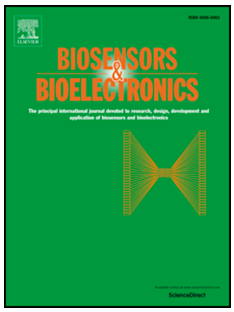

Single-molecule biosensors: Recent advances and applications

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Single-molecule biosensors: Recent advances and applications

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Abstract

Single-molecule biosensors serve the purpose of unmet need for real time detecting of individual biological molecules in the molecular crowd with high specificity and accuracy, uncovering unique properties of individual molecules which are hidden in ensemble methods under physiological conditions. Measuring a signal out of an individual molecule or their interactions with biological partners is not only crucial for early diagnosis of various diseases such as cancer and to follow medical treatments but also offers a great potential for point-of-care devices and personalized medicine in the future. This review summarizes and discusses recent advances in nanosensors for both in vitro and in vivo detection of biological molecules with the single-molecule sensitivity. In the first part, we focus on label-free platforms, including electrochemical, plasmonic, SERS-based and spectroelectrochemical biosensors. We review

fluorescent single-molecule biosensors in the second part, highlighting nanoparticle-amplified assays, digital platforms and the utilization of CRISPR technology. We finally discuss recent advances in the emerging nanosensor technology of important biological species as well as future perspectives of these sensors.

Keywords: single-molecule; biosensor; biomarker; label-free; electrochemical biosensor; optical biosensor; digital assay; nanoparticle; CRISPR

1. Introduction

An increasing number of intriguing studies shed light on the importance of monitoring individual events in the heterogeneous samples since the achievement of single-molecule limit in 1989 by Moerner and Kador in condensed matter [1]. Instead of absorption, for the first time, Orrit and Bernard detected a fluorescence signal from a single pentacene molecule in 1990 [2]. Since then, fluorescence became a main tool in single-molecule detection whilst opening new doors in many fields by simply removing the need for ensemble averaging. With the development of fluorescent probes and super resolution microscopy in 1990s, it has attracted many scientist in order to answer long-standing questions in complex biological systems [3], heterogeneous catalysis [4], biomolecular interactions [5], enzymatic systems [6] and conformational changes in real time [7]. The development of super-resolved fluorescence microscopy which surpasses the diffraction limit to achieve “super-resolution” led to wide-applications in many scientific fields including but not limited to biophysics, quantum optics and physical chemistry,

which was recognized by the Nobel Prize in Chemistry in 2014. Several remarkable applications of single-molecule microscopy realized in the biosensor field resulted in great collaborations between the academy and industry. Several companies started up to demonstrate the applications of single-molecule biosensors composed of newly developed nano(bio)materials or their biohybrids not only in fluorescent assays but also in several label-free biosensor platforms.

Measuring the concentration of a molecule can be vital for clinical samples (Figure 1). For instance, while protein biomarkers are found in low-picomolar concentrations in clinical samples, cancer biomarkers can be as low as low-attomolar concentrations, although this may not be stable in response to infection, or the type of tissue. Thus, developing interventions for acute conditions, such as infectious diseases and immune mediated disorders, is hampered in part by the tools available for sensitive biomolecule monitoring. Detection of single-proteins or cells in complex mixtures, for instance, can enable diagnosis or mapping of a disease at a very early stage. Measuring a signal from an individual molecule can enable creation of superior biosensors that can specifically recognize and quantify a biomolecule or characterize its properties in real time within the ensemble. For instance, single-molecule detection of biomolecular interactions, can demonstrate kinetic distributions in an heterogeneous sample or distinguish the specific from non-specific interactions which is essentially not possible with traditional ensemble techniques such as surface plasmon resonance (SPR), optical waveguide grating (OWG), biolayer interferometry (BLI) or enzyme-linked immunosorbent assay (ELISA). Besides their high sensitivity, specificity and selectivity, single-molecule biosensors consume less sample, display shortened analysis times and open doors to multiplexing with high-throughput assays in clinical environments and drug discovery. Most importantly, single

molecule biosensors may allow to track individual events in living cells or quantify biologically rare species in human body that can help to diagnose many diseases in early stage.

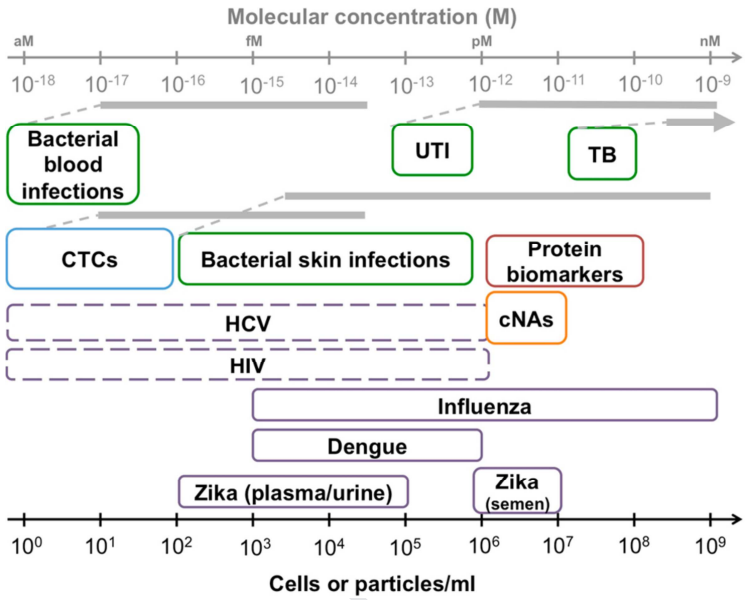

Figure 1. Concentrations of biomolecular analytes in clinical samples. Applications of bacterial detection are outlined in green, viral targets are outlined in purple (with a dotted line for indications where quantitative monitoring is required), and cancer biomarkers are shown in blue (circulating tumor cells), red (protein biomarkers), and orange (circulating nucleic acids). Reprinted with permission from [8]. Copyright 2017, American Chemical Society.

There are three critical aspects in order to produce a single-biosensor which requires to be at the high detection sensitivity, selectivity and preferably to be tightly localized. The detection sensitivity for instance can be increased by tagging biomolecules with a fluorescent probe, or integration with inorganic materials including (metallic) nanoparticles, graphene and carbon nanotubes. Hence, integration of different techniques such as electrochemistry [9], electrokinetic trapping [10], microfluidic platforms [11-12] and fluorescent methods [13], detection of one molecule

at a time gained a deserved attention in biosensor technology as all these methods may allow to monitor disease progression in heterogeneous samples, enabling personalized medicine and point-of-care devices [13]. It is clear that from a purely technological point of view, single-molecule biosensors may be even pivotal because of their potential applicability in medicine and drug discovery [14] as a consequence of their ability to reach superior detection limits as compared to traditional techniques.

In this review, we discuss these recent developments in single-molecule detection of biologically important species. In the first part, we will describe and highlight recently developed label-free assays based on electrochemical, field-effect transistor, plasmonic and surface-enhanced raman spectroscopy-based biosensing platforms. In the second part we will focus on fluorescent assays including DNA-based single-molecule biosensors, liposome and bead-based amplification strategies, quantification of exosomes and exosomal biomarkers, amplification strategies for nucleic acid detection, CRISPR-Cas platforms and single-molecule digital assays. We finally discuss the recent advances in the emerging sensor technology for in vivo monitoring of important biological species as well as future perspectives of these single-molecule biosensors.

2. Label-free assays

2.1 Electrochemical single-molecule biosensors

An electrochemical (EC) biosensor is an analytical device that can recognize a specific biological molecule on an electrode and transform the biological information into a measurable output signal by electronic transduction. It requires an electron flow that is mostly relies on an

enzymatic reaction, which can produce or consumes electrons on an electrode surface. As the EC sensors are the first generated sensors in the history [15], an excellent review on electrochemical biosensors can be found elsewhere [16]. Here we focus recently developed single-molecule electrochemical (SM-EC) assays that have the ability or potential to achieve highly sensitive, selective and rapid detection of biomolecular analytes one molecule at a time that are relevant to clinical diagnosis of various diseases.

While label-free detection of small molecules is the most remarkable advantage of electrochemical biosensors, these sensors require a well-oriented surface architecture that does not limit or perturb the electron flow rate. However, single-molecule electrochemistry (EC) [9, 17-18] is an extremely challenging for biosensor development which requires thousands of electron flow to obtain a reasonable signal. EC sensors requires a "homogeneous" surface electrode where the molecules attached on a well-defined orientation without perturbed by the molecular crowds.[19] Although detection is mostly limited by the lack of the instrument sensitivity, electric field drop, protein folding and nanomaterial incompatibility, SM-EC was achieved on (micro)electrodes [20-22] and in aqueous and ionic liquid solvents [23] for redox-active molecules. For instance, Zevenbergen et al. demonstrated the electrochemical detection of individual redox molecules while they freely diffuse in solution using a nanofluidic device [24].

A noticeable SM-EC biosensor was demonstrated for the detection of carcinoembryonic antigen (CEA) based on electrochemiluminescence (Figure 2A-B) [25]. This method is an electrochemically triggered optical radiation produced by the energy relaxation of excited species. The sensor consisted of a sandwich-typed immune complex which was able detect carcinoembryonic antigen down to 6-8 molecules in 20 μL of serum sample by employing dual-stabilizers capped,

CdSe nanocrystals as the electrochemiluminescence emitter. A necessity for SM-EC is signal amplification which for instance can be achieved with large quantities of cadmium ions (Cd^{2+}) that are mounted in titanium phosphate spheres. Using this approach Cheng et al. [26] detected miRNA-21 based on two DNAs as the target capturers with a detection limit of 0.76 aM. Here $Ru(NH_3)_6^{3+}$ molecules are introduced to interact with DNA base-pairs as an electron transfer mediator. Another promising technique towards SM-EC biosensors is electrocatalytic amplification (ECA)[27] which is based on the individual collisions of catalytic nanoparticles with a noncatalytic electrode surface through amplification of the current by electrocatalysis. Using the electrocatalytic amplification technique Castañeda et al. detected microRNA down to the 100 pM [28].

Recently, a digital single molecule electrochemical detection method (dSMED) was introduced by integration of digital analysis with signal amplification of enzyme-induced metallization (EIM) on microelectrode array [29]. This method can amplify the EC signals with about 100 times, thereby enabling alkaline phosphatase (ALP) detection in a single-liver cancer cells at about 12 aM [29] sensitivity. This concentration level indicates the superior sensitivity of dSMED that can measure very low target concentrations or even a single-molecule in complex samples (Figure 2C-E). Based on this principle, the same group proposed and developed a digital electrochemical enzyme-linked immunoassay (ELISA) for the detection of avian influenza virus (H7N9 AIV) with a very low detection limit of 7.8 fg/mL [30].

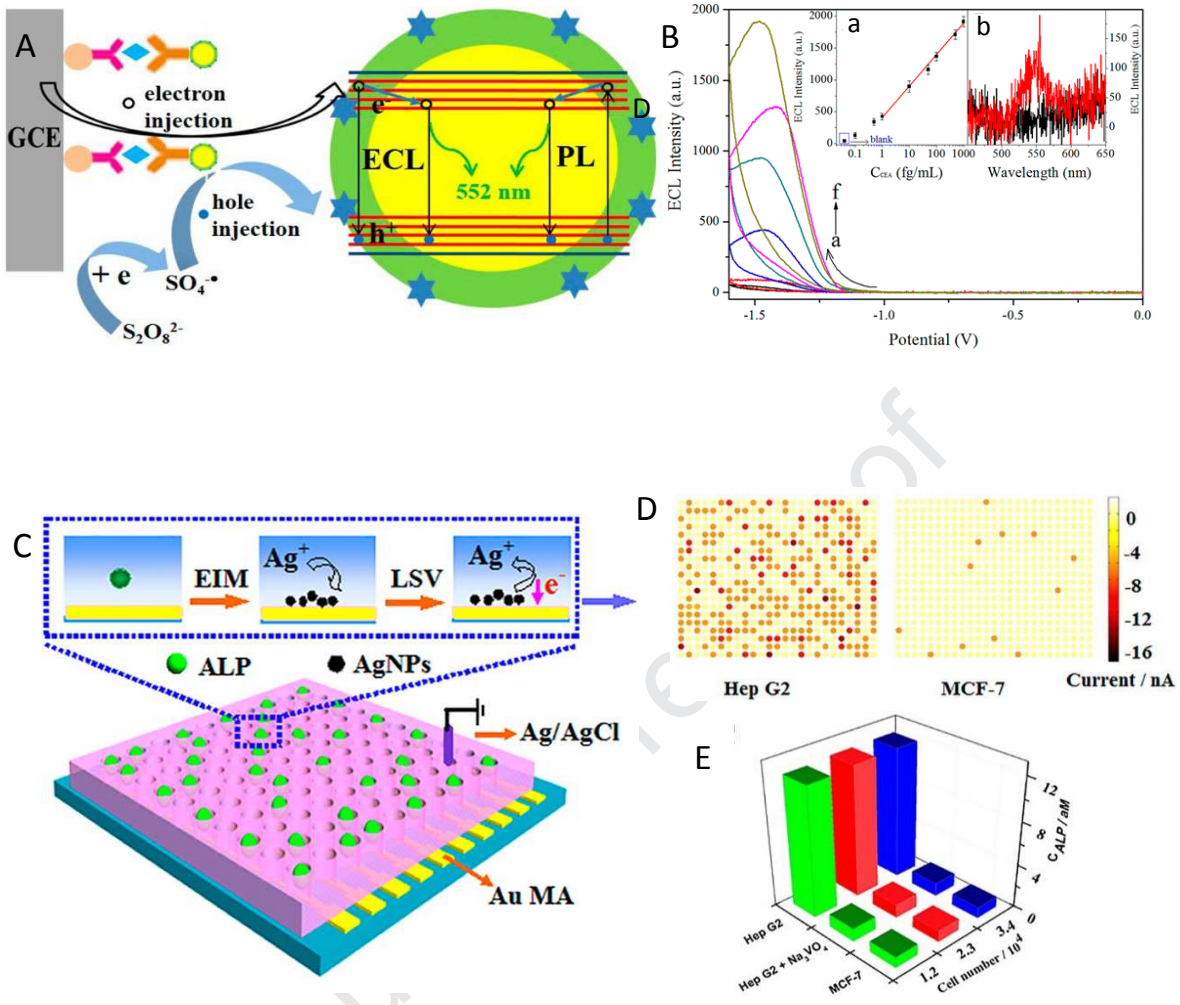

Figure 2. (A) Schematic illustration of electrochemiluminescent single-molecule immunoassay with dual-stabilizers-capped CdSe nanocrystals as labels. (B) Electrochemiluminescence (ECL) responses of (a) 0.0 to (f) 1000 fg/mL carcinoembryonic antigen samples (10-fold dilution) in 0.10 M pH 7.4 PBS containing 0.10 M $(NH_4)_2S_2O_8$ at 50 mV/s. Inset: (a) calibration curve, (b) corresponding ECL spectra of (a) 0.0 fg/mL and (b) 0.10 fg/mL CEA samples. (PL: Photoluminescence). Reprinted with permission from [25]. Copyright 2016, American Chemical Society. C) Diagram of the microelectrode array (MA) for digital single-molecule electrochemical detection method. About 100 times signal enhancement obtained through enzyme-induced metallization (EIM), e.g. single-ALP molecules has 8 nA current intensity while background noise is 1.8 nA. D) 500 electrochemical signals were transformed to a colorful imaging by a MATLAB program and the probability of "0" or "1" obtained by counting dark and bright balls. The integration of digital analysis solve the influence of current fluctuation and enzyme heterogeneity of SME as signals are denoted as "0" (containing 0 molecules) or "1" (containing 1, 2, or 3 molecules) without considering specific current intensities. (E) Histogram for the specificity of dSMED for ALP detection in liver cancer cells. Reprinted with permission from [29]. Copyright 2016, American Chemical Society.

To address the isolation and characterization of rare cells and molecules from a heterogeneous population Esfandyarpour et al. [31] developed a lab-on-a-chip (LOC) platform. It has been shown that this device can perform multiple functions such as single-bioparticle trapping, single-cell quantification and enumeration through impedance microcytometry, high-speed cellular manipulation, concentration, sorting, patterning, and selective analytical separation and isolation of the cells of interest. This label-free LOC platform is reusable and has ultra–low-cost ($0.01) which is made of flexible inkjet-nanoparticle-printed electronic apparatuses and disposable microfluidic biochips.

Although there are few examples to in vivo EC-biosensors at the ultra-high sensitivity, these sensors are highly promising to detect physiologically relevant concentrations [32] and having potential for label-free point-of-care devices. In combination with novel, advanced materials [33] such as molecularly imprinted polymers, they are highly promising for label-free quantification of clinically relevant biological species and offer the advantage of low cost and easy fabrication whilst being stable in harsh chemical environments. For instance, a mussel-inspired surface-imprinted sensor was developed for the detection of trypsin and yeast cells [34] at levels down to 0.03 U mL^{-1} and 50 CFU mL^{-1} (CFU, colony-forming unit is a measure of viable cells), respectively as well as azithromycin in pharmaceuticals and biological samples [35]. However, toxicity can be an issue

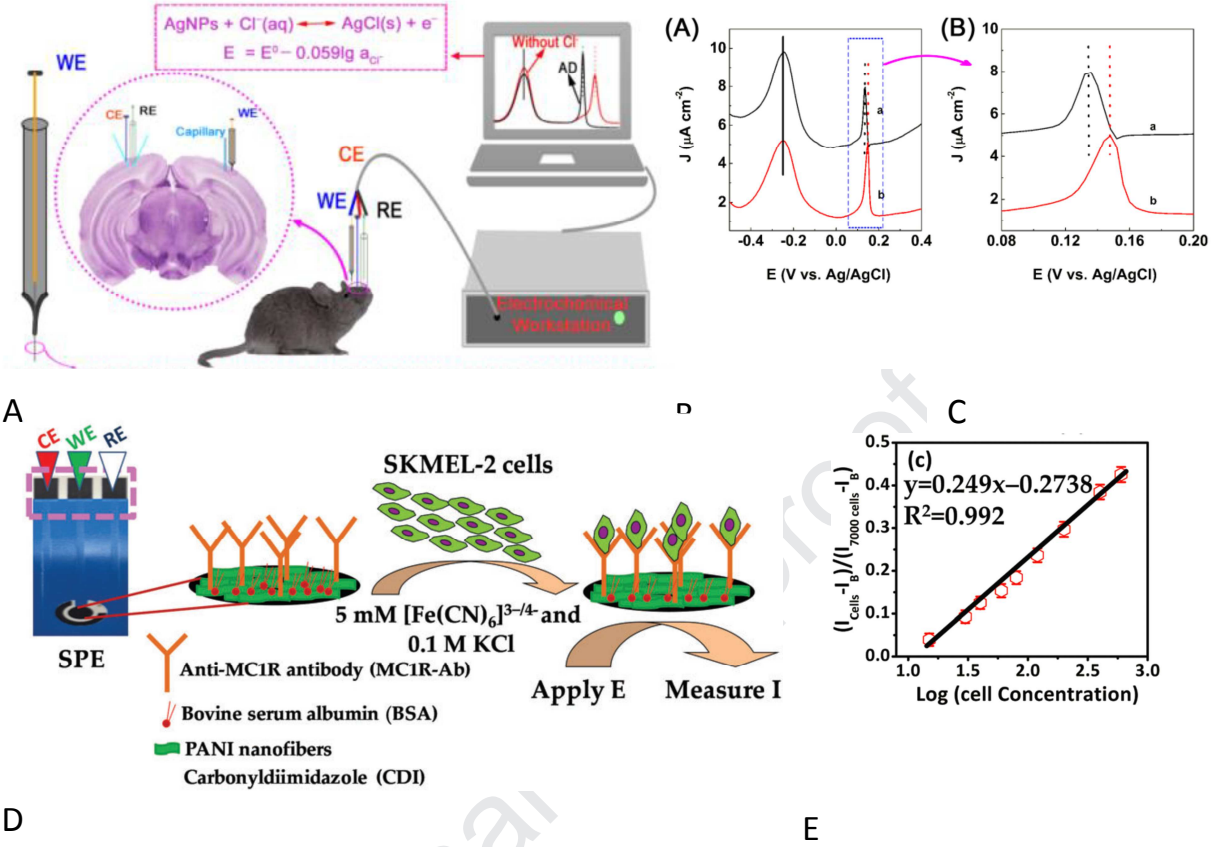

Figure 3. (A) Real-time monitoring of Cl^- levels in live mouse brain with an electrochemical biosensor. The response of the sensor is enhanced via single-walled carbon nanotubes (SWNT) and Au nanoleaves assembled on the carbon fiber microelectrode (CFME) surface. (B) DPV responses obtained at CFME/SWNT/Au/MB+HDT/AgNPs electrodes for determination of Cl- in the hippocampus of (a) normal mouse brain and (b) a mouse brain model of AD. (C) The enlarged view of labelled area in Figure B. Reprinted with permission from [36]. Copyright 2017, American Chemical Society." (D) Illustration of electrochemical immunoassay for melanoma (SKMEL-2) cell detection on a polyaniline nanofibers modified screen-printed electrode (SPE). (E) Calibration curves of peak current ratio versus the logarithmic value of SKMEL-2 cell concentration for 15–600 cells/5 mL. Reproduced from [37] with permission of the Royal Society of Chemistry. http://dx.doi.org/10.1039/C7CC09248B"

when producing such polymeric compounds and requires further investigations. An in vivo application to electrochemical sensor recently shown by Liu et al. [38] monitored nitric oxide, a key free-radical messenger, in rat brain following cerebral ischemia with a LOD of 10 nM. This ratiometric biosensor is based on a carbon nanotube fiber (CNF) modified with hemin which

greatly facilitated the electron transfer to the electrode surface [38]. For real-time monitoring of Cl^- levels in live mouse and rat brains and those in the mouse brain model of Alzheimer's disease an electrochemical biosensor was developed by the same group (Figure 3A-C) [36]. Here AgNPs were used as a selective recognition element for Cl^- and SH-DNA-MB (MB = methylene blue) was synthesized as inner reference element to prevent effects from the live brain. The sensitivity of the sensor was increased by functionalization of single-walled carbon nanotubes and Au nanoleaves on carbon fiber microelectrode surface. The sensor demonstrated presence of different Cl^- concentrations at different regions of the rat brains after global cerebral ischemia [36]. In a recent work by Prathap et al. [37] an EC-immunoassay developed that can detect melanoma cells with a limit of quantitation of 1 cell/mL using anti-MC1R antibody functionalized polyaniline nanofibers (PANI-NFs) (Figure 3D-E). This ultra-high sensitivity is attributed to PANI-NFs which are having large surface area and mesoporous structure to accommodate large number of antibodies as well as owing to unique electronic properties.

2.2 Plasmonic single-molecule biosensors

Nanoplasmonic sensing is an optical technique based on localized surface plasmon resonance (LSPR) with very high spatial resolution. This technique allows detection of changes in the collective electron charge oscillations in metallic nanoparticles that are excited by light upon binding of an analyte to the nanoparticle surface (Figure 4). The technique offers high sensitivity and selectivity due to tightly localized surface plasmons at the surface at a high signal-to-noise ratio. A detailed review on general principles, and surface functionalization and applications of single-plasmonic sensors can be found in refs [39-41]. Here we focus on the very

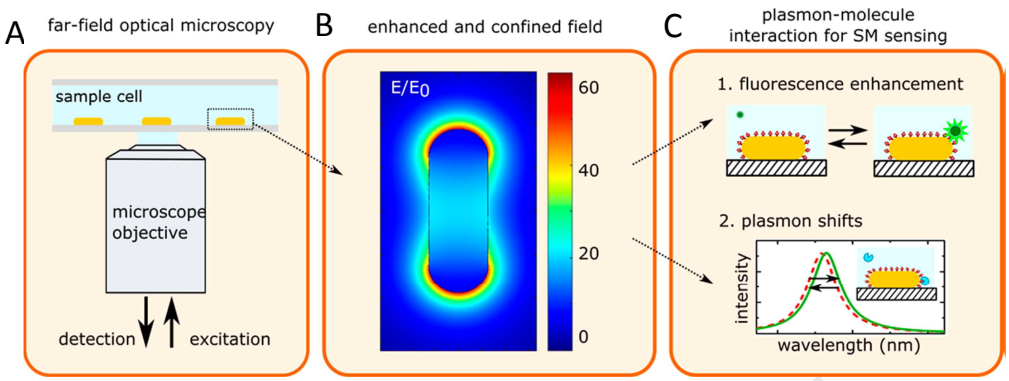

Figure 4. Principle of plasmon-enhanced single-molecule sensing using nanoparticles. (A) The sensor is typically probed in a far-field optical microscope, where single particles are interrogated. (B) The plasmon resonance induces a strongly enhanced and tightly confined local field around the particles. The field shown here is for a gold nanorod that is excited on resonance (calculated using the boundary element method). (C) This local field mediates plasmon–molecule interactions, enabling enhanced single-molecule detection by monitoring plasmon-induced changes of the molecule resulting in 1. fluorescence enhancement or by monitoring molecule-induced changes of the plasmon resulting in 2. frequency-shifts of the plasmon. Reprinted with permission from [41]. Copyright 2017, American Chemical Society.

recent biological applications of these type of sensors that are capable of detection of single-molecule targets.

In most of the LSPR sensors, gold nanoparticles and nanorods has been integrated in the plasmonic sensors. Importantly, detection of these nanoparticles at the single-molecule sensitivity can enable superior, label-free optical biosensing. Monroe et al. [42] applied Interferometric Reflectance Imaging Sensor (IRIS) to digitally detect and size single-gold nanoparticles to identify protein biomarkers in unprocessed serum and blood samples. This multiplexed immunoassay platform reached a detection limit of ~60 aM in serum and ~500 aM in whole blood for β-lactoglobulin. In a recent work it has been shown that by integration of the plasmonic gold nanoroads with IRIS, individual binding events can be monitored, at a limit of

detection of 19 fM based on this light scattering method.[43] Another plasmonic sensor developed for HIV virus through biotinylated-antibody functionalized gold nanoparticles demonstrated low viral concentrations in whole blood (98 ±39 copies/mL).[44] A similar immobilization approach combined with electrical field-enhanced resonating device (NE^2RD), allowed quantification of multiple biotargets in various clinical samples reaching to LOD of ~23 fM (400 fg/mL) for detecting interferon-γ in human serum [45]. For the first time, Ziljstra et al. [46] showed the possibility of detection of single non-absorbing molecules by monitoring the surface plasmon resonance of a gold nanorod through photothermal microscopy. They attached biotin at the tip of a single-gold nanorod and detected longitudinal SPR shift upon binding of a single-protein molecule to the biotin receptor. Remarkably their sensor showed ~700 times higher sensitivity than conventional plasmonic sensors. In another assay, gold nanorods integerated microfluidic device could detect cancer biomarkers (human alpha-feto-protein and prostate specific antigen) down to concentrations of 500 pg/mL in 50% human serum [47]. LSPR-based miRNA (miRNA-10b) biosensor with single nucleotide specificity developed by Joshi et al. on gold nanoprisms [48]. They recorded nanoprism's local dielectric environment upon an analyte absorption which boosted the detection limit to 83 aM with a 47 nm edge-length nanoprism in human plasma. The researchers employed their novel sensor to detect microRNA-10b not only in plasma but also in in vitro pancreatic cell lines, tissue culture media and plasma exosomes with attomolar sensitivity. This LSPR biosensor clearly differentiated, that miRNA-10b levels in plasma-derived exosomes are significantly higher in patients with pancreatic cancer than in patients with chronic pancreatitis as well as normal controls. Apart from gold nanoparticles and nanorods, several advanced materials are

developed in order to increase the sensitivity of plasmonic biosensors. Detection of lower-molecular-weight (<500 Da) biomolecules in highly diluted solutions is challenging due to their lower polarizability. To this end, Sreekanth et al. [49] developed a nanosensor to detect extremely small refractive index changes by exciting the high-k modes associated with hyperbolic metamaterials. This

microfluidics-integrated plasmonic platform based on hyperbolic metamaterials applied for biotin (244 Da) detection at picomolar concentrations.

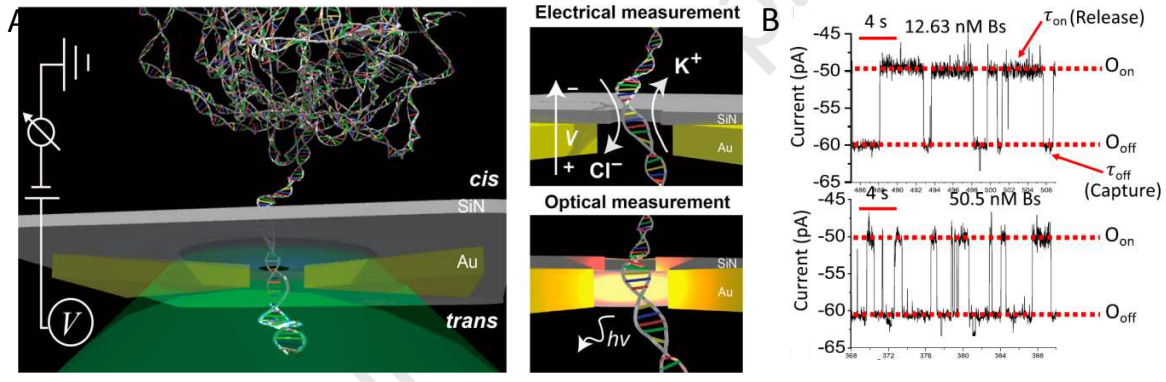

Figure 5. (A) Plasmonic nanopore-based electrical and optical measurement. (Left) DNA translocation through a plasmonic nanopore. Coiled double-stranded DNA that enters the pore from the *cis* chamber passes through the pore equipped with a plasmonic nanostructure comprising two gold triangle plates. An electromagnetic field is generated at the exit of the pore through the visible laser (green) illumination on bottom side of plasmonic nanopore. (Top right) Principle of electrical measurement. When voltage is applied across the plasmonic nanopore, DNA and ions (K^+ and Cl^-) are driven through the pore, producing a strong, pore-localized electric field. (Bottom right) An illustration of a plasmonic-enhanced localized optical measurement. DNA traversing the hotspot between two gold nanostructures leads to emission of enhanced optical signals containing information about the molecule. Reprinted with permission from [50]. Copyright 2019, American Chemical Society. https://pubs.acs.org/doi/10.1021/acs.nanolett.9b02759. (B) Representative single-channel electrical traces obtained from a nanopore biosensor. The ionic current decreases when an analyte translocated through the pore (off-times), followed by a signal recovery when the molecule exits the pore (on-times). Shown traces are sensing of barstar (Bs) protein in the presence of 12.63 nM Bs (top) and 50.5 nM Bs (bottom) at an applied transmembrane potential of −40 mV. Reprinted with permission from [51]. Copyright 2019, American Chemical Society.

Recently plasmonic biosensing with nanopores,[50, 52-54] nanowells[55-56] and nanopipettes[57-58] gained rapid increase due their sensitivity at the single-molecule detection limits. Plasmonic nanopores with its nanometer-sized pores can be fabricated at a low cost, achieve high stability and allow modification of the channel size. These properties make them ideal for biosensor device integration and commercialization. As shown in Figure 5, a nanopore consist of an electrically insulating thin membrane in which the pores with 1-100 nm in diameter are formed. In nanopore biosensing, an electric field is generated by the applied potential through the electrodes which are inserted across the membrane at two electrolyte-filled compartments. This applied potential between the two electrodes changes the ionic current, and the later, thus, moves the charged biomolecules to the pore by displacing the ions. Such modulations of the ionic charge current can be measured as an electrical or optical signal. Plasmonic nanopore biosensors allow for measuring the size and concentration of the molecules and reveal the dynamic process of the molecule translocation behavior. To date, single-molecule nanopore biosensing are applied to detect single protein,[51, 58-66] enzyme[67-68], polysaccharide[69] molecules, protein-protein interactions,[70-71] folding/unfolding and translocation of single proteins,[72-74] size of short peptides with a single amino acid resolution,[75] enzymatic kinetic reactions,[74, 76-77] single nucleotide polymorphisms[78], DNA knots,[79] single-molecule DNA sequencing[77]. The rapid progress of the nanopore-based single-molecule biosensors was established for the first time in an electrode-free nanopore biosensor. In the so called DiffusiOptoPhysiology (DOP) method, single-molecule events are monitored optically without any electrical connections to detect

small molecules, macromolecules, and biomacromolecules.[80] Moreover, nanopore biosensing is highly promising for digital counting of single molecules.[81]

Periodic nanohole arrays applied to several SPR applications due to their intense resonances at specific wavelengths based on extraordinary optical transmission. Jackman et al. [82] created such nanoholes for capturing single virus-like particles and evaluating virucidal drug candidates. Using the plasmonic nanowell-nanopore configuration, Assad et al. [55] recorded the optical translocation signal. Their electro-optical detection in solid-state nanopores (4 nm) enabled signal enhancement by a factor of 10 as compared to the standard nanochip device configuration which could eventually measure single-DNA translocation events.

2.3 SERS-based single-molecule biosensors

Surface-enhanced Raman spectroscopy (SERS) is a label-free, powerful vibrational spectroscopy technique that enhances Raman scattering by molecules adsorbed on plasmonic surfaces such as gold, silver or magnetic silica nanotubes. SERS is highly promising for ultra-sensitive detection of biomolecules through the amplification of electromagnetic fields that are generated by the excitation of localized surface plasmons at single-molecule level without being destructive and photobleaching [83-84].

To this end, Sun et al. [85] developed a SERS-based magnetic immunosensor to detect avian influenza virus H3N2 by constructing a sandwich complex. This immunosensor was able to detect H3N2 down to 10^2 $TCID_{50}$/mL ($TCID_{50}$ refers to tissue culture infection dose at 50% end point). By coating AuNPs on 96 well plate, influenza virus A (H1N1) and clinically isolated virus A (H3N2) were detected down to the 10 pfu/ml (pfu=plaque forming units are number of

infectious virus particles) with a SERS-based immunosensor, a sensitivity that is 116-fold higher than the conventional ELISA method [86]. Vijayakumar et al. [87] developed a SERS-based biosensor with a LOD of 1 pM for the detection of crystal violet and glutathione at excitation wavelengths of 532 and 785 nm, and thus having the possibility to both in vivo and in vitro sensing. Liu et al. [88] recently developed a biosensor based on gold-silver core-shell SERS nanotags and photonic crystal beads to detect IgG and C reactive protein (CRP), a biomarker for cardiovascular disease and inflammation. The sensor had a LOD of 672 fg/mL and 478 fg/mL for IgG and CRP, respectively, and showed good correlation with reference method ELISA in real serum samples.

SERS is becoming demanding technique for multiplexed sensor applications with high sensitivity owing the 'fingerprint' Raman spectra from every molecule. For instance multiplexed assays were developed for the detection of multiple liver cancer related microRNA (miRNA) biomarkers [89], α-fetoprotein and Glypican-3 [90], prostate specific antigen [91] and several tumor markers [92-95] at the sub-picomolar concentrations in vitro as well as in vivo [94]. To detect neurotransmitter secretion events, a SERS nanosensor developed by Lussier et al. [96] that measured ATP, glutamate, acetylcholine, GABA and dopamine in close proximity to neurons.

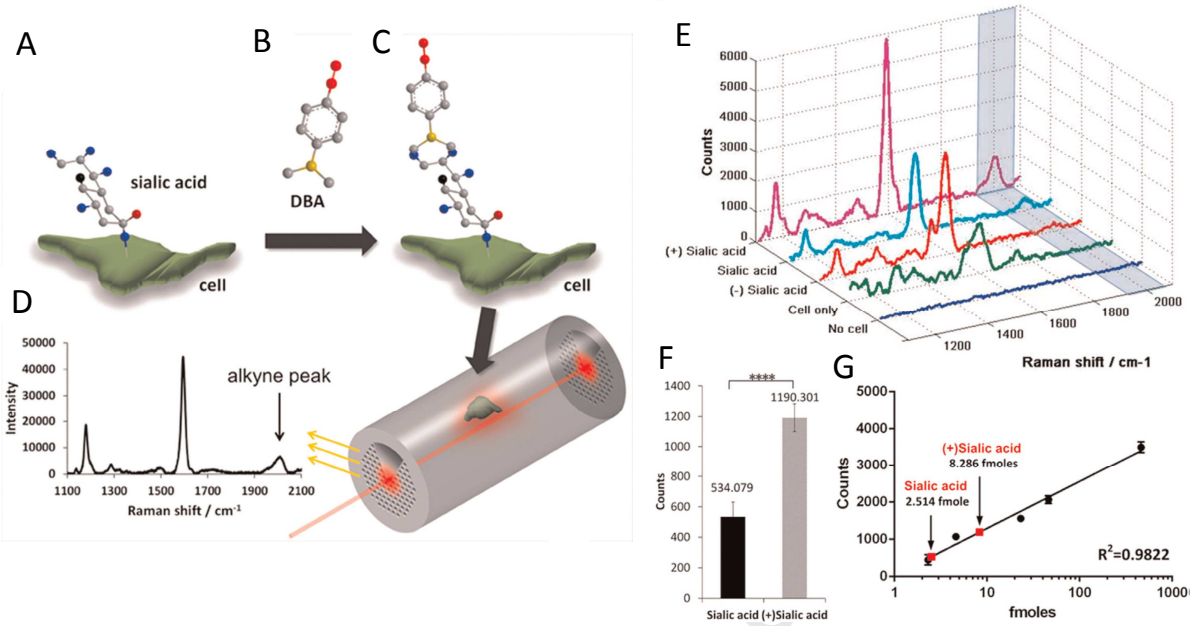

Figure 6. Illustration of the detection of sialic acid on a single-cell. (A) Chemical structure of sialic acid on cell surface. (B) The chemical structure of DBA. (C) DBA-bonded sialic acid on the membrane of a cell at the 8,9 diol of sialic acid. (D) Raman spectrum detected from DBA tagged cell in SC-PCF, the 2000 cm-1 peak arises from alkyne group from DBA. (F) SERS detection and quantification of single Hela cell. Raman spectra of (+) Sialic acid sample, (-) Sialic acid sample, positive control (sialic acid), negative control (cell only) and Raman signal from the fiber when no cell was loaded. (F) 2000 cm^{-1} peak intensity of (+) sialic acid and sialic acid samples. (G) Calculated number of sialic acid molecules (red square for sialic acid and (+) sialic acid samples) from plotted trendline (black line) of the calibration points (black dots). Reprinted with permission from [97]. Copyright 2018, Elsevier.

The superior sensitivity of the SERS based sensing platform is capable of detecting single-amino acid mutations in a mixture of peptides unambiguously [98] and sialic acid (tumor-associated carbohydrate antigen) on a single-cancer cell [97] as shown in Figure 6. Lussier et al. [99] developed a SERS nanosensor to monitor extracellular metabolites (e.g. pyruvate, lactate, ATP, and urea) near living cells simultaneously. Importantly, this sensor depicted a sensitivity at the single-molecule regime to locally probe cellular secretion events as well as monitored the distance dependent sensing of the extracellular medium. Raman fingerprint can also be used for sorting and identifying the single-amino acids adsorbed onto gold nanoparticle [100]. SERS signals can be

enhanced by creating hybrid materials. A good example for this was demonstrated by Fan et al. [101] where hybrid graphene oxide and popcorn-shaped Au NP-based SERS probes enhanced the SERS signals 11-orders of magnitudes, which resulted in the detection of HIV-1 gag-gene DNA with a sensitivity at the femtomolar level and 10 CFU/mL for methicillin-resistant Staphylococcus aureus bacteria.

2.4 Spectroelectrochemical single-molecule biosensors

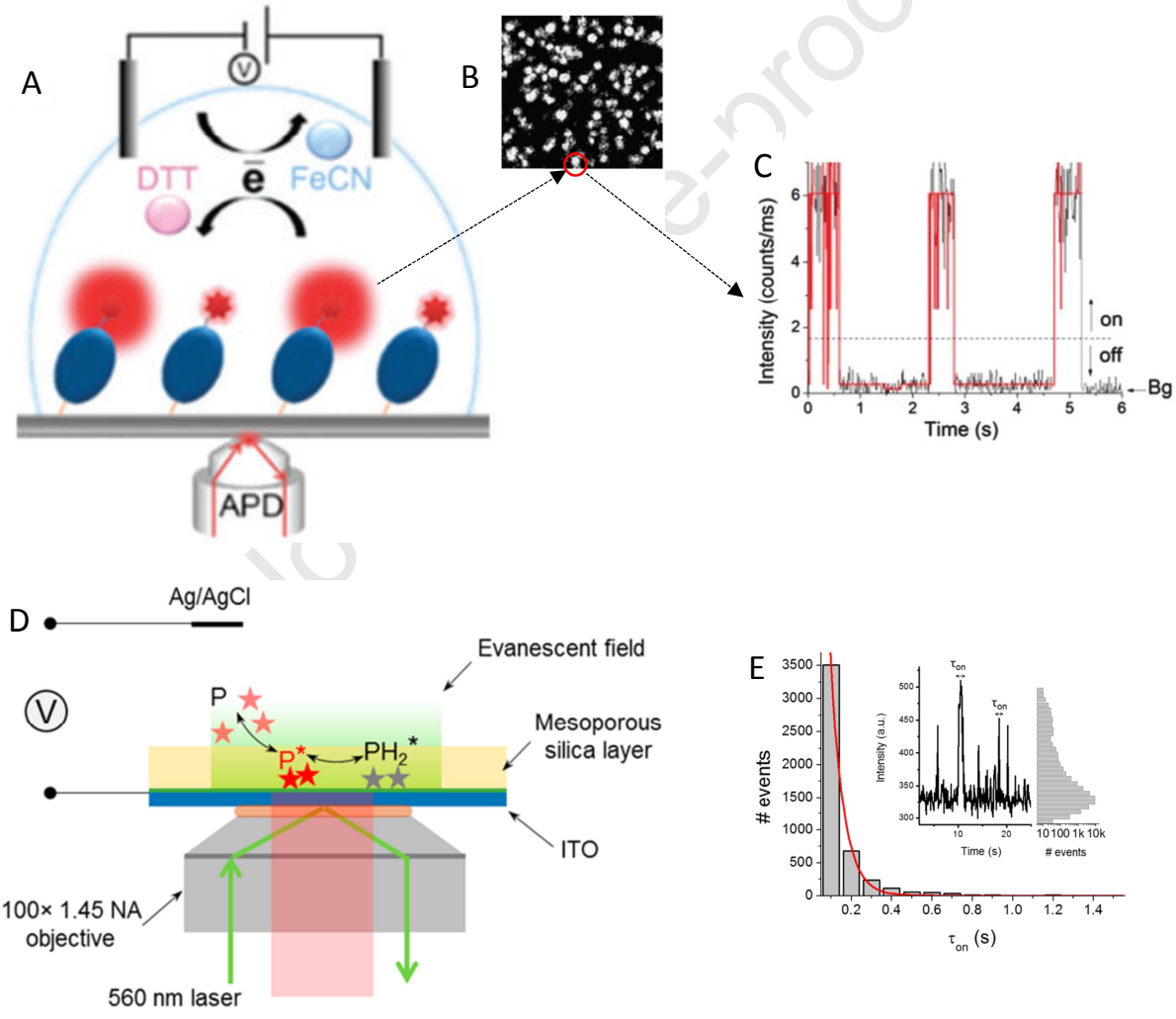

Figure 7. (A) Single molecule fluorescence detection of azurin molecules. The redox state of the protein was monitored using a mixture of DTT and $K_3[Fe(CN)_6)$ as a reductant and an oxidant in the buffer solution. The redox potential of the solution was determined with a reference electrode (SCE) and counter electrode (Pt wire) connected to a voltmeter. (B) Confocal image of single Cy5-labeled azurin molecules immobilized on a glass surface. The real time fluorescence intensity traces of a single azurin molecule with a 10 ms bin size (black) and calculated intensity changepoint states (red) overlaid as a function of time. They exhibit an on–off switching behavior which depends on the redox potential in solution E = 60 mV (P_{ox} = 0.76) vs. SCE. Reproduced from [102] with permission from the Royal Society of Chemistry. (D) Schematic illustration of single-molecule electrochemistry on a porous silica-modified ITO electrode. (E) Distribution of τ_{on} of single resorufin molecules. Red line is a single-exponential decay fit with a time constant of 0.064 ± 0.003 s. Inset: (left) the fluorescence trajectory from one fluorescent spot; (right) histogram of the fluorescence trajectory. Reprinted with permission from [20]. Copyright 2017. American Chemical Society."

In 2006 single-molecule sensitivity was achieved with spectroelectrochemistry [103] opening the doors to investigate thermodynamics and kinetics of electron transfer of redox-active molecules that have great potential for sensor applications [9]. In this technique fluorescence intensity from the single molecules of the organic conjugated polymer F8BT was read out to study the electrochemical oxidation [103]. This first spectroelectrochemical approach has been conducted on an indium tin oxide (ITO) electrode and fluorescence emission was monitored simultaneously with a wide-field microscope. Following this novel approach, electrochemistry integrated optical detection [104] have been applied to study electron-tranfer dynamics of several redox-active molecules and fluorescent probes [105-112]. For instance, electrochemical properties of low quantum yield redox dye methylene blue was determined using fluorescent single-molecule electrochemistry by using individual gold nanorods as fluorescence enhancer [113]. However, a real contribution to biological electron-transfer has been demonstrated with fluorescent-electrochemistry which applied to monitor redox properties of a metalloprotein down to single-molecule regime on a semi-transparent gold electrode [114-115]. While the single-

electron-transfer reactions from a redox-active protein can be measured on a “non-conductive” surface by the aid of mediators using confocal microscope [102] (Figure 7A-C), Lu et al. demonstrated the possibility of single-molecule electrochemistry on a transparent ITO electrode for fluorogenic redox species using TIRF microscopy [20] (Figure 7D-E). In a new approach based on collision-based bioelectrocatalysis monitored the electrocatalytic current from single-laccase molecules in solution [116] which eventually can be integrated with optical detection.

Another spectroelectrochemical technique to probe the redox reactions has been demonstrated by surface- and tip-enhanced Raman spectroscopy (SERS) at the molecule-metal interface [84, 117-118]. One of the first electrochemically-induced single-electron transfer events with SERS was demonstrated on rhodomine 6G and Nile Blue [83, 119-120]. Very strong SERS signals can be for instance obtained by creating plasmonic hotspots (via creating molecular junctions through separating the gold surfaces by a self-assembled monolayer (SAM)) of molecules that enables to follow redox processes in real time at the single-molecule level [121]. To the best of our knowledge, SERS-electrochemistry-based redox reactions is not yet applied to biologically relevant molecules as compared to fluorescent-electrochemistry. One of the drawbacks of the technique is that the signals from the SERS reporters can be heavily influenced by interactions with the metal. All in all, these spectroelectrochemical techniques developed in recent years offer great possibilities to measure the local redox potential in biological species at the single-molecule regime in the very near future.

3. Fluorescent assays

3.1 DNA-based single-molecule biosensors

Emerging new fluorescence microscopy techniques has been established that are surpassing the diffraction limit of the traditional optical microscopes such as those called stimulated emission depletion (STED)[122] and photoactivated localization microscopy (PALM)[123] or stochastic optical reconstruction microscopy (STORM) [124]. Optical techniques and experimental methods have been evolved over the years, resolving nanostructures at sub-nanometer in size and thus became an invaluable tool in single-molecule biosensing.[13, 125-126] Most widely, confocal microscopy and total internal fluorescence (TIRF)-based biosensors employed to detect target biomolecules that are fluorescently tagged.[14, 127-129]

In this respect, Lu et al. [129] developed an immunobiosensor showing that antibody (antiBSA IgG) binding to antigen (BSA) modified ITO surface is dependent on the concentration of both molecules. The researchers display the analyte binding events at the single-molecule level using direct stochastic optical reconstruction microscopy (dSTORM) which is superior over the conventional microscopes in terms of spatial resolution and sensitivity. In order to monitor polymerase/DNA interactions Kim et al. [130] developed an optical, label-free method which is capable of observing enzymatic activity on a single-molecule regime by monitoring the kinetics and conformational transitions of DNA polymerases. Aptamers are known to be employed as novel recognition receptors and are typically composed of short single stranded DNA (ssDNA) or RNA sequences that enable to produce sensitive biosensors. One of the advantages of using aptamers is that it is possible to integrate them with various materials. In this respect, a remarkable contribution to single-molecule biosensor reported by Landry et al. [131]

demonstrated a label-free detection of individual proteins from Escherichia coli (bacteria) and Pichia pastoris (yeast) immobilized in a microfluidic chamber, . Selective protein recognition and secretion of single-proteins from individual microorganisms were achieved through coupling of aptamer-anchor polymers to semiconducting single-walled carbon nanotube (SWNT) near-infrared emitters. Alternating AT nucleotide repeats are the anchor segments which can adsorb strongly to the SWNT surface, and the molecular recognition is provided by a folded polynucleotide aptamer. The proximity of the protein to the SWNT surface produces a change in the local dielectric environment of the SWNT, which produces a near-infrared optical signal in the form of a SWNT fluorescence increase. The work measured protein efflux from single organisms in real time as well as selectively detected unlabeled RAP1 GTPase (a vital cytosolic protein for T-cell receptor signaling) and HIV integrase proteins from various cell lines, through a large near infrared fluorescent turn-on responses [131]. In another work, nanofluidic chips (<10 nm) developed for detecting and manipulating single-DNA molecules with fluorescence imaging [132].

To achieve single-molecule detection of polynucleotide kinase, Wang et al. [133] developed a TIRF-based method based on the phosphorylation-directed recovery of fluorescence quenched by gold nanoparticles in combination with lambda exonuclease-mediated cleavage reaction. Same group developed a multiplex sensor to detect single-histone-modifying enzymes through the integration of antibody-based fluorescence labeling [134] and detected cellular activity of single-quantum dot-based DNA glycosylase [135] with TIRF microscopy. Another interesting application to fluorescent biosensor developed by Yu et al. [136] could monitor the enzymatic

activity of matrix metalloproteinases at the single-cell level. Their approach is based on the single cell-encapsulated microdroplets integrated with microfluidic technology. In each microdroplet, matrix metalloproteinases can rapidly cleave the peptides modified with a pair of fluorophore/ quencher molecules, therefore producing a fluorescence "on" signal. In another DNA sensor, fluorescence resonance energy transfer (FRET) has been used to monitor DNA polymerization in real time at a single-molecule sensitivity. This biosensor is based on the conformational change of the double stranded DNA from a random to canonical structure upon extension of the primer using a DNA polymerase. The FRET efficiency alters based on the conformational change allowing to monitor DNA synthesis, and kinetics between DNA polymerase and DNA [137].

3.2 Liposome and bead-based amplification

Among others, single-molecule technology have found potential applications in drug screening context because of its low sample consumption and possibility of determining affinities at a high sensitivity compared to traditional surface-based techniques, especially for weakly binding biomolecules [138]. To this end, Gunnarsson et al. [14] developed a single-molecule inhibition-in-solution assay (ISA) which is based on time-resolved total internal reflection fluorescence imaging [139] to monitor small molecule inhibitors directed against the β-secretase 1 (BACE1). Figure 8 shows liposomes containing BACE1, an attractive therapeutic target for potential Alzheimer's disease treatment, interacting with a ligand modified biosensor surface thereby enabling their monitoring by TIRF microscopy. The assay allows to extract the binding affinity (Kd) of different inhibitors by analyzing the change in the binding rate upon challenge with a suspended competitive inhibitor. This single-molecule TIRF assay showed superior sensitivity

over the conventional SPR spectroscopy, increasing the dynamic window of molecular binding down to low-nanomolar affinities (Figures 8B-C) [14]. Particularly, this method can track both weak (> μM) and strong (< pM) interactions due to its ability to visualize binding events individually. This single-molecule sensitive method was successfully applied to detect human norovirus (NoV), a small nonenveloped RNA virus of the Caliciviridae family which causes acute gastroenteritis, based on a sandwich-type configuration [140]. The virus particles captured onto a non-fouling supported lipid bilayer which is detected after recognition by a glycolipid-containing fluorescent vesicle at a limit of detection of 16 fM. In a recent work it was shown that liposome-amplified detection platform can mimic the natural membrane fusion while having possibilities for biosensing applications. By employing FRET donor and acceptor pairs in the membrane of DNA-functionalized liposome, flu biomarker microRNA (miR-29a) can be detected with a LOD of 1.2 nM using a 2-color fluorescence correlation microscopy [141].

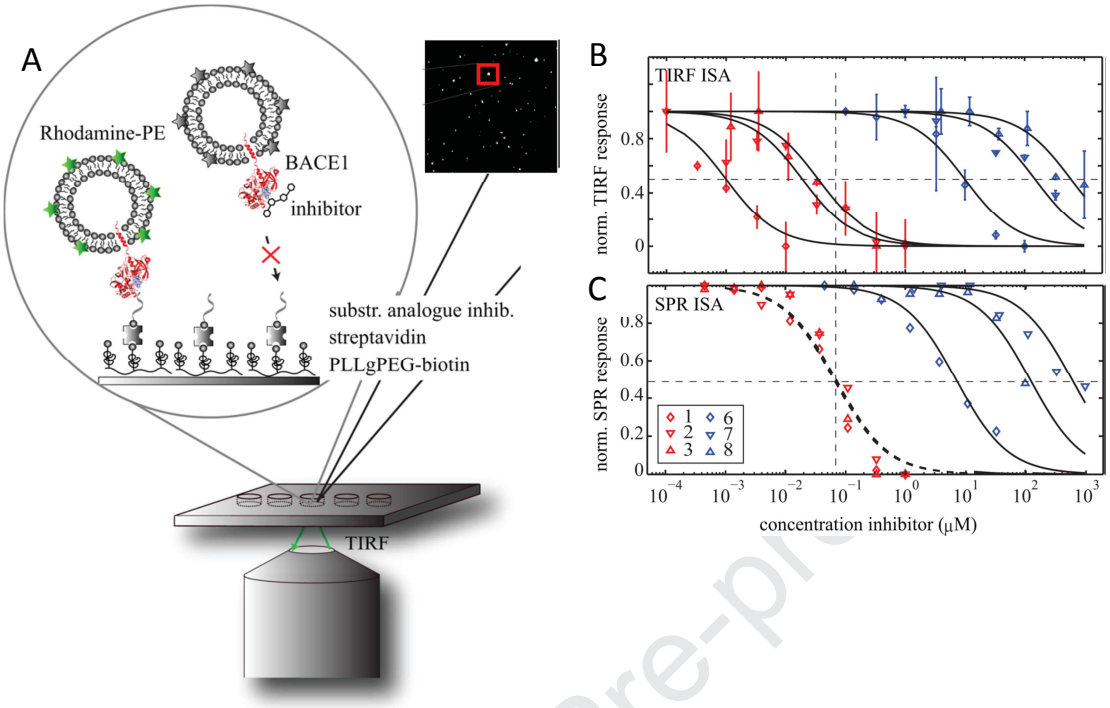

Figure 8. (A) Schematic illustration of the methodology. Fluorescent liposomes containing, on average, <1 reconstituted BACE1 per liposome associate (and dissociate) from the surface-immobilized ligand under equilibrium binding conditions. Each individual association is monitored by time-resolved TIRF microscopy, in the presence of varying concentration of inhibitor. (B) Dose–response curves for selected inhibitors using flBACE in TIRF and (C) ecBACE in SPR. Dotted curve fits indicate compounds with potencies below the assay limit (<140 nM) for SPR but still accessible using the single-molecule TIRF assay. Reprinted with permission from [14]. Copyright 2015, American Chemical Society.

Similar to liposome enhancement, in a bead-based microfluidic assay developed by Singhai et al. [142] successfully demonstrated antibody-antigen binding kinetics with a sensitivity and sample consumption of 4 orders of magnitude compared to surface plasmon resonance (SPR) on the single-cell level. In another bead-based fluorescence assay an 15000, 60 picolitre-sized

microwells created which is capable of multiplexing up to 12 different proteins by using four bead

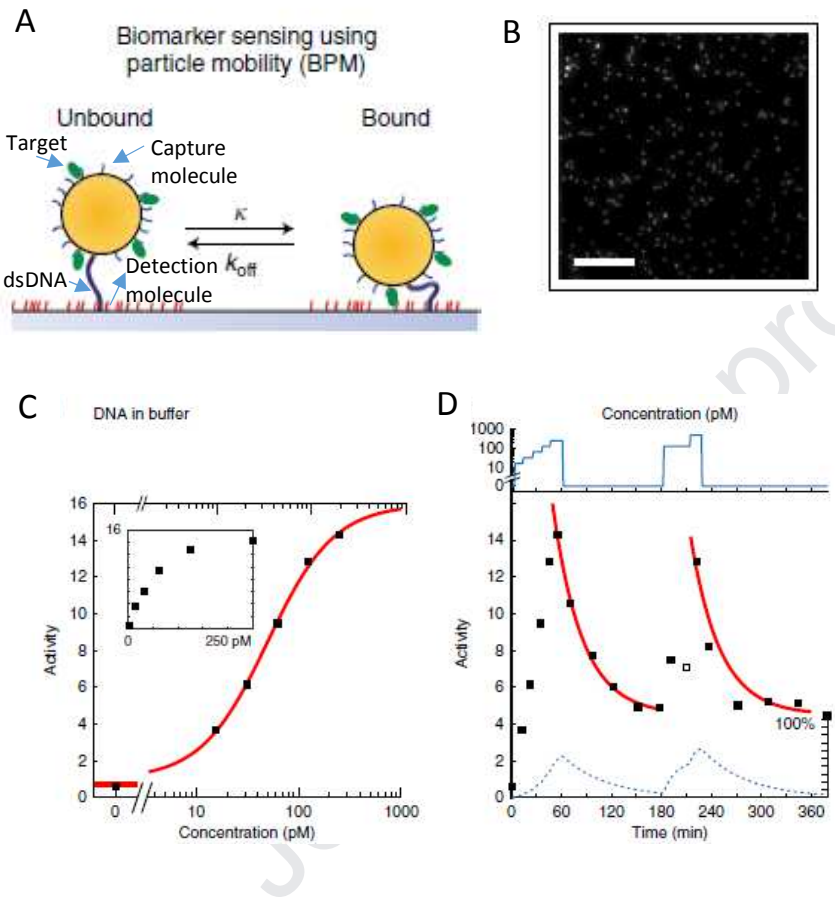

Figure 9. Nanoparticle mobility dependent biomarker detection. (A) Particles (orange) are tethered to the substrate via a 40 nm dsDNA strand (black). The particles are functionalized with capture molecules (blue), the substrate is functionalized with lower-affinity detection molecules (red), which create short-lived target-induced bonds between the particle and the substrate. Target molecules (green) are either 22-nucleotide ssDNA molecules or thrombin proteins. Target binding causes the particle to become intermittently bound to the substrate resulting in switching between different mobilities and motion patterns. The effective association rate of the particle to the substrate is indicated as κ, the dissociation rate is given by koff. (B) The particles are detected using darkfield microscopy. The scale bar represents 50 µm. (C) Average number of detected motion changes per particle per 5 min as a function of the ssDNA concentration. The data is fitted by the Hill equation with a baseline offset (red line). The insets show the dose–response curves on double-linear axes. (D) Sensor reversibility is shown by measuring particle activity as a function of time vs. target molecule concentration. Reprinted from [143] under the Creative Commons Attribution 4.0 International License http://creativecommons.org/licenses/by/4.0/.

sizes (1.75, 3, 4.5, and 6 μm) and three colors (blue, green, and yellow) [144]. This microchambers can isolate single cells for cell lysis and the subsequently detect proteins, including organ-specific markers and drug targets. Another method developed based on the optical scattering signals from streptavidin-coated magnetic particles has been applied for biomarker detection. These particles and substrate are kept close to each other by a flexible dsDNA tether as shown in Figure 9. As a result of an analyte binding a compact molecular sandwich can be formed, and the Brownian motion of particles becomes restricted (bound state). Having more analyte in the solution increases the chance of binding of the analyte to its substrate and thus the frequency of reversible binding events, which eventually can be converted to dose-response curves. The proof-of-principle of this technology has been demonstrated with DNA and thrombin showing a sensitivity down to the picomolar concentrations [143].

3.3 Detection of exosomes and exosomal biomarkers

During the past two decades detection of exosomes, 40-120 nm size extracellular vesicles, gained remarkable attention since they are thought to be responsible of regulation of vital biological processes through enabling cell to cell communication [145]. Understanding their role in carrying and transferring of nucleic acids, cytoplasmic and membrane proteins, lipids, is highly critical in order to have them such as for drug delivery applications but most importantly to understand the disease mechanism and for emerging therapeutic opportunities [145-146].

As they are considered to be diagnostic markers detecting and quantifying of exosomes and the biological molecules that are transported by exosomes in body fluids can lead to understand many unresolved problems in complex biological systems. The concentration of exosomes for instance can be determined by employing label-free, surface-based single-molecule biosensing

with surface plasmon resonance (SPR)[147-149] and microtoroid optical resonators[150] which can distinguish the healthy exosomes from the cancer patients [148-149, 151]. With this technique total mass including lipids, proteins, and nucleotides can be measured which can be selective to one of the target molecule with low limit of detection (0.194 μg/ml [151]). On the other hand, with an electrochemical detection 10-fold higher sensitivity (100-200 exosomes/μL) [152-153] can be obtained through quantum dot-functionalized antibodies that are specific to cancer [152] or antibodies directly attached on gold surfaces via 11-mercaptoundecanoic acid [153]. A much more sensitive detection of exosomes was achieved through fluorescence-based ELISA assay. Zhang et al [154] detected 50 exosomes/μL (80 aM) on layer by layer formed graphene oxide/polydopamine (GO/PDA) interface in combination with a microfluidic platform.

3.4 Amplification strategies for ultra-sensitive detection of nucleic acids

Naturally detection of exosomal RNA/proteins biomarkers such as microRNAs (miRNAs) gained rapid attraction as they are potential for diagnostics, especially for cancer. To this end, several miRNA biosensors have been proposed including fluorescence [141, 155-158], LSPR [48], SERS [89, 159] and electrochemical [160-161] platforms. Several strategies were introduced to amplify the signal enormously such as isothermal amplification, rolling circle amplification or strand displacement amplification. Among those attomolar sensitivity was achieved, for instance, by ratiometric electrochemical biosensor based

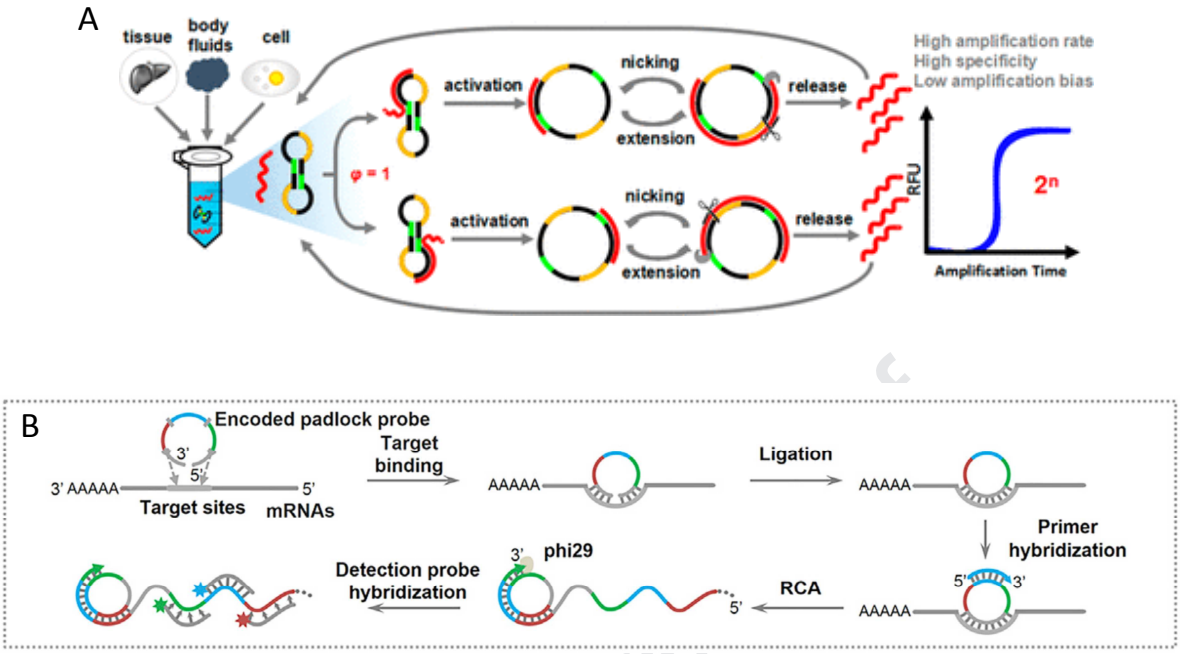

Figure 10. (A) Scheme of isothermal symmetric exponential amplification reaction (SEXPAR). The reaction involves five steps: hybridization of target miRNA (primer) with STD-template; activation of STD-template from dumbbell-shape to circular-shape; extension of primer from its 3' terminus along template by DNA polymerase; cleavage of the upper strand DNA by NEase; and release of the amplification product from template for the next SEXPAR. Reprinted with permission from [162]. Copyright 2018, American Chemical Society. (B) Schematic representation of mRNA detection in single cells by Rolling Circle Amplicon (RCA) and an encoded padlock probe. Reprinted with permission from [163]. Copyright 2018, Elsevier.

on bipedal DNA walkers and locked nucleic acid (LNA) modified toehold mediate strand displacement reaction [160]. An example to toehold mediated strand displacement was shown on a photonic crystal biosensor surface to detect miRNA.[164] This plasmonic biosensor, conjugates gold nanoparticles with miRNA-specific DNA probe and employs photonic resonator absorption microscopy (PRAM) to monitor surface-captured particles over time. PRAM achieved a LOD of 100 aM miRNA with single-base discrimination in a 2 hours kinetic discrimination assay.

DNA nanotechnology enables to create nanomachines inspired by nature. As an example, Ma et al. [157] developed miRNA sensor using a fluorescently labeled hairpin probe and exonuclease I activity. When there is no target miRNA, the 2-aminopurine fluorescence quenches upon incorporated within DNA. The hairpin probe opens when the target miRNA hybridizes with the hairpin, forming a double-stranded structure with a protruding 3'-terminus, which triggers the digestion of the protruding 3'-terminus by exo I. This digestion releases the fluorophore free in the solution and thus leads to an enhancement in the fluorescence signal [157]. A similar approach based on the RNA-cleaving DNAzyme probe as the molecular recognition element and enzyme-responsive plasmonic nanoparticles LSPR as the signal readout used to detect Escherichia coli down to 50 bacteria/mL [165]. A remarkable sensitivity for miRNA (let-7a) has been achieved by exponential isothermal amplification by Jia et al. [166] using a fluorescent reporter. Authors reached the limit of detection of 0.1 zeptomole, corresponding to less than 100 copies of a miRNA molecule per 10 μL. This method further developed by addition of a structure-switchable symmetric toehold dumbbell-template in the so-called isothermal symmetric exponential amplification reaction (SEXPAR) method that could measure miRNA let-7a down to 0.01 zeptomole (~6 copies per 10 μL). The single-molecule sensitivity of this novel isothermal amplification method can discriminate one-base-mismatched miRNAs, and quantify let-7a in human tissues, sera and even single-cell lysate [162] (Figure 10A). Another label-free fluorescence method based on the phosphorylation-triggered isothermal exponential amplification reached a detection limit of 0.0002 U/mL and enabled the detection of endogenous polynucleotide kinase (PNK) activity at the single-cell level [167]. The amplification strategy was applied for multiplexed imaging of RNA in single cells at single-base resolution using a DNA-sequence-

encoded fluorescence barcoding method entitled as sequence-encoded amplicon (SeqEA) [163]. SeqEA is based on thermodynamically tuning the DNA hybridization approach for precisely encoding rolling circle amplicons with discrete fluorescence intensities as fluorescence barcodes (Figure 10B). This method was applied to detect nine mRNA species simultaneously and evaluated the coordination and spatial pattern of breast-cancer-associated oncogene expression.

We further highlight an in vivo biosensor developed by Harvey et al. [168] based on a carbon nanotube photoluminescence as the reporter which demonstrates miRNA hybridization events and other oligonucleotides transiently. Here, DNA wraps the nanotube which has a single oligonucleotide sequence consisted of two domains: (1) nanotube binding sequence that can impart nanotube colloidal stability and (2) miRNA capture sequence with a sequence complementary to a target oligonucleotide. By using different nanotube chiralities and real-time monitoring of toehold-mediated DNA-strand displacement, which causes a reversal of the signal response, the sensor enabled multiplexed detection of several miRNA sequences. Moreover, to detect miRNA non-invasively via an implantable device in vivo, this sensor placed medially over the intestines of mice. The sensor established a detection limit of miRNA in biofluids in the picomolar range and down to 100 pmol in vivo as well as measured single-nanotube responses representing the binding of 1–60 miRNA copies [168].

3.5 CRISPR-Cas platforms for single-nucleic acid detection

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology repurposed as a tool for genome editing together with Cas9 (CRISPR associated protein 9), works like a scissors

to cut any desired DNA sequence [169-170]. CRISPR technology attracted many laboratories worldwide in the last 5 years, albeit mainly centered around editing or modifying human genome for cell therapy which are currently limited to only developed countries due to costly high-tech requirements. However, new class of diagnostic tool via CRISPR-Cas systems for the detection of virus DNA in bacteria has been reported by 3 groups in 2018 [171-174], offering superior sensitivity and inexpensive production compared to the amplification strategies for ultra-sensitive detection of nucleic acids discussed in section 3.4. Single-molecule sensitivity reached through CRISPR-based biosensors are urgently needed for early medical diagnostics, such as for parthenogenic viruses, which is extremely difficult to detect as it contains one virus copy/µL in blood serum of infected individuals [175].

One of the first of these reports about CRISPR diagnostics shown by Chen et al. discovered that Cas12a displays collateral cleavage against single-stranded (ss) DNA after binding to its specific double-stranded (ds) DNA target [171]. The reporter ssDNA which is tagged with a fluorescent probe at one end and a quencher at the other end is cleaved by Cas 12a which then can be readily detected by the “free” probe. To amplify the signal authors boosted the sensitivity by recombinase polymerase amplification (RPA) increasing the number of the target viral DNA. Using this novel technology which is termed DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter), the authors were able to achieve attomolar sensitivity as well as distinguish two distinct genotypes of human papillomavirus in crude DNA extracts from patient samples with high accuracy.

In the second report by Gootenberg et al., authors used Cas13 instead of Cas12a as in the DETECTR, that binds and cleaves ssRNA in combination with recombinase polymerase

amplification [176]. This method named as SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing) (Figure 11), not only enabled detection of specific strains of Zika and Dengue virus at attomolar sensitivity but also distinguished pathogenic bacteria, genetic variability in human DNA and identified mutations in cell-free tumor DNA. In the follow-up work SHERLOCKv2 [172] (Figure 11B), the researchers performed biochemical analysis of 17 Cas13a and Cas13b proteins and determined their sequence preferences. Then they selected four of these Cas13 proteins that have distinct cleavage properties which enabled detection of four viruses at once. Hence, they discovered that a poly-adenylate (polyA) reporter which is the product of Cas13 cleavage, can activate another CRISPR-associated nuclease Csm6. By combining Cas13 with Csm6, in which the activated Csm6 can cleave the second reporter ssRNA construct, authors boosted signal sensitivity by 3.5-fold in the SHERLOCKv2 reaction. They could not only detect RNA but also viral DNA sequences with a transcription step added in the recombinase polymerase amplification reaching a sensitivity of 2 copies/mL. As a result of the developed paper-based test, authors were able to determine the presence of viral RNA with a colorimetric readout.

The third biosensor application of CRISPR technology by Myhrvold and colleagues [173] demonstrated a new approach to SHERLOCK [176] termed as HUDSON (heating unextracted diagnostic samples to obliterate nucleases) in order to protect degradation of viral DNA from clinical specimens (Figure 11F). HUDSON applied to detect(1) Dengue (DENV) and Zika (ZIKV) flaviviruses directly from body fluids such as urine, saliva, plasma, serum and whole blood (2) in less than 2 hours (3) with minimal equipment (4) at concentrations as low as 1 copy/µL. Moreover, authors can distinguish region specific ZIKV by detecting single-nucleotide

polymorphisms and four DENV serotypes. CRISPR-Cas9-based biosensing, moreover, has been applied to understand the accumulation of mitochondrial DNA (mtDNA) mutations in single cells [177]. Authors developed a CRISPR/Cas9-mediated proximity ligation assay (CasPLA) and in situ rolling circle amplification (RCA) which enabled visualization of individual wild-type and mutated mtDNA in single cells. All these developments demonstrate the high potential of CRISPR-Cas assays that can enable the rapid and early diagnosis with high accuracy which can be beneficial for the laboratories with restricted access to costly equipment.

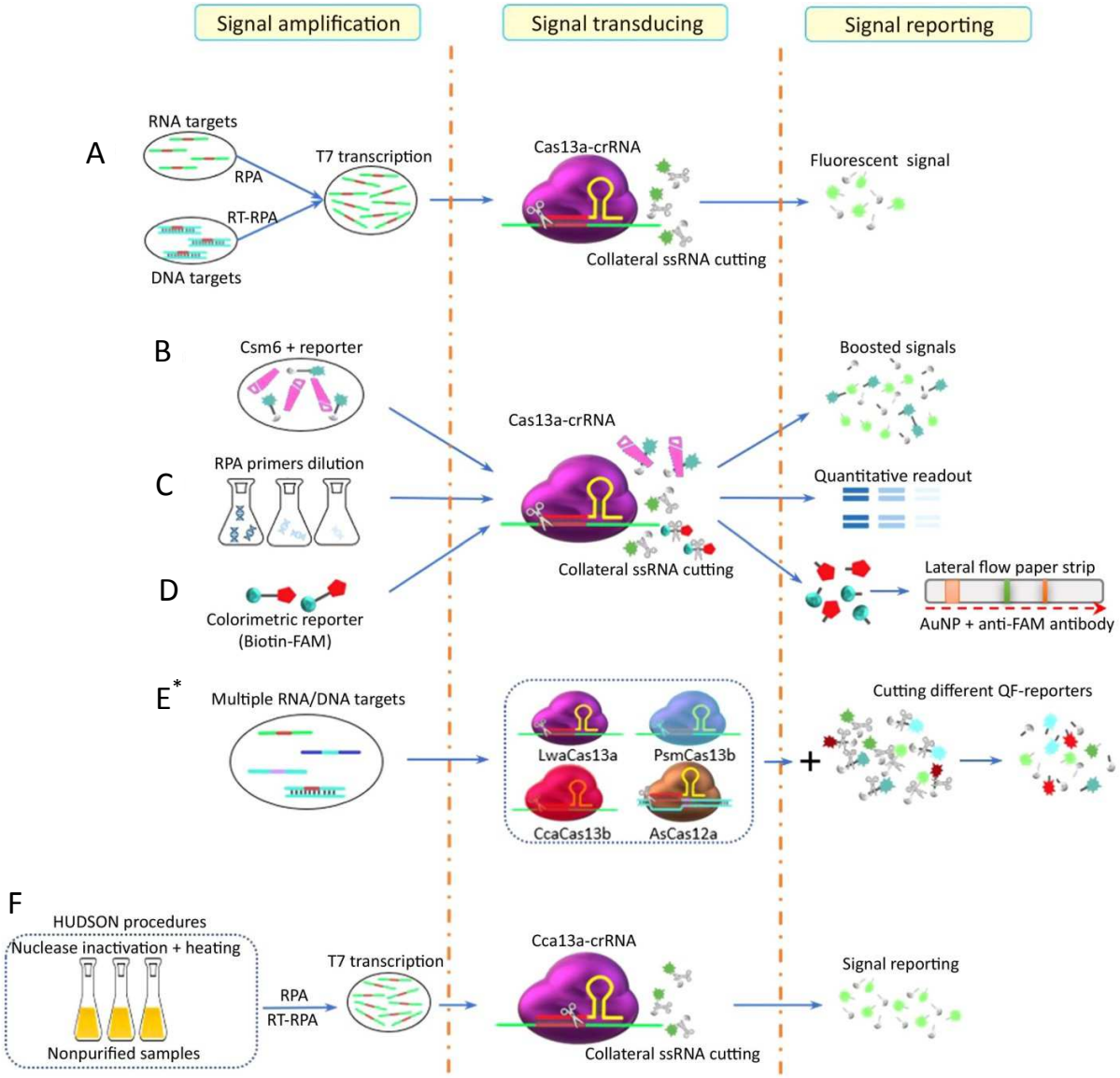

Figure 11. (A) The first SHERLOCK system. (B) SHERLOCK system with Csm6 sensitivity boosting. (C) Quantitative SHERLOCK system. (D) SHERLOCK system combining colorimetric reporter for visualized readout. (E) Four-channel multiplex detection of SHERLOCK system. (F) SHERLOCK system combing with HUDSON for eliminating the nucleic acid extraction and purification procedures. *In four-channel multiplex target detection. A Cas12a effector was used. crRNA, CRISPR RNA; HUDSON, heating unextracted diagnostic samples to obliterate nucleases; RPA, recombinase polymerase amplification; RT, reverse transcription; SHERLOCK, specific high sensitivity enzymatic reporter unlocking; ssRNA, single-stranded RNA. Reprinted with permission from [178]. Copyright 2019, Elsevier.

3.6 Single-molecule digital assays

The enzyme-linked immunosorbent assay (ELISA) has been used for decades for detecting and quantifying antibodies and antigens as well as proteins, peptides and food allergens. However, its detection limit is typically around 100 pg/well which requires large sample volumes owing weak specificity whereas in most cases analytes compete with other serum proteins. To overcome the sensitivity and specificity issues in conventional ELISA, an enzyme-linked digital immunoassays platform with a fully automated single-molecule array (Simoa) technology was introduced in 2010 [179]. Simoa sensitivity over the conventional ELISA is >1200-fold with coefficients of variation of <10% [180]. This digital assay builds on and is enabled by femtoliter-sized reaction chambers. Each chamber is loaded with one magnetic bead (2.7 μm diameter) which is covered with a pair of capture and fluorescently labeled detection antibody as in a conventional sandwich ELISA to detect single-target molecules (Figure 12). At the low concentrations of protein, only a small fraction of beads demonstrates catalytic enzyme activity, which is the signature of that beads carry either a single immunocomplex or none. This extremely sensitive digital immunoassay reached the limit of detection for streptavidin-β-galactosidase to 220 zM (~10–20 enzymes in 100 μl) as well as for prostate-specific antigen (PSA) and tumor necrosis factor-α (TNF-α) to ~200 aM (6 fg/ml) and ~600 aM (10 fg/ml) in whole serum samples, respectively [179]. Moreover, PSA in serum samples of patients who had undergone radical prostatectomy were detected at concentrations as low as 14 fg/ml (0.4 fM) [179] and PSA levels were detected in mice after tumor formation [181]. Most recently, this bead-based ELISA technology has been applied to detect various analytes at the superior sensitivity,

including, miRNA-122 [158], amyloid-β 1–42 peptide [182], ricin toxin [183], botulinum neurotoxin [184], influenza A virus [185], anti-dengue virus [186], cytokines (TNF-α, IFN-γ, IL-1β, IL-2, IL-6, and IL-10) with the capability of multiplexing [187-192] in human plasma in the range of 0.01–0.03 pg/mL [193]. However, one of the drawbacks of SIMOA is the processing of the whole blood samples prior to testing and having the detection limitation to high affinity interactions.

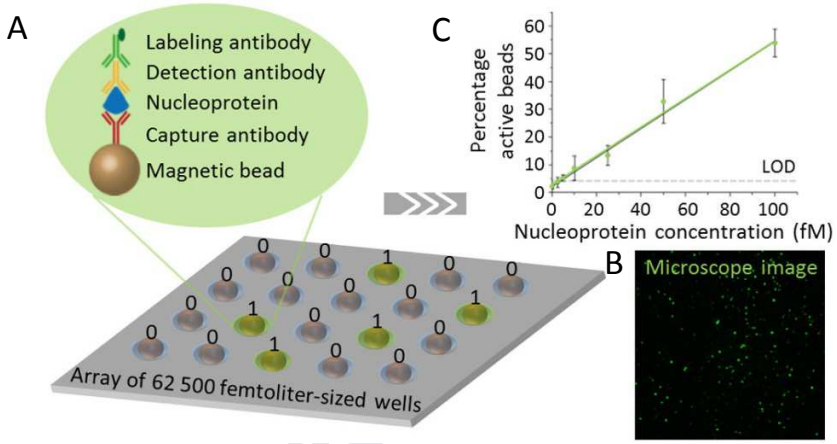

Figure 12. Schematic representation of digital ELISA. (A) Antibody-coated beads captures the single target biomolecules, which are then detected by another antibody conjugated with a labeled antibody. Loading of beads into femtoliter well arrays for isolation and detection of single molecules. (B) Fluorescence image of a small section of the femtoliter well array after signals from single molecules are generated. While the majority of femtoliter chambers contain a bead from the assay, only a fraction of those beads possess catalytic enzyme activity, indicative of a single, bound protein. (C) The concentration of protein in bulk solution is correlated to the percentage of beads that have bound a protein molecule. Reprinted with permission from [185]. Copyright 2016, American Chemical Society.

Another digital assay having a microfluidic platform that combines digital and analog detection is based on the mechanically induced trapping of molecular interactions (MITOMI). The assay

can measure broad range of molecular interactions and capable of obtaining kinetic information of several hundred independent measurements in a single experiment simultaneously [194-195]. First demonstration of MITOMI enabled characterization of the DNA binding energy landscapes for eukaryotic transcription factors [196]. In a recent work by Piraino et al. [197] MITOMI is capable of detecting down to 3−4 biomarkers in quadruplicate. The performance of the assay measured ∼10 fM (330 fg/mL) GFP in buffer and ∼12 fM GFP in human serum in only 5 µL sample with the capability of multiplexing [197]. All in all, MITOMI is promising for future SM kinetic studies while this digital immunoassay requires a fluorescently labeled molecule of interest to detect molecular interactions. Albeit all these digital platforms require biomolecules tagged with fluorescent dyes or quantum dots to quantify each molecule. However, these fluorescent probes can generate complications due to their photophysical properties and eventually can for instance, alter the molecular interactions. Hence, antibody specificity and cross-reactivity needs to be taken into careful consideration, especially when designing multiplexed assays which can be a major limiting factor in order to obtain high sensitivity and multiplexing degree.[198]

A new microscopy technique called interferometric scattering mass spectrometry (iSCAMS) has the capability of real-time imaging of single, unlabeled biomolecules in solution based on the interferometric scattered light [199] (Figure 13). Naturally, larger molecules scatter more light which enables to build a correlation between the measurements of the light with the mass of each individual protein. The researchers could measure the molecular weight of single molecules in solution ranging from about 50 to 800 kDa with 2% sequence mass accuracy, up to 19 kDa resolution and 1 kDa precision [199]. The authors were able to resolve oligomeric

distributions at high dynamic range, detected small-molecule binding events, and mass-imaged proteins with associated lipids and sugars. They could also characterize the molecular dynamics of processes as diverse as glycoprotein cross-linking, amyloidogenic protein aggregation, and actin polymerization in real time. The fascinating piece of the technique is despite the ability to measure the molecular weight of single molecules, being completely label-free as it only uses the back-scattered light to determine the molecular weight of the different species. The method has recently been commercialized by Arago Biosciences, a spin-up company from the Kukura group in Oxford.

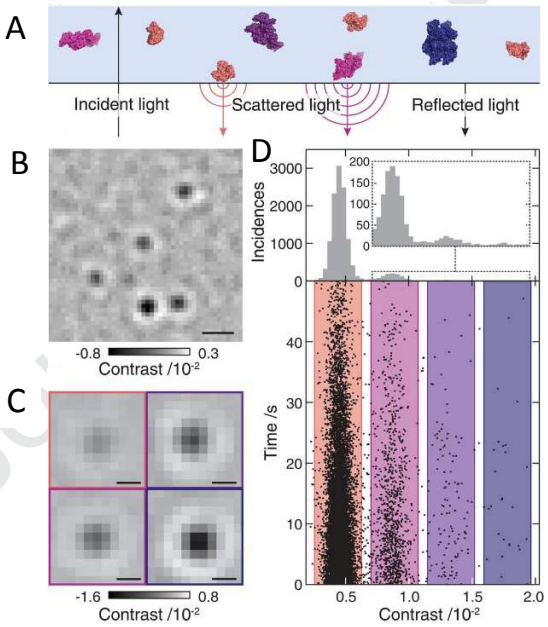

Figure 13. (A) Schematic of the experimental approach relying on immobilization of individual molecules near a refractive index interface. Oligomeric states are colored differently for clarity. (B) Differential interferometric scattering image of BSA. Scale bar, 0.5 µm. (C) Representative images of monomers (top left), dimers (bottom left), trimers (top right), and tetramers (bottom right) of BSA. Scale bars, 200 nm. (D) Scatter plot of single-molecule binding events and their scattering contrasts for 12 nM BSA from 14 movies (lower panel). Corresponding histogram (n = 12,209) with a zoomed-in view of the region for larger species (upper panel). The reduction in landing rate results from a drop in BSA concentration with time owing to the large surface-to-volume ratio of our sample cell (supplementary materials). Reprinted with permission from [199]. Copyright 2018, The American Association for the Advancement of Science.

Other potential approaches for high-throughput single-molecule biosensing, for instance, can be achieved by label free, quantum-enhancement [200] and integration of microtoroid optical resonators into the sensor arrays [150]. Noticeably, an evanescent biosensor enabled quantum noise-limited tracking of single BSA biomolecules as small as 3.5 nm. [200] Using the plasmonic effect near the microtoroid optical resonators, mass of single molecules in solution can be estimated[150] or single-exosomes can be detected[201].

4. Conclusion and perspectives

Albeit we have mainly discussed label-free and fluorescent biosensors, we highlighted novel biosensing technologies in sub-sections that can enable superior sensitivity for the detection of biologically important analytes at the single-molecule level. We furthermore pointed out recently developed emerging technologies as well as the integration of more than one technique (e.g. electrochemistry with fluorescence and SERS) which helps to increase the detection sensitivity tremendously. Fluorescent single-molecule biosensing allows tracking of individual molecules and cells with high localization, multicolor imaging, as well as monitoring single-molecule binding events, and can be extended to even three dimensions. Unless its naturally fluorescent, a non-fluorescent or weakly fluorescent molecule requires to be labeled with a high quantum-yield fluorophore or quantum dot. These sensors, however, mostly require signal amplification, which in some cases may alter the photophysical properties of the fluorescent probes. To overcome this limitation, development of novel probes is essential to overcome their short life time. One solution could be signal amplification (see section 3.4), that

can greatly enhance the desired sensitivity and selectivity such as demonstrated in isothermal amplification, rolling circle amplification or strand displacement amplification assays. Novel fluorescent biosensors such as DNA-based CRISPR technology holds great potential for on-site detection of biologically relevant analytes, especially for nucleic acid detection and single nucleotide polymorphism identification. CRISPR-based biosensing can be enhanced by combining with isothermal amplification and nucleic acid hybridization methods, making CRISPR as a powerful tool for next generation single-molecule biosensor technology. Although there are several advantages of the CRISPR/Cas system for biosensing applications such as being rapid, low-cost, highly sensitive and specific, it currently only allows for multiplexing of up to four targets. Moreover, they are not capable of real-time quantification or in vivo biosensing yet. Further efforts, however, need to be taken to translate this technology into portable device arrays with high multiplexing capability as well as their integration into the medical devices needs careful considerations in terms of biocompatibility with sensor surfaces. To date, several biotechnology companies established digital assays for high-throughput detection of target molecules at one-molecule sensitivity, albeit mainly employing nanobeads and fluorescent probes as the read out. As new technologies are emerging towards label-free detection, such as interferometric scattering spectroscopy, these assays may need to be re-designed towards more efficient assay steps, for instance reducing the washing steps and having a shorter time for assay development. Hence, employing new nanoparticles or carbon-based nanomaterials may help them to eliminate the limits of fluorescent detection especially at low-concentration regimes, and allow detection of low-affinity molecules at a higher sensitivity.

Label free single-molecule biosensing, on the other hand, showed promising and rapid developments towards miniaturized device integration due to their high sensitivity, thanks to novel nanomaterials developed during recent years. Most electrochemical biosensors developed to date are limited to detect ensemble molecules with an ultimate goal of reaching single-molecule distribution in heterogeneous samples. However, single-molecule electrochemistry alone is generally limited to detect redox current of short-lived intermediates and redox-sensitive fluorescent labels. Challenges in single molecule electrochemistry remain through the limit of current amplification strategies, albeit integration with other techniques such as fluorescence or plasmonic biosensing are highly promising to detect redox activity of a single-protein or enzymes, revealing complex kinetic rates which are hidden in ensemble measurements. Plasmonic single-molecule biosensing with nanoparticles is one to of the most studied label-free platforms to date because of their tremendously enhanced sensitivity owing to the reduced surface area as compared to the 2-D metallic films. On the other hand, plasmonic sensing with nanopores expand their applications not only for DNA sequencing which is commercially available, but also to study protein binding and enzyme reaction kinetics. Moreover, with their tunable pore sizes and easy fabrication and integration with microfluidics, nanopore-based single-molecule biosensors may open the door for high throughput multiplexing of several proteins simultaneously and single-cell sorting in the pharmaceutical companies.

Overall, the future direction of single-molecule biosensors relies on the enhancement of detection sensitivity that requires development of smart technologies with high spatial and time resolution which eventually can achieve single-molecule sensitivity not only in in vitro

bioassays but also for the purpose of uncovering heterogeneity and molecular interactions in the living organisms. Equally important, with the development of biocompatible, long-term stable nanomaterials and engineered biomolecules, we believe that it will be possible to monitor the target molecules at the single-molecule level, that will eventually open new doors in an area of highly emerging diagnostics, drug discovery and personalized medicine.

Declarations of interest

None.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

AstraZeneca authors are current or former salaried employees of AstraZeneca and have/had stock or stock options in AstraZeneca.

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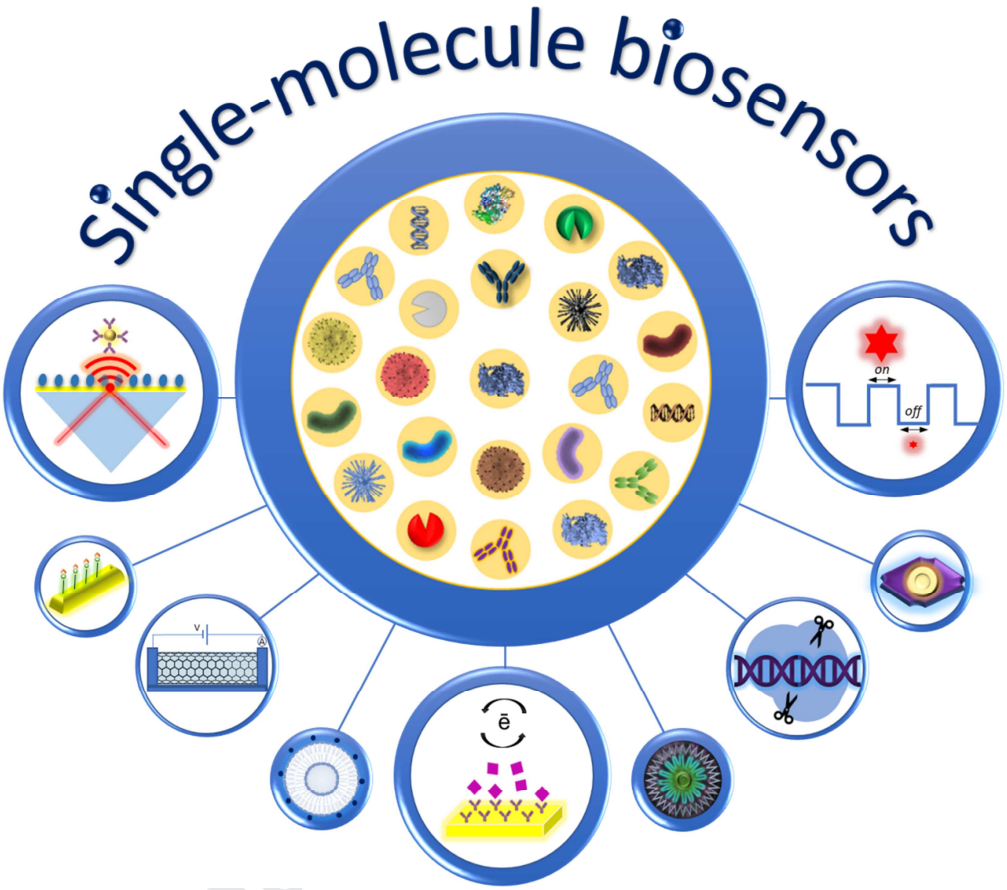
TOC Figure


Single-molecule biosensors
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Highlights

- In-depth review of major breakthroughs in single-molecule biosensors for both in vitro and in vivo detection of biologically important molecules.
- Recent advances in electrochemical, plasmonic and SERS-based biosensors as well as spectroelectrochemical biosensors with single-molecule sensitivity are discussed.
- Recent advances in single-molecule fluorescent biosensors.
- Focus on nanoparticle-amplified assays, digital platforms and CRISPR technology.
- The advantages, drawbacks and future perspectives of single-molecule biosensors were discussed.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: