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Nanotechnology and Microfluidics for Biosensing and Biophysical Property Assessment: Implications for Next-Generation *in Vitro* Diagnostics

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3.1 Introduction

Medical tests provide important clinical information on patients and serve as the first step toward successful clinical diagnosis and treatments. Generally, medical tests start with a routine physical examination (known as checkup), which measures patient temperature, blood pressure, pulse, etc., to gather basic knowledge of the patients' physical status [1]. Besides these preliminary tests, *in vivo* diagnostics and *in vitro* diagnostics (IVDs) are the two more sophisticated types of tests performed to obtain more specific patient information.

In vivo diagnostics are the tests performed on live bodies. These tests mainly include radiological imaging [2], such as X-ray radiography, magnetic resonance imaging (MRI), positron emission tomography (PET), computed tomography (CT), etc., and tests that are performed by administering a diagnostic agent and measuring the body response, such as penicillin skin test, gluten challenge test, etc. These tests have been the primary tests for the diagnosis of many diseases such as tumor and allergy. However, because these tests act directly on the body, some may be invasive and have side effects. For example, X-ray radiography generates ionized radiation. When performed at high dosages during pregnancy, it comes with the risk of miscarriage and birth defects [3]. The associated risks must be adequately assessed before prescribing these tests.

In contrast, IVDs are the tests performed on bodily samples, such as blood, saliva, urine, tissue biopsy, etc., taken outside the body. Glucose test, which is normally performed by extracting a blood sample and analyzing the blood glucose concentration, is one of the most commonly seen IVD tests in daily life. Compared to *in vivo* diagnostics, these tests do not directly contact the body and thus have much lower associated risks. As such, IVD has a much wider range of tests with more flexibility on the instrumentation techniques. It has been estimated

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that the overall global diagnostics market in 2016 worth US$40–45 billion with point-of-care diagnostics contributing US$12–13 billion [4]. The annual growth rate of the total IVD market was expected to be 5% over the next a few years [4].

Given the importance of IVD in healthcare, there has been steady growth in the IVD industry and consistent technological innovations for emerging IVD tests with improved performances. There innovations have been focused on the following a few aspects [5].

The first innovation needed is to improve the limit and sensitivity of the detection. Many of the currently available IVD tools are limited by their limit and sensitivity of detection. For example, the current gold standard of immunoassay, enzyme-linked immunosorbent assay (ELISA), has a detection limit of 0.1 ng/ml for prostate cancer biomarker detection, which is higher than the concentrations in most patient serum samples especially at early stage of prostate cancer [6, 7]. It is therefore important to develop new sensing techniques with improved detection performance in this aspect and expand the diagnostic capability.

The second innovation needed is to improve the test speed and throughput. In many clinical settings, patients' physical status changes quickly and timely assessments are critical for informed decision-making. For example, in perioperative care, quick test results on blood coagulation can guide physicians on types and dosages of anticoagulating plan, greatly lowering the risk of over-bleeding as well as the need of blood transfusion [8].

The third innovation needed is to miniaturize the tests and develop point-of-care tests. There is a need of decentralizing medical tests from central laboratories. By developing point-of-care tests through test miniaturization, many tests can be performed at bedsides and even at homes. Consequently, the efficiency of healthcare would be significantly improved at lowered costs. With improved test efficiency and affordability, testing tools with multiplexed test capability would also be possible.

The demand for IVDs with these improved features has been driving the academia and industry to develop fast, accurate, and affordable tests with high throughput. To this end, emerging micro/nanotechnology offers great potential in achieving these goals.

3.1.1 Nanotechnology and Microfluidics

Compared to bulk phase, materials with nanometer scale exhibit unique physical and chemical properties; some of these properties are extremely desirable for highly sensitive IVD tests. First, nanomaterials have a large surface-to-volume ratio, termed specific surface area. The large specific surface area enables versatile surface functionalization, which enhances the rate of biochemical reaction on the surface as well as the sensitivity of detection. Second, leveraging the recent development in nanomaterial synthesis, the size and shape of nanomaterials can be finely tuned, providing an opportunity to precisely design the size and shape with optimal detection performance. Lastly, nanomaterials offer special electrical and optical properties. Such properties enabled development of qualitatively different technologies such as quantum dots and nanoelectronics that are not possible to be implemented in bulk phase.

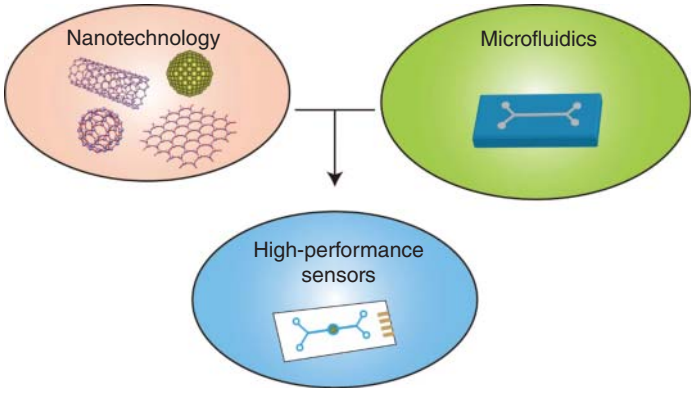

Figure 3.1 The scope of this chapter: leveraging the advances in nanotechnology and microfluidics to develop emerging sensors with high performance.

Microfluidics is the technology of manipulating fluids at micron scale. Fluids at micron scale exhibits unique flow patterns. First, Reynolds number, which is a dimensionless number indicating the ratio between inertial force to viscous forces, is typically smaller than 1 (Ref. [9]). It suggests that the flow is normally strictly dominated by laminar flow [10], which is much more predictable than turbulent flows. Strictly laminar flow in microfluidics allows better control over the fluid flows. Second, the emerging techniques to control flow developed in microfluidics, such as microvalves and micropumps [11], laid the foundation for high integration and high automation. Thus, it is possible to integrate sample preparation and tests into a single microfluidic chip [12]. Last but not the least, tests based on microfluidics consume only a small volume of samples, typically in picoliters [13]. Tiny sample consumption is extremely desirable for repeated tests as well as tests where samples are very precious.

Nanotechnology enables biosensing techniques with enhanced performance, whereas microfluidics offers automated sample preparation and exquisite fluid handling. Taken together, these two emerging technologies could potentially lead to next-generation IVDs with unprecedented performance.

This chapter aims to review the emerging techniques on biosensors that were based on nanotechnology and microfluidics (Figure 3.1). Instead of delving into details of each technology, we aim to provide an overview of a broad range of techniques, covering the underlying mechanisms and discussing representative works. Nevertheless, readers are encouraged to read the reviews referenced in the discussion of each technique for a more comprehensive understanding.

We first present the basics of nanotechnology and microfluidics, including properties and synthesis techniques. We then discuss the reported works on biomolecule sensing based on sensing readouts including optical readout, electrical readout, and other readouts. After that, we discuss the techniques on biophysical property sensing, based on the targets measured including cell contractility, cell deformability, cell electrophysical property, and bodily fluid rheology. We conclude this chapter with a discussion on the outlook and perspectives of nanotechnology-embedded microfluidic systems on IVDs.

3.2 Fundamentals of Nanotechnology and Microfluidics

3.2.1 Nanotechnology

Nanotechnology can be broadly defined as the techniques of manipulating matters on nanometer scale, typically with at least one dimension within 100 nm range. Although the fields of science of nanotechnology can be very broad, including surface science, organic chemistry, molecular biology, etc., majority of the studies have been focused on nanomaterials.

Materials at micrometer scale mostly show similar physical properties as the bulk phase. However, nanomaterials, with dimensions much closer to atoms, exhibit drastically different physical properties [14]. Such unique physical properties essentially come from the nanometer dimension of nanomaterials and the large specific surface areas that small dimension directly brings about. First, large specific surface areas allow more flexibility in surface functionalization and modification [15]. Functionalized nanomaterials play critical roles in biochemical reactions such as immunoassays. Modifiable surfaces also allow the formation of stable suspensions such as colloids. In addition, large specific surface areas allow efficient catalysis, rendering some nanomaterials, such as gold nanoparticles (AuNPs), an effective catalysts. Second, nanomaterials, with their nanometer dimensions, possess the opportunity to confine their electrons and produce quantum effects. Electron confinement leads to unique band structure, generating unique optical and electrical properties that could not be easily observed with bulk materials. For example, AuNPs can generate surface plasmon with much higher intensity, allowing for more sensitive molecule detection based on localized surface plasmon resonance (LSPR) and surface-enhanced Raman spectroscopy (SERS). Special electron arrangement also leads to new semiconducting materials such as carbon nanotube and molybdenum disulfide. Last, as these unique properties largely depend on the size and shape of the nanomaterials, by tuning the size and shape, nanomaterials with the most desirable properties could be obtained.

Based on the number of dimensions in their geometries, nanomaterials can be grouped into zero-dimensional, one-dimensional, two-dimensional, and three-dimensional nanomaterials. Zero-dimensional nanomaterials have all the dimensions measured within nanometer scale. Thus, from a macroscopic view, the materials are essentially dots with zero degree of freedom. Examples are nanoparticles, such as nanorods, nanospheres, etc. Similarly, one-dimensional nanomaterials, such as nanotubes and nanowires, have two dimensions within nanometer scale, exhibiting one degree of freedom. Two-dimensional nanomaterials are single layer of atoms with two degrees of freedom, with the most important representations being graphene and the emerging transition metal dichalcogenide (TMDC) monolayers. Three-dimensional nanomaterials are essentially bulk materials with features on nanometer scale, including nanocomposites, nanostructured surfaces, etc.

From material perspective, nanomaterials made of metal and semiconductor have been intensively studied. Gold and silver are of special interests as metal nanomaterials, given their high conductivity and biocompatibility. Carbon and silicon are examples of semiconductor nanomaterials because of their superior

properties and easy access [16]. Recently, two-dimensional inorganic compounds based on transition metals, such as TMDC monolayer and MXenes [17], have gained great attention. With their unique transport, optical, and surface properties, these materials have found great applications in transistors, optoelectronics, and energy storage.

The synthesis of nanomaterials or nanostructures can take two different approaches: bottom-up approach and top-down approach. Bottom-up approach is relatively more commonly used in nanomaterial synthesis, although such synthesis methods have existed for decades in conventional chemical industry, instead of being exclusively adopted for nanomaterials. Bottom-up synthesis takes an atom-by-atom, molecule-by-molecule, and cluster-by-cluster manner. Examples include chemical vapor deposition, laser ablation, and solution precipitation [18].

Top-down approach, or nanofabrication, uses larger initial structures and subsequently form the desired nanostructure from there. Nanofabrication, sometimes interchangeably termed by nanolithography, was derived from the microfabrication process in the semiconductor industry. The basic concept is to pattern substrates using light and predesigned photomasks. Following the steps of deposition and etching, nanostructures can be formed on the substrate. Conventional photolithography used ultraviolet light with wavelength ranging from 100 to 400 nm. Because of diffraction effects, it was very difficult to fabricate nanostructures smaller than 50 nm. As such, lithography techniques based on electron beam, X-ray, and focused ion beam were developed, which advanced the fabrication limit down to 10 nm. A major challenge that nanofabrication faces is the fabrication throughput and cost. To address it, many studies have been reported to pave the way toward nanomanufacturing, including nanoimprint [19], nanocontact printing [20], and roll-to-roll fabrication [21].

3.2.2 Microfluidics

Microfluidics provide a technique to precisely control fluid flows. As described above, at micrometer scale, viscous force and surface tension dominate inertial force and gravity; thus, fluids remain in the laminar flow regime without developing into turbulent. As a result, the flow field can be readily predicted and reproducibly controlled.

Fabrication of microfluidic devices with well-defined geometry and surface properties is important for its functions. Two types of fabrication techniques are most commonly adopted. Glass capillary microfluidic devices have been widely used for droplet generation. Briefly, two or more glass capillaries, each serving as either injection tube, transition tube, or collection tube, are coaxially aligned and assembled to form microfluidic channels [22, 23]. The injection tube could be pulled to form micronozzles with desirable diameter using micropipette puller machine. Monodisperse droplets can be generated at the nozzle where continuous phase and disperse phase meet. By adding additional collection tube, higher-order emulsion can be generated through sequential emulsification. Glass capillary microfluidic devices do not require complicated fabrication equipment and have played a significant role in the development of droplet microfluidics, particularly for material applications. However, such devices have limited design

flexibility. To achieve more complicated flow control, polydimethylsiloxane (PDMS) microfluidics represents an alternative for more sophisticated flow controls.

The fabrication techniques of PDMS microfluidic devices were also derived from microfabrication in the semiconductor field. Briefly, photolithography generates patterns on a silicon wafer, commonly by either patterning photoresist or directly etching silicon substrate, and the generated patterns are subsequently transferred to PDMS through replica molding, termed soft lithography. Chemically bonding a PDMS slab to a flat substrate, such as a glass slide, generates the final microfluidic device. Desired microfluidic channels can be achieved by custom-designing the photomask for the photolithography. Although the generated patterns are generally two dimensional, multiple photolithography steps can lead to structure in the vertical axis. In addition, microfluidic devices with multiple layers of patterned PDMS slab can be fabricated and form microvalves, with one layer serving as the flow layer and other layers serving as control layers [24]. Such design flexibility is extremely useful to design integrated microfluidic devices as biosensors with high degree of automation. Therefore, microfluidic systems, with nanotechnology-embedded biosensing units, could potentially lead to next-generation biosensors with high sensitivity and high throughput.

3.3 Biomolecule Sensing

Most disease diagnosis requires the detection of molecular signatures. For example, in the diagnosis of cancers, the overexpression of certain biomarkers is tested. As another example, liver function is tested by examining the concentration of a few enzymes including alanine transaminase, aspartate aminotransferase, and alkaline phosphatase [25]. Measuring the concentration of biomolecules within bodily fluids in a fast and accurate manner is the key to developing successful IVD tools.

Biosensing typically involves two major components: biorecognition and signal transduction. In biorecognition, the analytes are recognized by specific molecular interactions, commonly through one of the three strategies. A strategy is based on specific binding between proteins, such as antibody/antigen binding in nature. Such protein interactions have high binding affinity and high specificity, which could serve as reliable analyte recognition scheme. With the advance of protein engineering, synthetic proteins with optimized binding specificity and affinity have been achieved. Another strategy is based on biochemical reactions, often facilitated by enzymes. By supplementing particular chemical reagents, the existence of specific analytes induces specific chemical reaction, generating colorimetric or electrical readouts, which are transduced and acquired. A third strategy involves the complementary base pairing of nucleic acids. Hybridization probes are designed to detect the target sequences within a body fluid.

Signal transduction converts the biorecognition into detectable signals, most commonly with an optical readout or an electrical readout. Optical readouts can be further divided into readouts with monochromatic light, such as fluorescence,

and full-spectrum light, such as scattering spectrum. In this section, we discuss the emerging biosensing techniques based on the nature of the readout, including optical readout and electrical readout.

3.3.1 Techniques Based on Optical Readout

3.3.1.1 Localized Surface Plasmon Resonance

When a metallic–dielectric surface is subject to electromagnetic wave, the electron cloud oscillates along with the frequency of the electromagnetic wave. At a certain frequency, the oscillation reaches resonance and the electron cloud oscillates at the largest amplitude, creating plasmons at the interface. The excitation, or surface plasmon polariton, including the electron motion and associated electromagnetic field, propagates in a direction parallel to the interface and is confined on the boundary. Thus, the oscillations are very sensitive to changes on the interface, such as adsorption of molecules to the metallic surface. This phenomenon forms the fundamental principle of surface plasmon resonance (SPR) detection of biomolecules [25].

Replacing bulk metal with metal nanoparticles can prevent the surface plasmon polariton from propagating and localize the plasmon to the nanoparticles, forming localized surface plasmon (Figure 3.2a). Because of the intrinsic size limit of LSPR, the excitation decays rapidly away from the surface into the background, giving LSPR very high spatial resolution. Compared to SPR, nanoparticle-enabled LSPR has several advantages. First, the optical hardware has been significantly simplified because no prism is needed to couple the light and the angle of the incident light does not need to be finely adjusted. Second, the sensor chip can be manufactured at a much lower cost. Third, the sensitivity is improved.

The absorption bands of AuNPs and silver nanoparticles (AgNPs) are generally within the visible light region, making AuNPs and AgNPs the most commonly used nanomaterials for LSPR. However, it has been reported that the surface stability and the biosafety of AgNPs can be problematic [32, 33]. Thus, LSPR studies have been mostly focused on using AuNPs as the substrates. The binding of analytes on the nanoparticles changes the surface of the nanoparticles, leading to a shift in the adsorption spectrum. Detecting the LSPR peak shift serves as an efficient way to measure the analyte concentration.

Sensors based on LSPR have been developed to detect different types of analytes. For example, a work reported the detection of streptavidin using biotin-conjugated gold nanorods by detecting the peak shift based on LSPR [34]. Streptavidin detection was also achieved in a AgNP-based LSPR system [35].

LSPR-based immunoassays have also been reported. For example, researchers fabricated a LSPR-based microfluidic chip for the detection of cancer markers, namely human alpha-feto-protein and prostate-specific antigen [36]. In another work, a microfluidic chip was developed to detect serum cytokines, including IL-2, IL-4, and TNF-α, using gold nanorods as the sensing unit (Figure 3.2a) [37, 38]. To improve the sensing speed, AC electroosmosis was incorporated into the microfluidic channel, which enabled efficient mixing and faster cytokine detection [27].

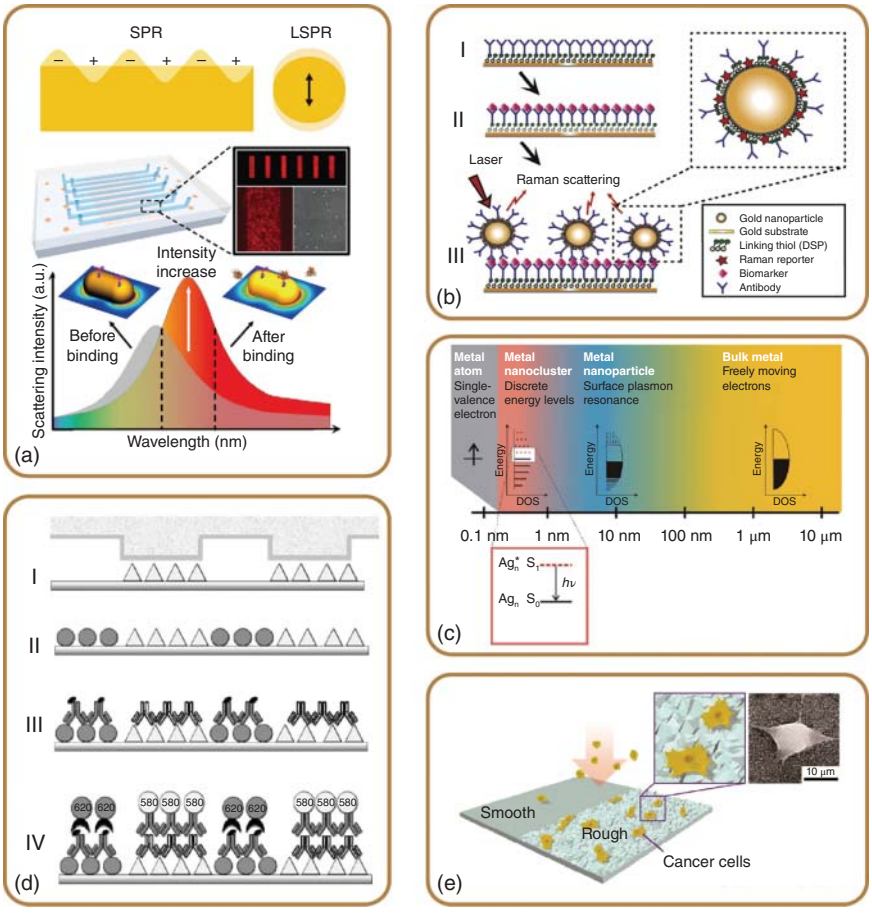

Figure 3.2 Biomolecule sensing techniques based on optical readouts. (a) Schematics showing the mechanism of surface plasmon resonance and localized surface plasmon resonance and a representative work on LSPR for biosensing. Adapted from Chen et al. 2015 [37] and Mayer et al. 2011 [43]. (b) A schematic showing a study using surface-enhanced Raman spectroscopy for immunoassay. Adapted from Banaei et al. 2017 [47]. (c) A schematic showing the mechanism of metal nanocluster-based fluorescence. Source: Adapted from Díez et al. 2011 [49]. (d) A schematic showing a representative study using quantum dots in immunoassays. Adapted from Li et al, 2013 [58]. (e) A schematic showing the capturing of circulating tumor cells using nanoroughened surfaces. Source: Adapted from Chen et al, 2012 [26].

LSPR-based sensors for the assessment of other substances have also been reported, including gas [39], pH [40], ammonia [41], and copper ions [42]. Focused reviews on LSPR can be found in a few review papers [43, 44].

3.3.1.2 Surface-Enhanced Raman Spectroscopy

Raman spectroscopy is another standard technique to identify molecules in chemistry. When monochromatic light illuminates on a sample, both elastic scattering and inelastic scattering take place. Elastic scattering, or Rayleigh

scattering, has the same energy level and thus the same wavelength as the incident light. In contrast, inelastic scattering, or Raman scattering, has different wavelength and is largely sample dependent, which serves as a signature of the molecule. Despite the unique chemical and structural information it provides, Raman signals are inherently weak, especially when visible light is used for excitation. To obtain good signal-to-noise ratio, Raman spectroscopy needs to be carefully designed and aligned.

SERS improves the sensitivity by employing nanoparticles in the detection. Similar to LSPR, nanoparticles form surface plasmon upon light excitation, which magnifies the intensity of incident light. In addition, the magnitude of Raman cross section, which is required for Raman scattering, is also greatly enhanced when molecules are placed on the surface of nanoparticles. As a result, Raman scattering is enhanced and Raman signal can be improved by factors up to 10^{14} (Ref. [28]).

Electrostatic models explain that silver and gold are the ideal materials for SERS because they achieve plasmon resonance with excitation lights in the visible and near-infrared range [45]. Indeed, most of the existing works on SERS have been based on AgNPs or AuNPs. In addition, AuNPs are a more preferred candidate for biomedical applications because of their long-term stability and good biocompatibility. Consequently, many SERS sensor designs based on AuNPs have been reported. For example, a reported work demonstrated the implementation of AgNP-based SERS with single rhodamine 6G as reporters [29]. The intrinsic Raman enhancement factors were shown to be on the order of 10^{14}. In another work, researchers synthesized silica nanotubes with embedded solid nanomagnets and AgNPs for SERS and analyzed the membrane composition of mammalian cells [46]. To move one step closer to clinical applications, SERS-based immunoassay using AuNPs was developed, which demonstrated the detection of a panel of pancreatic biomarkers, suggesting the great potentials of AuNP-based SERS for label-free cancer diagnostics (Figure 3.2b) [47]. Comprehensive details on SERS can be found in a review paper [48].

3.3.1.3 Nanoengineered Fluorescence Probes

Fluorescence has been widely used for biological imaging techniques, such as epifluorescent microscopy and laser confocal fluorescence microscopy. The basic principle is that when a fluorescent molecule, or a fluorophore, is illuminated by light at a certain wavelength, the electrons are excited. Upon relaxation, a photon, normally at a longer wavelength than the excitation photon, is emitted. This technique has served as a labeling method for imaging. The commonly used fluorophores are organic molecules such as fluorescent proteins and cyanine. However, many of these fluorophores suffer from suboptimal photostability and relatively high background fluorescence (autofluorescence). Emerging nanomaterials have been adopted to develop stable and brighter fluorescent assays, such as metal nanocluster, nanoparticle surface energy transfer (NSET), and quantum dots.

Nanomaterials exhibit distinct optical and electrical properties than the bulk phase. As the size of metal particles becomes as small as 1 nm, fluorescence can be observed [49]. This is because around 1 nm or less, the nanocluster consists of

only a few atoms and the band structure becomes discontinuous, resembling the energy levels of fluorescent molecules, as illustrated in Figure 3.2c. By changing the size and chemical compositions of the nanoparticles, their band structure, and thus their optical properties, can be tuned. Given the mature techniques for surface functionalization of metal nanoparticles, fluorescent metal nanoclusters serve as a powerful reporter for biomolecule recognitions; surface functionalization also ensures good stability of the resultant nanoparticles by adjusting the charge, hydrophobicity, functional groups, etc. [50] It is worth noting that without stabilization, metal nanoclusters would aggregate with each other and form larger clusters. As such, there have been intensive studies on the scaffolds as a nanocluster stabilizer [49].

Biosensors based on gold nanoclusters (AuNCs) have been developed to detect the concentration of various analytes. For example, researchers developed a sensor to assess glucose concentration by detecting H_2O_2-induced luminescence quenching of 11-mercaptoundecanoic acid-bound (11-MUA) AuNCs [51]. In the presence of H_2O_2, 11-MUA was oxidized and AuNCs exhibited reduced luminescence. Similar results were observed in a study using bovine serum albumin stabilized AuNCs [52]. Detections of dopamine [53], blood urea [54], and protein kinase [55] were also reported.

NSET is a fluorescence detection mechanism that is similar to Forster resonance energy transfer (FRET). FRET describes the energy transfer between two photosensitive molecules through nonradiative dipole–dipole coupling. The first molecule serves as the donor chromophore and transfers energy to the acceptor chromophore upon excitation, resulting in photoemission from the acceptor chromophore at a wavelength that is distinctive from that of the excitation light. FRET provides a powerful tool in biological fields. However, the FRET efficiency is highly dependent on the distance between the two chromophores, typically requiring the distance to be within 10 nm. It fails to monitor biochemical interactions with a distance greater than 10 nm.

Instead of dipole–dipole coupling, NSET is based on dipole–surface energy transfer, with chromophore serving as the dipole and metal nanoparticle, commonly AuNPs, serving as the surface. Because of the continuous electron–hole pair excitation on AuNP surfaces, which enhances the possibility of the dipole–surface interactions, NSET has higher energy transfer efficiency and can occur with a distance up to 25 nm.

The application of NSET has been demonstrated in the detection of various analytes. For example, a sensing strategy based on PEGylated pyrene-AuNPs dyads was developed to detect biothiols. PEGylated pyrene was noncovalently adsorbed onto AuNPs with quenched fluorescence due to NSET. In the presence of thiols, the energy transfer was interfered and the fluorescence of pyrene was restored. This mechanism served as a way to sensitively detect thiols [30]. Detection of glucose using NSET was also reported [56, 57].

Quantum dots are similar as nanoparticle clusters in some way. However, instead of using metal, quantum dots are semiconductor particles of several nanometers in size. Nanometer-scaled size gives quantum dots different optical and electronic properties when compared to the bulk phase. When a quantum dot is illuminated by light, the electrons are excited from the valence band to the conduction band, forming an electron–hole pair. When the excited electron and

hole recombine, energy is released and emitted as fluorescent light. As the band structure depends on the quantum dot size, both the excitation and fluorescence emission can be tuned by adjusting the size and the semiconductor materials, providing flexibility in the fluorescence design.

Quantum dots have been intensively studied in nanotechnology with a myriad of applications, including transistor, solar cells, diode lasers, quantum computing, and medical imaging. Given their superior fluorescent properties, quantum dots have also been applied in different types of biosensors.

Quantum dots have also been used as labels in immunoassays. For example, researchers used quantum dots as fluorophores and conjugated them to secondary antibodies, as shown in Figure 3.2d. Using the same procedure as immunostaining, they demonstrated the multiplex detection of 3-phenoxybenzoic acid and atrazine-mercapturate [58]. Quantum dots have also been used as donors for FRET/NSET. A detailed review on quantum-dot-based FRET can be found in this review [59].

3.3.1.4 Nanotopography-Based Cell Capturing

Nanotechnology allows the fabrication of nanostructures on the surface using nanofabrication techniques such as photolithography and nanoimprint. Nanopatterned surfaces have shown applications in various biomedical studies such as guided cell migration and elongation [60]. In terms of biosensing, nanotopography has shown interesting applications in cell capturing.

Anchor-dependent cells require a substrate to adhere to maintain viability and routine functions. In cell culture, a plastic dish, sometimes treated with plasma to modify the surface wettability, is used as the adhesion substrate. For cell isolation, a biomolecule-functionalized substrate is often used to bind certain cell membrane protein based on binding affinity. For example, extracellular matrixes (ECMs), such as collagen and fibrinogen, were patterned on a coverslip using microcontact printing to spatially confine cells within certain areas. In addition to biochemical modification of the substrate, it has been observed that nanotopography of the surface also affects cell adhesion [61]. Although the molecular mechanism for topological sensing by adherent cells is largely undetermined, integrin-mediated focal adhesion plays an important role. The interaction of integrin with nanotopological features remodulates the integrin distribution and alters the affinity of the cell adhesion to the substrate [31].

Using reactive-ion etching (RIE), surfaces with tunable nanoroughness were fabricated on a glass substrate [26]. Researchers showed that cancer cells had a higher preference of adhering to nanoroughened surfaces. They further demonstrated the successful capturing of circulating tumor cells (CTCs) from blood samples of cancer patients using nanoroughed surfaces (Figure 3.2e). Nanotopography was also shown to play a role in the self-renewal of human pluripotent stem cells [62].

3.3.2 Techniques Based on Electrical Readouts

3.3.2.1 Electrochemical Reactions

Biochemical reactions generally involve bond break/formation as well as electron transfer. Many proteins, especially enzymes, contain a metal ion cofactor

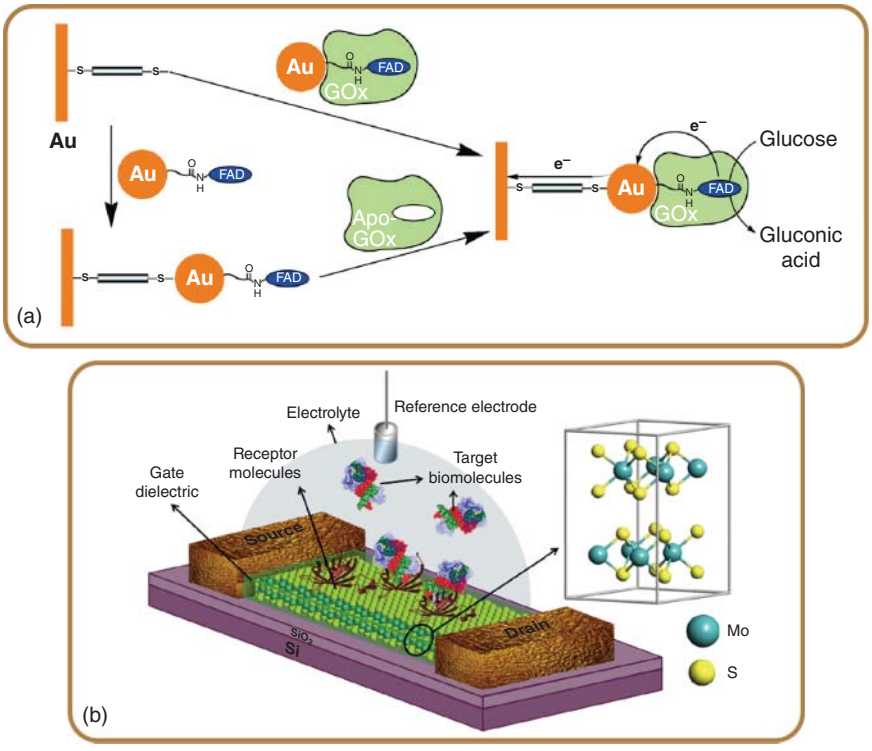

Figure 3.3 Biomolecule sensing techniques based on electrical readouts. (a) A schematic showing a work using gold nanoparticles as conductive wire in enzymatic reaction. Source: Adapted from Xiao et al. 2003 [63]. (b) A schematic showing the mechanism of nanotransistor-based biomolecule Source: Adapted from Sarkar et al. 2014 [64].

and the rate of enzymatic reaction largely depends on the electron transfer efficiency through these metal ions. However, the electron transfer can be hindered because of the surrounding globular protein structure. Therefore, facilitating the electron transfer can potentially accelerate the enzymatic reaction and build fast biosensing mechanisms.

Because of their superior conductivity and biocompatibility, AuNPs have been widely used as electron wires in such assays to facilitate the electron transfer between electrode and reaction substrates and accelerate the reaction rate. For example, researchers incorporated AuNP into apo-glucose oxidase in the reconstitution [63], as shown in Figure 3.3a. The existence of AuNP greatly facilitated the electron transport during reaction and served as a bioelectrocatalyst, increasing the turnover rate by about sevenfolds.

3.3.2.2 Nanotransistor-Based Assays

Field-effect transistors (FETs) have a few properties that are extremely desirable for label-free biosensing applications. FETs offer rapid electrical detection, which enables quick real-time biosensing. FETs have a submicron dimension with high sensitivity, and it thus allows single-cell detection and even single-molecule

detection. In addition, because the fabrication can adopt the highly mature semiconductor manufacturing technology, it can potentially lead to low cost, highly integrated, and highly portable biosensors.

In conventional FET, drain and source electrodes are built to connect the semiconductor material channel. In addition, gate electrode, which is built on top of a dielectric layer covering the channel, is used to electrostatically modulate the current flowing through the channel. To build a biosensor, the gate electrode is replaced with a receptor that specifically binds biomolecules of interest. The charged biomolecules introduce a gating effect, which can be detected by the drain-to-source current (Figure 3.3b).

Carbon nanotubes and silicon nanowires have been tested as the materials to build FET-based biosensors [65, 66]. Given their one-dimensional nature, the fabricated FET had efficient electrostatics and high sensitivity. However, fabrication process was challenging and costly. Two-dimensional materials also provide superior electrostatics, yet are easy to be integrated into device fabrication. As such, two-dimensional semiconductor materials such as TMDC have been adopted as the FET materials. For example, in a work, researchers built an FET using molybdenum disulfide (MoS_2) and demonstrated its application for pH sensing and protein sensing [64]. In another work, MoS_2 transistor biosensors were fabricated to detect interleukin-1β in serum and saliva [67]. Detection of cancer biomarkers, namely prostate-specific antigens, was also achieved using MoS_2 transistor biosensors [68, 69].

3.4 Biophysical Property Sensing

In addition to molecular signatures, biophysical properties of bodily fluids are another important biological aspect that can provide valuable information for IVDs. On the cellular level, transcription factors activate or mute some genes, giving cells of different lineages different gene expressions and thus different functional proteins. Among the expressed proteins, some are involved in cell mechanics, including focal adhesion, cytoskeleton, and other mechanosensitive proteins on the mechanotransduction signaling pathway. As a result, different cells exhibit distinctive mechanical properties, including size, shape, contractility, and deformability [70]. By accurately measuring these cellular properties, one can potentially assess the status of some diseases.

Indeed, biophysical properties have been assessed in clinics to gather information for the diagnosis of some diseases. For example, in hematology characterization, the populations of red blood cells and platelets, along with the subpopulations of white blood cells, are determined based on the cell size distribution. In addition, in blood coagulation, an important test named thromboelastography is used to assess the dynamic viscoelasticity of blood during clotting, providing information on the functionality of blood clotting [71].

The emergence of nanotechnology and microfluidics offers powerful tools for more precise biophysical probing on the cellular level. In the following sections, we discuss recent advances in this field.

3.4.1 Cell Contractility Measurement

Muscle cells, including skeletal, cardiac, and smooth muscle cells, contracts to assume their basic duties such as pumping and weight lifting. Additionally, blood platelets contract during blood clotting to achieve the mechanical integrity of blood clots. Nonmuscle cells also contract and apply contractile force to the substrate to migrate and change the shape. Some intracellular activities, such as intracellular transport and genome replication, also require contractile stress [72]. Disease states are sometimes associated with abnormal cell contractility. Therefore, cell contractility is a biophysical state that can provide important physiological information for diagnosis.

Using nanotechnology and microfluidics, a few methods have been developed to measure cell contractility on single-cell level, including micropost array detector (mPAD), traction force microscopy (TFM), and atomic force microscopy (AFM) [73–75]. Despite that these methods have the complicated fabrication, data acquisition, and data analysis process, the underlying principles are quite simple: displacement is proportional to the exerted force within the measured range of the force. By acquiring the displacement of the substrate that the cells are in contact, force can be deduced.

mPAD is a technique developed to assay single-cell contractility (Figure 3.4a) [76, 80]. These micropillars typically had diameters of about 1 μm and spacings of 1 μm, and the tips were functionalized with ECM proteins for cell adhesion and fluorophores for imaging. While the cells were cultured on the mPAD, the cells resided on the tip and applied contractile force, deforming the micropillars and displacing the tips. By recording and analyzing the fluorescent images of mPAD from the top, a force map showing the cell contractility could be generated.

mPAD was applied in various mechanobiology studies, including neural differentiation of pluripotent stem cell [81] and osteogenesis of mesenchymal stem cell [82]. The contractility measurement helped unveil the underlying mechanotransduction pathway involved in the lineage specification of these cells. Micropost array technique was also applied to test the contractility of platelets, which could be an indicator of blood coagulation functionality. Researchers showed that by functionalizing the micropost tips with fibrinogen, platelet aggregates adhered to the tips and the contractile force were successfully captured by the platform [83].

Because mPAD fabrication is based on soft lithography, it can be integrated into microfluidic devices. For example, the shear-induced platelet activation was investigated by embedding mPAD in microfluidic channels [84]. Flow-mediated mechanotransduction of endothelial cells were studied using the same strategy [85].

TFM is another cell contractility assay technique [86]. Soft substrate, normally made of hydrogel with Young's modulus lower than 100 kPa, was embedded with fluorescent marker beads. As cells were cultured on the substrate, the substrate deformed in response to cellular contraction. The fluorescence images of the marker beads were recorded and analyzed to generate force field. Compared to mPAD, the fabrication process of TFM is more straightforward and can be easily adopted by different laboratories. A detailed summary on the fundamental studies based on TFM can be found in this review [86].

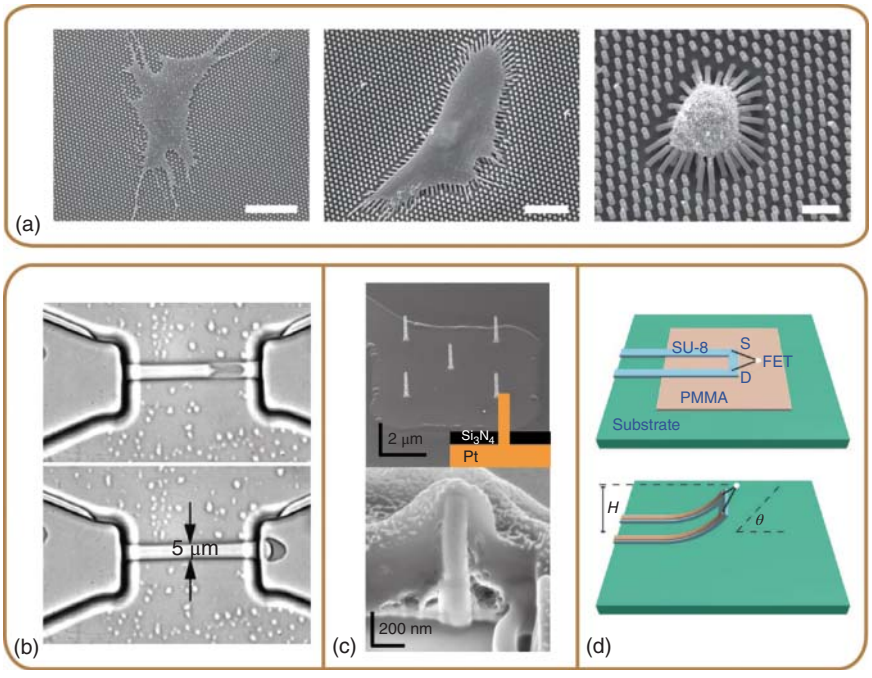

Figure 3.4 Biophysical property sensing techniques. (a) Scanning electron microscopy images showing cells contracting on micropost arrays. Scale bars are 50, 30, and 10 μm. Source: Adapted from Fu et al. 2010 [76]. (b) Bright-field images showing red blood cells going through constricted channels for deformability measurement. Source: Adapted from Zheng et al. 2012 [101]. (c) Scanning electron microscopy images of metal nanoelectrodes and cell membrane contact. Adapted from Xie et al, 2012 [112]. (d) A schematic showing three-dimensional transistor for cell electrophysical measurement. Source: Adapted from Tian et al, 2010 [113].

AFM is a scanning probe technique. AFM is composed of a microcantilever with a mechanically sharp probe contacting with the surface of substrates. The microcantilever equipped with a probe, which has a nanofabricated nanosized tip, is the core part of AFM. When the probe tip approaches the sample surface, the force from the sample surface can cause offset bending of the tip following Hooke's law [87]. Then, the force variations would be detected by a sensor and the detected signals could be interpreted into information of the surface morphology and surface roughness. This technique has been applied to measure the contractility of endothelial cells [88] and platelets [89].

In contrast to single-cell measurement, tissue-level measurement provides a relatively more global assessment of the contractility and thus can be more readily adopted for clinical diagnosis. Using nanotechnology and microfluidics, a few techniques have been developed for the measurement of tissue contractility.

A tissue gauge was fabricated using microfabrication techniques [90]. They used soft materials and generated microwells with cantilever beams suspended inside. Cardiomyocytes were cultured in the microwell, and upon maturation, cardiomyocytes contracted, pulling the cantilever beams. The displacement of the cantilever beams was recorded for the calculation of contraction forces.

This platform was further applied in the study of the validation of antifibrosis drugs [91].

Using carbon nanotube network as a piezoresistive strain sensor, a device was developed to measure the contractile force generated by blood clot during clotting [92]. Carbon nanotube network exhibited good piezoresistivity with high gauge factor (~70) and the strain sensor provided good sensitivity [93]. This device successfully captured the dynamics of blood clotting, showing the potential as a point-of-care blood coagulation testing tool.

3.4.2 Cell Deformability

The molecular composition makes up a cell and thus directly determines the mechanical properties of the cell. In particular, the structure of the cytoskeleton, such as actin filaments and microtubules, is modulated when the cell undergoes physiological processes, such as endothelial–mesenchymal transition that requires morphological change. Therefore, by examining the deformability, we can potentially probe the functional status of the cells. Cell deformability is a cellular-level signature and can serve as an important biomarker [94].

Detection of CTCs in patients with metastatic cancer showed good application of cell deformability. CTCs are cells that shed from primary tumor site, invade into surround tissues, and intravasate into the bloodstream. They are the precursors of the secondary tumors at distal organs. CTC capturing from blood samples provides a mean to noninvasive metastasis evaluation. In addition, the genetic signatures of CTC contain important clinical information for personalized cancer treatment considering the heterogeneity of cancers [95].

For a long time, CTC capturing was based on the expression of surface antigens, particularly epithelial cell adhesion molecule (EpCAM) [96–98]. Numerous works based on microfluidics with EpCAM-functionalized capturing sites have been reported. However, because of the variability in cell surface markers and heterogeneity of cancers, some CTCs may have lost expression of EpCAM, leading to failed capturing of some subpopulations of CTCs. To this end, biophysical property sensing had been investigated to develop label-free and highly efficient separation of CTCs from blood. For example, researchers designed microsieves in microfluidic channels and successfully demonstrated CTC capturing based on size and deformability. In another study, researchers developed a resettable cell trap with adjustable apertures and demonstrated successful deformability-based CTC separation [77].

Appropriate cell deformability is also critical for the function of red blood cells. When red blood cells go through constricted vessels, poorly deformable red blood cells result in microcirculatory occlusion and potentially tissue ischemia [99]. Additionally, red blood cells with poor deformability can lead to high clearance by the spleen and contribute to respiratory distress and systemic sepsis. Assessing the deformability of red blood cells has great clinical applications.

Researchers developed microfluidic channels that adhered red blood cells on the channel bottom, maintained a constant oxygen level, and applied shear stress on the red blood cells. The deformability of the red blood cells was then analyzed based on the deformation induced by the shear stress [100]. Another study

adopted the concept of Coulter counter, as shown in Figure 3.4b: a constricted channel that connected two chambers was designed to squeeze red blood cells while simultaneously measuring the electrical resistance across the constricted channel [101]. When a red blood cell travelled through the channel, it occupied a portion of the channel cross section, thus altering the electrical resistance. This resistance alteration was dependent on the deformation of the red blood cells and served as a mean to deformability measurement. The same device also showed the application of testing the deformability of lymphocytes [102].

3.4.3 Fluid Rheology

The rheological property of bodily fluids depends on the fluid contents and thus can provide useful information for diagnosis. In addition, the rheological property affects some physiological functions. For example, the viscosity of blood affects blood pressure as well as overall circulation. As a result, many diseases, such as obesity and diabetes, are correlated with elevated blood viscosity. In the context of blood coagulation, the viscoelasticity of the blood is often measured over the course of blood clotting to assess clotting functionality. Although benchtops have been developed for this purpose, nanotechnology and microfluidics offered means of device miniaturization and developing point-of-care testing tools.

A study integrated a magnetoelastic transducer within the microfluidic channel to monitor the viscoelasticity of blood during clotting [103]. Another work fabricated microelectromechanical system (MEMS) cantilever beams and captured blood viscoelasticity change during clotting based on resonance oscillation [104]. In another study, an optical method, namely laser speckle rheology, was implemented to measure blood viscoelasticity in a microfluidic chamber [105]. Miniaturized tools based on acoustic resonator [106], ultrasound radiation [107], and dielectric sensor [108] were also reported.

3.4.4 Electrophysiology

The electrical property of cells is another important aspect of cell biophysics. Ions are transported in and out of cells constantly, altering the electrical potential on the cell membrane. In particular, neurons use action potential to relay signals. Additionally, the heart generates electrical signals that activate cardiac tissues to synchronize the pumping activity. Therefore, monitoring the electrophysiological characteristics of cells has important clinical implications.

Nanotechnology can fabricate small electrodes that allow high-resolution recording of the electrical signals from single cells [109]. For extracellular recording, nanotransistors have been applied based on a similar mechanism for FET-based biomolecule sensing, for example, nanowire-based transistor arrays for sensitive detection and stimulation of neurons [110] and cardiomyocytes [111]. Nanotube-based transistors were also shown to be able to record neuronal signals [78]. Good coupling on the transistor/cell interface and well-defined orientation of cells over the nanoscale devices were very critical for reliable signal measurement. To address these issues, researchers developed a flexible

scheme by first culturing cells on thin and optically transparent PDMS sheets and then bringing cells into contact with the transistor arrays [79]. As a result, the signal-to-noise ratio has been significantly reduced.

Unlike extracellular recording, intracellular recording requires the electrode to penetrate through the cell membrane, which is more technically challenging. A study fabricated Pt nanopillar arrays serving as electrodes and cultured cardiac muscle cells on them (Figure 3.4c). By nanoelectroporation on the cell membrane, it was possible to switch between extracellular and intracellular recording [112]. FET has also been applied in the intracellular recording. A 3D-FET device with kinked nanowire probes was fabricated to allow penetration through cell membranes and thus offered intracellular recording (Figure 3.4d) [113].

3.5 Concluding Remarks

Accurate, fast, reliable, and affordable IVD tools have tremendous values in efficient disease treatment. Currently, many IVD tests are performed with benchtops in central laboratories with limited portability and suboptimal sensitivity and efficiency. Nanotechnology, with the ultrahigh-specific surface areas about and versatile surface functionalization methods it brings, offers a way to develop highly sensitive tests for biomolecular and biophysical property sensing. Microfluidics, on the other hand, offers exquisite flow control with high degree of automation, enabling sample preparation and handling with minimal human intervention. Combined together, these two emerging technologies could lead to next-generation IVD tests that are suitable for highly sensitive point-of-care testing.

Although many exciting works on this research junction have been reported, it is worth noting that very few of the academic progress have been capitalized into products. A few factors contributed to this predicament. First, even though the sensors were miniaturized and the sample consumption was significantly reduced, many tests required complicated supporting equipment for data acquisition. For example, spectroscopy-based techniques generally required dedicated optical apparatus, making the analyzers (as opposed to sensor chips) difficult to implement. Second, the reproducibility and reliability could be a major issue. To reach the requirement of quality control in industry, many techniques need to go through a prolonged troubleshooting stage before production. This long period of time could be fatal for start-ups with limited financial support. Third, the manufacturability could be a deal breaker in technology commercialization. Most techniques developed in academic laboratories involved manual steps, especially in the assembly process, making it very difficult for high-volume production. To develop high-performance sensors with better applicability, these aspects must be considered.

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