*71*). Aptamers are nucleic acid sequences that can selectively bind various analytes, including proteins. They have several advantages over antibodies (e.g., compactness) and make excellent sensing elements (Fig. 4F) (*79*).

4) Redox reactions: The intrinsic redox properties of certain biomolecules allow nanoparticle biosensors to measure pH. This is useful for intracellular biosensing: for example, where pH can modulate many cellular events. An example is dopamine, which can directly react with

O_2 to convert to quinone under basic pH. As quinone is an electron acceptor, it can quench QDs by charge transfer (Fig. 4D) (*80*).

Moving toward applications

Fundamental nonclinical research is likely to be one of the first areas where nanoparticle biosensors will have a substantial impact given the fewer restrictions and regulatory hurdles compared to clinical application. We can envision nanoparticle biosensor-based assays as replacements for many standard research tools. For example, ELISAs allow quantification of proteins in complex media but are slow, with many incubation and washing steps. Instead, nanoparticle biosensors added to a sample could readily output a quantifiable optical signal. This has been demonstrated with QDs, where prostate-specific antigen (PSA) was detected in serum by using antibody fragments, long fluorescence–lifetime lanthanide dyes, and time-gated fluorescence detection (*77*). PCR is robust and reliable for DNA or RNA detection but requires sophisticated equipment and lengthy protocols. Instead, a one-pot nanoparticle biosensor assay could detect and quantify targets in complex mixtures, which has been demonstrated with superquenching AuNPs (*81*). Such assays could be kit-based, and users would generally be well skilled; therefore, these types of application are easy to envisage and would fit in easily alongside other research tools and methodologies.

Understanding of biology, and ultimately disease, diagnosis, and cure, is reliant on understanding what transpires in and around cells. This requires sensitive, reliable, reproducible, and highly stable tools. The application of nanoparticle biosensors as active cellular probes is a highly promising area, and they are beginning to achieve success (Fig. 5A) (*78*, *82*). Getting nanoparticles into intracellular regions of interest is a major challenge. Cellular cytosol, the intracellular fluid in which substructures (organelles) are suspended, is a major target for intracellular sensing. Cells most commonly take up matter by endocytosis, enveloping it in endosomes and not exposing it to the cytosol. To reach the cytosol, nanoparticles must break out of endosomes or find alternative internalization pathways. Standard delivery methods include microinjection and electroporation, but these can affect cell viability. Cell-penetrating peptides (CPPs) offer a nonmechanical “lock and key” type of entry (*83*). Peptides containing His-tag QD-binding motifs and CPP segments have been used to achieve endosomal escape and cytosol entry (*84*), and intracellular detection of Ca^{2+} ions has been demonstrated with QD-CPP biosensing constructs (*85*).

Analytical tools developed for research can translate into in vitro clinical diagnostics, though complications regarding cost, regulation, and rapidity make this very challenging (*86*). A particular area of interest is in nanoparticle biosensors for point-of-care diagnostics—for example, in microfluidic “lab-on-a-chip” devices (*87*) or merging with technologies such as smartphones (Fig. 5B) (*88*) and Google Glass (*89*). For such applications, nanoparticle biosensors must be stable enough to withstand long-term storage and fluctuations in environmental conditions. These are challenges for all reagents but will be particularly so for nanomaterials given their complex chemistry. These applications are a mid-to-long-term goal, and advances in the synthesis and surface engineering of nanoparticles are imperative.

Using sophisticated “turn-on” in vivo biosensors that could signal upon binding specific targets (e.g., cancer cells) would be a far-reaching development, and the high absorptivity

and strong fluorescence of many nanoparticles make them highly suitable. In vivo translation introduces many complexities regarding regulation, short- and long-term toxicity, undesired rapid capture by the immune system, routes of degradation or escape, and environmental impacts of cleared nanoparticles, and nanoparticle biosensors must be carefully designed to circumvent these issues. There are also physical limitations, such as autofluorescence and low tissue penetration by light due to high scattering and absorbance, which make probing biological structures and tissues with light problematic. However, these impediments can be sidestepped with the use of certain materials and techniques. By working in the near-infrared energy range, where scattering and absorbance of tissue are at a minimum, tissue penetration can be maximized. This can be done with UCNPs, which can undergo excitation and emission in the red–infrared bioimaging window (Fig. 5C) (*11*, *90*). Furthermore, by using long-lifetime fluorophores, such as lanthanide dyes and time-gated fluorescence spectroscopy (*91*), it is possible to discard the initial short-lifetime autofluorescence burst and detect only the long-lifetime fluorescence signal that is reporting on the target. Another exciting prospect is in vivo photoacoustic sensing, which combines NIR excitation with ultrasonic detection based on the photoacoustic effect and yields higher spatial resolution and deeper tissue penetration than fluorescence techniques. For example, low–band-gap semiconducting polymer nanospheres (SPNs) with strong NIR absorbance have been used for in vivo sensing of reactive oxygen species (ROS) in live mice. Immobilization of a ROS-responsive cyanine dye derivative on the particle surface, and comparison of the photoacoustic response of the SPN at 700 nm and the dye at 735 nm, yielded a ratiometric response (*10*). Looking toward application of these techniques, clinical in vivo sensing still remains a long-term goal, but solid foundations have been built for this field.

It is useful here to reference a nanomaterial that has had great success in many applications. Spherical nucleic acids (SNAs) are nanoparticles, commonly AuNPs, with a dense DNA “shell” that is simultaneously a highly protective capping layer and an active biosensing construct (*92*). In the original work (*93*), a target DNA sequence induced AuNP aggregation, inducing an LSPR shift. This was developed into a microarray scanometric assay (*94*), which evolved into the Verigene System that can detect analytes for many different diagnostic ends, including clinical microbiology, as well as human genetic and pharmacogenetic testing (*95*). With a LOD of 100 aM for DNA targets, target amplification is not required as it would be for PCR-based methods, and the system has also been adapted to detect proteins (*96*) and microRNAs (miRNAs) (*97*). SNAs also allow intracellular biosensing and can enter cells in considerable numbers without transfection agents (*98*). Hybridizing dye-functionalized nucleic acid strands (nanoflares) to the shell turns the dyes “off” as a result of superquenching by the AuNP core. A toehold region in the probe allows a target nucleic acid to hybridize and remove the flare, whereupon the dye turns “on.” These are effective for measuring intracellular mRNA levels (*71*).

A perspective on the future of nanoparticle biosensors

Technology transfer is a great challenge for all applied research. Commercialization of biosensors in general has been slow, with glucose monitoring and pregnancy testing remaining the only large-scale consumer markets. Since the advent of functional bionanomaterials in the 1990s, there has been much excitement that combining these with

advanced biosensing strategies could revolutionize many research and clinical practices. While the abundance of proof-of-principle studies proves that nanoparticle biosensors can deliver, we still await effective technology transfer. Such transfer is absolutely vital for nanoparticle biosensors to achieve widespread use and impact.

We can identify five key areas on the materials front that will substantially influence the short-term development and translation of nanoparticle biosensors: (i) Nanoparticle cores with minimal toxicity and optimal optical properties are imperative for wide-scale applications. New candidates are constantly emerging and evolving, and the likes of heavy metal–free QD alloys, carbon dots, and metal nanoclusters may supersede Cd-QDs. (ii) Robust synthesis of whole nanoparticle biosensors is required for the manufacture of standardized products. Systems with precise control of all reaction parameters for multistep procedures are required: for example, continuous-flow reactors with built-in analytical and feedback mechanisms, and microfluidic systems for optimization and discovery. (iii) Compact capping agents that yield highly stable and resistant nanoparticles are essential: for example, modular small-molecule or polymer ligands including units such as zwitterionic groups, ethylene glycol segments, and imidazole groups, enhancing colloidal stability, nonspecific adsorption resistance, and surface binding. (iv) Bioconjugation methods are needed for fine control of number and orientation of surface-conjugated biomolecules. Ongoing transfer of ideas from highly selective and efficient protein-labeling chemistries that do not perturb structure or function will have a massive impact: for example, enzyme-mediated ligation and recombinant proteins with non-natural amino acids. (v) Robust RET agents are required that are more effective and stable than organic dyes, such as graphene and AuNPs acting as quenchers, or long-lifetime agents like lanthanides that allow time-gating (*63*).

Beyond materials development work, we identify five areas that we believe will be at the forefront of nanoparticle biosensor development:

1) Computer simulations to aid development and predict performance: Simulation will become a vital tool for understanding phenomena and optimizing performance in all areas of nanoparticle biosensor development (e.g., structure–property, surface–ligand–environment, and target–probe relationships). Simulation currently provides validation of experiment, but the increasing sophistication of algorithms and modern supercomputers will give these techniques predictive capabilities and great potential for success within the next decade. An example application is analyzing the structure of the metal-sulfur interface of thiolate-protected particles, which is being studied using DFT modeling in combination with scanning tunneling microscopy, low-energy electron diffraction, and surface-sensitive x-ray spectroscopic techniques (*99*). Such studies have revealed, for example, the importance of the complex RS–Au–SR in forming continuous “polymeric” structural features on the surface of AuNPs. Another area of importance will be elucidating the complex phenomena that occur at the nano-bio interface (*100*): for example, understanding the specific and nonspecific interactions between proteins and ligand-capped nanoparticles, where MD simulations have already revealed how amphiphilic residues (e.g., arginine, tyrosine, tryptophan) play a key role in protein-particle interactions for mixed-monolayer–protected metal nanoparticles (*101*).

2) Bioinformatics approaches for predictive modeling: Identification of structure–activity relationships (SARs) helps predict biological activity from structure. Considering the complexity of biological environments, the vast array of engineered nanomaterials, and the emergent phenomena at the nano–bio interface, approaches that can handle vast amounts of experimental data are needed to create SARs outputs for nanoparticle-biomolecule constructs. Bioinformatics approaches will prove vital for this purpose, and recent work has demonstrated the power of turning systematic combinatorial experimental data into quantitative models with predictive power (*102*). Here, the interaction of 105 types of unique serum protein–coated AuNPs with human lung epithelial carcinoma cells was characterized and used to create a multivariate model that uses the protein corona fingerprint to accurately predict cell association (nanoparticle internalization and cell membrane adhesion). Such models will direct nanoparticle biosensor design to maximize specific and minimize nonspecific interactions to optimize performance in physiological fluids, and we expect to see work in this area increase rapidly.

3) Merging with developments in molecular diagnostics: There is intense research to identify panomic (genomic, proteomic, etc.) molecular markers of disease for screening, diagnosis, prognosis, and staging. The trend is toward identifying biomarker "panels" from large-cohort data sets to increase sensitivity and specificity versus single biomarkers. For example, a recent study revealed a panel of 10 lipids from peripheral blood that predicted, with >90% accuracy, phenoconversion to amnestic mild cognitive impairment or Alzheimer's disease within 2 to 3 years (*103*). To use such panels for clinical diagnostics, or even rapid point-of-care testing, technologies that can simultaneously detect multiple analytes with ultralow LOD are vital. Ultrasensitive nanoparticle biosensor assays using the multiplexing capabilities of QDs (*88*), or fluorescent barcode nanoparticles for high-density multiplexing (*104*), are ideal for such application, and merging these fields will yield high-impact technologies and clinical approaches in the medium-to-long term. Furthermore, discovery of new types of biomarkers can feed back into biosensor development with the integration of the newly discovered target-analyte interactions into colloidal particle systems.

4) Integration of nanoparticle biosensors into assays and devices: The question of how nanoparticle biosensors fit into larger systems that go from raw sample to result is of paramount importance and the focus of much current research activity. An example is combining nanoparticle biosensors with advanced target amplification protocols for the detection of nucleic acids, which can further decrease LOD. For example, a QD biosensor has been incorporated into an exponential amplification reaction to achieve a 0.1-aM LOD for miRNA (*105*). The U.S. Food and Drug Administration–cleared Alere i system (*106*), which provides detection and differentiation of influenza A and B in 15 min from a nasal swab, has demonstrated that isothermal amplification and fluorescence-based detection are viable in point-of-care technologies. We anticipate that integrating sophisticated nanoparticle biosensors into such systems will yield extremely high-performance technologies. Furthermore, given that nucleic acid targets can be easily amplified whereas other targets cannot (e.g., proteins, metal ions), it is likely that nanoparticle biosensors will have a more rapid impact in genomics than in other fields.

5) Application as intracellular sensors: We anticipate that this will be the first area of high-impact application of nanoparticle biosensors. We expect to see some high-profile examples

appearing in the next few years, followed by extensive usage and ground-breaking work when nanoparticle biosensors break through as commercially available tools. A possible breakthrough application could be in probing cell-signaling networks, which are of critical importance in cancer. The healthy cell to cancer cell transformation involves many changes in behavior—for example, in proliferation, motility and survival—which are underpinned by complex alterations in cellular signaling. Understanding such pathways is important for both fundamental understanding of cancer progression and identifying specific drug targets. Conducting these studies with traditional techniques is laborious and slow. Instead, multiplexed “turn on” nanoparticle biosensors could allow extended monitoring of signaling events in live cells. Multiple branches or points in a signal cascade could be monitored simultaneously, with highly specific reporting on events such as phosphorylation or dimerization, to follow the flow of information and elucidate the nature of the cascade. Furthermore, such sensors could form the basis of next-generation theranostics, providing site-specific treatment with feedback mechanisms: for example, monitoring drug effects in real time and informing adjustment of dose and rate. The opportunities in this area represent probably the greatest potential for successful application of nanoparticle biosensors in biomedicine.

Nanoparticle biosensor research stands at a critical juncture, with a vast amount of excellent research that forms a solid foundation for future work. The field must push on to take its technologies from being lab curiosities to achieving large-scale application and impact, and we are now very well equipped with the tools necessary to do this.

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