Biosensor platforms for rapid HIV detection

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Abstract

Human immunodeficiency virus (HIV), a type of lentivirus (a subgroup of retrovirus), causes acquired immunodeficiency syndrome (AIDS). This pathophysiologic state destroys the immune system allowing opportunistic infections, cancer and other life-threatening diseases to thrive. Although many analytic tools including enzyme-linked immunoassay (ELISA), indirect and line immunoassay, Western blotting, radio-immunoprecipitation, nucleic acid amplification testing (NAAT) have been developed to detect HIV, recent developments in nanosensor technology have prompted its use as a novel diagnostic approach. Nanosensors provide analytical information about behavior and characteristics of particles by using biochemical reactions mediated by enzymes, immune components, cells and tissues. These reactions are transformed into decipherable signals, i.e., electrical, thermal, optical, using nano to micro scale technology. Nanosensors are capable of both quantitative and qualitative detection of HIV, are highly specific and sensitive and provide rapid reproducible results. Nanosensor

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technology can trace infant infection during mother-to-child transmission, the latent HIV pool and monitor anti-HIV therapy. In this chapter, we review nanosensor analytics including electrochemical, optical, piezoelectric, SERS-based lateral flow assay, microfluidic channel-based biosensors in the detection of HIV. Other techniques in combination with different biorecognition elements (aptamers, antibodies, oligonucleotides) are also discussed.

1. Introduction

HIV has become pandemic infecting 36.9 million people worldwide as of 2017 with 1.8 million being infected in that year alone [1]. The disease has higher incidence in sub-Saharan Africa (~25.7 million) outnumbering America and South-East Asia severalfold [2]. Despite its prevalence, only 59% of adults and 52% of children received antiretroviral therapy (ART) for treatment of HIV infection [1].

HIV mainly targets T-helper lymphocytes of the human immune system by attachment to $CD4^+$ surface protein (Fig. 1). Following incorporation, HIV uses the cell machinery for reproduction via reverse transcription, replication and multiplication. After proliferation it destroys the host cell.

HIV infection progresses through three stages.

Stage 1: Acute HIV infection occurs usually 2–4 weeks after the initial viral introduction. This stage can be characterized by high viral load in blood and increased transmission risk. Although a flu-like illness may occur, many individuals cite no or minimal clinical symptoms.

Stage 2: Clinical latency (HIV inactivity or dormancy) is the symptomatic or chronic phase of the infection wherein HIV reproduces, but at a very slow rate. This phase can persist for a decade or longer. In this phase, HIV can be transmitted.

Stage 3: AIDS, the most severe phase of infection, is when the immune system gets immensely damaged as the increased viral load lowers the $CD4^+$ cell counts. Symptoms of AIDS include chills, fever, sweats, swollen lymph glands, physical weakness and weight loss. Survival is approximately 3 years without treatment.

Based on pathogenesis, the possibility to spread the HIV infection is very small if the patient has an undetectable viral load due to long-term treatment. Antiretroviral therapy (ART) is most effective prior to the patient becoming symptomatic. Early treatment results in stabilization an improved life span. In response to ART, the HIV concentration drops to an undetectable level (~90%) in most individuals. In a community setting, all the HIV positive

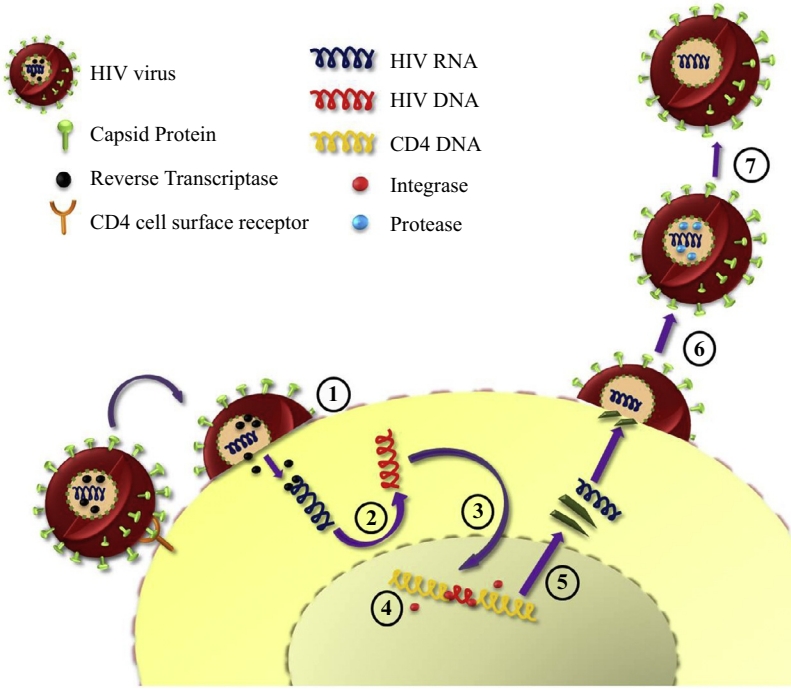

Fig. 1 Life cycle of HIV virus; (1) fusion to the host cell membrane through the $CD4^+$ cell surface receptor, (2) reverse transcription of viral RNA into DNA by reverse transcriptase of virus particle, (3 and 4) entry into the nucleus followed by the integration in the host genome by the enzyme integrase and replication by host cell enzymes, (5) transcription, (6) assembly and subsequent maturation in a virus particle and (7) finally budding out of the host cell.

people should be targeted and treated simultaneously to reduce the overall viral pool thus decreasing the potential for subsequent transmission. Unfortunately, one main drawback of ART is untracked reservoirs.

Transmission from non-diagnosed individuals substantially contribute to the viral pool. As such, improvement in HIV diagnostics has focused on early detection, i.e., before seroconversion, to increase the efficacy of the most advanced ART. It is also equally necessary to assess disease progression following ART.

Detection of HIV infection, irrespective of subtype, can be categorized into a variety of approaches relevant to disease stage. These include:

Antigen-based detection
Antibody-based detection
$CD4^+$ cell count-based detection
Viral load-based detection

Apart from these widely used approaches, another potential target in early diagnosis is the provirus stage, wherein the viral genome is integrated into host DNA. Despite its limited usefulness, it has been proven to be significant in early diagnosis of newborns, i.e., where mother-to-child transmission is suspected. This proviral testing approach also provides a viable alternative to target infected adults in the 2–8 weeks window prior to seroconversion [3]. The most common testing strategy is to identify viral antigen and/or antibodies in the circulation. Detection can also be performed by molecular analysis. Viral antigen peaks several weeks post-infection and becomes undetectable thereafter whereas the antibody can be detected in the 3–6 weeks span post-infection, i.e., the diagnostic window or serologic latency. In rare scenarios, it may take three or more months for the antibody to become detectable in blood.

Interestingly, HIV may remain undetected even in late stages potentially limiting development visible disease symptoms. Although transmission of this disease occurs through various voluntary and/or accidental body fluid exchanges, the prominent route to spread infection is blood transfusion. Although HIV testing of blood products has become routine, the lack of rapid sensitive inexpensive diagnostics has contributed to increased transfusion-related disease transmission in developing countries. As such, it is very important to detect HIV at an early stage and take consecutive preventive measures to improve survival.

Polymerase chain reaction (PCR) is a common well-standardized technique to detect HIV [4,5]. Fresh whole blood or blood dried on a filter paper can serve as specimens [6,7]. PCR results may be confirmed by immunohistochemical assays using monoclonal primary antibodies to leukocyte specific antigens and horseradish peroxidase-linked secondary antibodies [8]. Alternatively, PCR products may be hybridized to fluorescein-labeled probes in situ and individually analyzed by flow cytometry [9]. False-negative PCR results may occur when target nucleic acid is limited.

Enzyme-linked immunosorbent assay (ELISA) and agglutination assays are widely used to screen HIV. Third generation assays using recombinant proteins and peptides have largely replaced older whole virus lysate assays [10]. Unfortunately, ELISA demonstrate variable cross-reactivity with HIV variants, i.e., the O subtype tends to be missed in late infection leading to false-negative results [11,12]. It should be noted that ELISA is highly sensitive in the early phase of seroconversion only. False-positive and -negative results can occur when rapid HIV ELISA is used. As mentioned above, false-negative results are most common in specimens containing low

HIV. In contrast, false-positive result occur following administration of flu vaccine, in the presence of HLA-DR antibodies (multigravida women) or rheumatoid factor, in patients with a positive RPR test, hypergammaglobulinemia (multiple myeloma) or in autoimmune hepatitis [13].

Western blotting is a well-established confirmatory test for HIV. Unfortunately, it too is subject to the same interferences listed above. Although radio-immunoprecipitation is highly specific for viral glycoproteins, it is too labor intensive and may not detect recently identified HIV variants [14]. In fact, most standard diagnostic assays for HIV detection are not amenable in a point of care test (POCT) setting given their technical complexity, cost and personnel requirements. The ultrasensitive p24 assay, the reverse transcriptase (RT) assay and reverse transcription-quantitative PCR (RT-qPCR) have been developed to reduce expense, but are generally incompatible as a POCT [15,16].

Potential biomarkers detectable in early stage HIV infection have come to light and may be targetable by advanced versions of current devices or new-age nanosensor technology. Nanosensors (10–100 nm) are highly compact nanometer-scale devices that use optical, chemical, mechanical approaches or combination thereof to detect the presence or absence of various targets. These include chemical or biologic targets (antibodies, antigens, DNA and enzymes), physical parameters (temperature, pressure, force and mass) or particles. Biosensor technology is composed of three basic units, i.e., biorecognition, signal amplifier and transducer elements. Biosensors with nano-sized material, i.e., metallic nanoparticles (NPs), nanopolymers, etc., achieve higher sensitivity because NPs are capable of signal amplification. The high surface to volume ratio of NPs enables increased conjugation of biorecognition molecules such as oligonucleotide detection probes as well as capture probes and antibodies to target various analytes (Fig. 2).

As mentioned above, POCT platforms for HIV detection need to be simple, require low sample volume, minimal personnel training and be low cost. The first biosensors can be traced to those developed by Leland Clark for measurement various analytes including glucose and oxygen [17,18].

2. Biomolecular interaction for biosensor development

The specific sequestration of target analytes on the biosensor surface is the function of the biorecognition element. As with any analytical device, important biosensor characteristics include specificity, sensitivity, reproducibility and reusability. As such, selection of the most appropriate

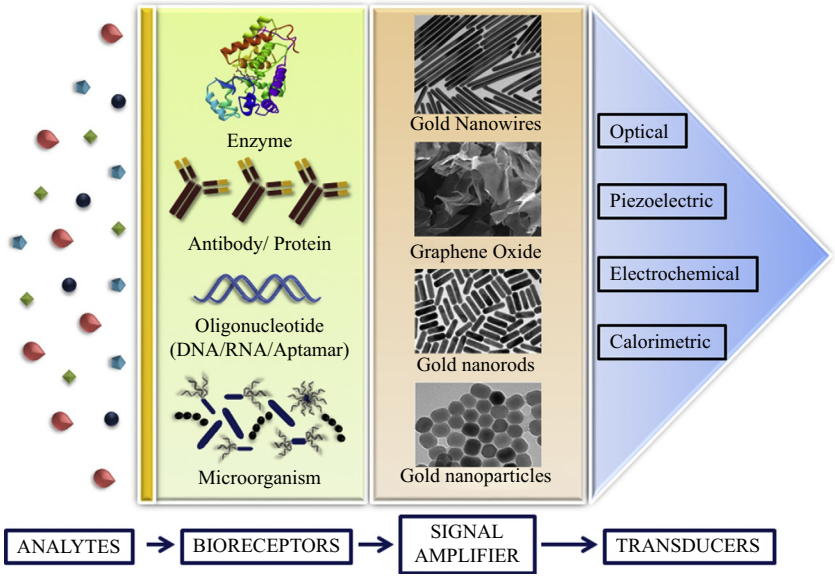

Fig. 2 Schematic structure of a biosensor; attachment of analytes via different bioreceptors such as enzymes, antibody, oligonucleotide, microorganisms, etc.; amplification of the signal by signal amplifiers; optical, piezoelectric, electrochemical, calorimetric systems which generate transducer signals.

biorecognition element is paramount to achieving these objections. The subsequent strength and specificity of the biomolecular interaction with target analytes significantly influence biosensor performance. Biomolecular interaction can be categorized as natural (antigen-antibody, enzyme, nucleic acid), synthetic (molecularly imprinted polymer (MIP)) and pseudo-natural (aptamer) (Fig. 3) [19].

2.1 Antibody

Immunodiagnostics and biomarker detection are analytical approaches for which poly-, mono- and recombinant antibodies have frequently been used.

2.2 Polyclonal antibodies

Polyclonal antibodies (pAb) are widely used in immunosensor-based assays for pathogen detection. A variety of sources (rabbits, goats, sheep) have been successfully employed to immunize and generate pAbs to target molecules [20]. Although useful for broad epitope range targets, pAbs on have limited application for assays requiring high specificity.

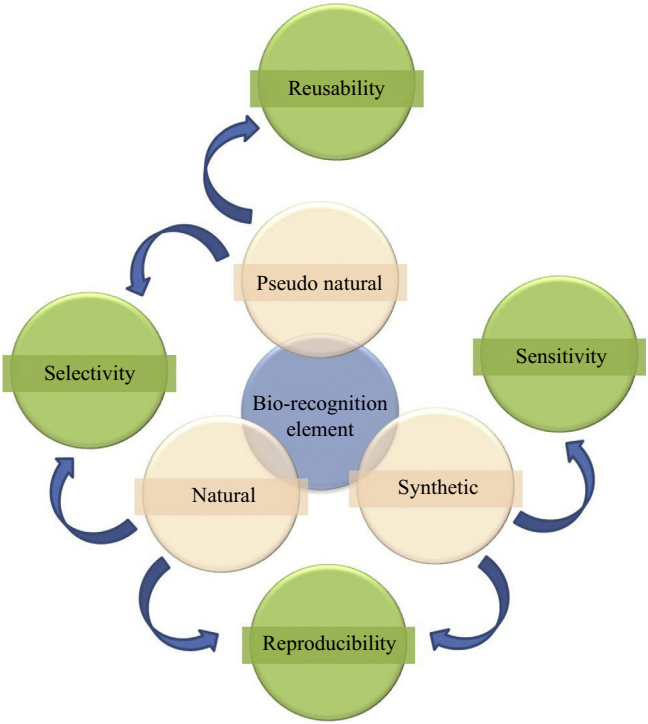

Fig. 3 Schematic diagram of types of biorecognition elements and their advantages.

2.3 Monoclonal antibodies

Hybridoma technology is exclusively used for the generation of monoclonal antibodies from murine hosts [21,22]. A single cell is "cloned out" from the hybrid colony (fusion of antibody-producing B cells from spleen or bone marrow of the immunized murine donor with immortal myeloma cells). This clone produces an antibody specific for a single epitope. Because of this uniqueness, mAbs excel in biosensors requiring a specific, sensitive and differentiating platform for closely related pathogens or structurally related molecules.

2.4 Recombinant antibodies

Recombinant antibodies are generated by a phage-display library. Three types of recombinant antibodies exist. They are naive, synthetic and immune. These can be selected to detect a wide range of the biomolecules including protein, hapten and carbohydrate-based moieties. Although recombinant antibodies offer unique advantages, most immunorecognition-based

biosensors utilize m- and pAbs. For example, one can fine-tune the selectivity of a recombinant antibody for a particular antigen by targeting the CDR regions via molecular engineering such as chain shuffling or site-directed mutagenesis [23,24]. Other flexibilities such as incorporation of tags for isolation and subsequent immobilization and availability of a range of antibody formats (Fab, scFv, re-engineered IgG dimers, etc.) makes recombinant antibodies a potentially viable alternative to traditional antibody-based approaches [25,26].

2.5 Aptamers

Aptamers are synthetic oligonucleotides (DNA and RNA) selected for specificity against a wide variety of targets, i.e., metal ions (Pb^{2+}, Hg^{2+}, K^{+}), small organic molecules (amino acids, ATP, vitamins, antibiotics, drugs) organic dyes, peptides and proteins (growth factors, thrombin, and HIV-associated peptides) as well as whole cells and microorganisms (bacteria). The selection and enrichment of aptamers does not rely on animal host systems but on the systematic evolution of ligands by exponential enrichment (SELEX). Aptamers are advantageous for detecting non-immunogenic and toxic targets and can overcome the difficulty in targeting specific biomarker regions.

2.6 Peptides

Anti-microbial peptides (AMPs) are host-derived peptides produced by a large number of living systems to protect against invading pathogens. Structurally, they are generally short (<40 amino acids), amphipathic (combine positively charged residues with hydrophobic residues) and can adopt widely-applicable secondary structures. AMPs have the potential to play a role as a selective recognition molecule in biosensors designed/fabricated for microbial diseases [27,28].

3. Signal amplifiers

To obtain necessary sensitivity, amplifiers must be employed to increase the generated signal many fold. This is especially important for very low abundance analytes in specimens of highly complex composition. A variety of configurations including capped, coated, metallic or non-metallic NPs/nanopolymers have been used as signal amplifiers (Table 1). These NPs also act as a immobilization platform for the

Table 1 Nanoparticles as signal amplifiers in biosensors for HIV detection.

Nanoparticles	Biorecognition element	Transducer	References
Colloidal gold	Capture probe for HIV oligonucleotide	Optical (lateral flow)	[29]
	HIV-1 anti-p24 antibody	Optical (ELISA)	[30]
Gold (in combination with silicon microcantilever)	HIV-1 anti-p24 antibody	Piezoelectric	[31]
Gold nanorods	Capture probe for HIV oligonucleotide		[32]
Quantum dots	Capture probe for HIV oligonucleotide	Optical	[33]
		Microfluidics	[34]
Chitosan/Fe_3O_4	Capture probe for HIV oligonucleotide	Electrochemical	[35]
Silicon nanowires	HIV-1 Rev response element (RRE) RNA	Electrochemical	[36]
Nafion-graphene composite	Capture probe for HIV oligonucleotide	Electrochemical	[37]
Graphene stabilized gold nanoclusters	Complementary oligonucleotide against HIV DNA	Electrochemical	[38]
Reduced graphene oxide	Complementary oligonucleotide against HIV-1 DNA	Electrochemical	[39]

biorecognition element. Different types of NPs in various configurations with different transducers and recognition elements together have the potential to serve as ultra-low level detectors for HIV.

4. Transducers for biosensor fabrication

4.1 Optical

Optical biosensors are generally qualitative in nature when used in a POCT setting, i.e., color change obvious to the naked eye. Although fluorescent- or chemiluminescent-based optical systems significantly improve sensitivity

Table 2 Optical transducers as HIV detection platform.

Biosensor	Biomarker target	Limit of detection (LOD)	References
Nanostructured optical photonic crystal	HIV whole virus particle	10^5 copies/mL	[40]
Single quantum dot	*gap* gene for HIV-1; *env* gene for HIV-2	–	[33]
QD (new fluorescent NP)	HIV protease	–	[41,42]
Fluorescence	Envelope protein gp120	–	[43]
Quantum dot microfluidic channel	Envelope protein gp120 and ConA lectin	–	[34]
SERS lateral flow	HIV-1 oligonucleotide	8 pg/mL	[29]
Gold nanoparticle	HIV-1 RNase H	27 units/mL	[44]
Quantum dot	HIV-1 viral DNA sequences	100 pmol/L	[32]
Quantum dot	HIV-1 protease (PR) activity	–	[45]
Fluorescent nanoparticle	HIV p24 antigen	–	[46]
Biobarcode amplification assay (BSA)	HIV capsid-p24 antigen	–	[47]

and quantitation, these instruments tend to be much more complex. Many POCT have, however, been introduced to specifically serve this purpose (Table 2).

4.1.1 Nanoplasmonic

Nanoplasmonic systems have been developed for detection and quantification of HIV and its subtypes in whole blood. These optical biosensors exploit plasmonic behavior based on two phenomena. The first involves surface plasmon resonance (SPR) using a planar, thin-film gold surface. The second involves localized surface plasmon resonance (LSPR) using confined gold NPs. SPR is a phenomenon that occurs when a metallic surface, i.e., gold, is illuminated by visible or near-infrared radiation from a monochromatic light source which first passes through a hemispherical prism and exits to a detector (photodiode array) at an angle related to the refractive index (RI). This causes free electrons to oscillate and generate surface plasmons/electromagnetic waves which resonate resulting in light absorption.

The specific wavelength/angle at which this absorption occurs is a function of the RI in the proximity of the gold surface related to the mass on the chip surface. Change in mass results from analyte interaction with the immobilized ligand causing a shift in the sensor surface resonance to a longer wavelength and change in RI [29]. Although LSPR uses the same principle, gold NPs possess high surface to volume ratio which greatly improves sensitivity and decreases signal-to-noise ratio vs conventional SPR.

Bio barcode amplification assay (BAA) is a modified version of ELISA. This approach uses NP probes, oligonucleotides (bio barcodes) and a silver amplification process for optical readout. This system targets the HIV capsid-p24 using anti-p24 antibody-coated microplates and subsequent streptavidin-coated NP-based bio barcode DNA amplification. A chip-based scanometric method is used for signal detection. Although somewhat lengthy ($2-3$ h), this approach is about 150-fold more sensitive than traditional ELISA-based methods [30].

To improve sensitivity and specificity, a sensor was developed to detect HIV-1 viral DNA without modification using the second-order non-linear optical (NLO) property of gold nanorods [31]. Signal intensity increased 45-fold increase when the *ss-gag* gene was hybridized to target DNA. Complementary matching region of the DNA sequence was found within the nucleocapsid portion of the HIV *gag* gene and tagged with gold nanorods (probe). Fragments containing different lengths of *gag* region of HIV-1 DNA were used as targets. The dipolar and quadripolar contributions created by the harmonic energy of electric dipoles and higher multipoles gave rise to hyper-Rayleigh scattering (HRS) light as the detection signal. The HRS intensity varied after the addition of target DNA into the probe HIV-1 *gag* gene DNA (400 pmol/L). Distinct HRS intensity change ($\sim$10%) after hybridization with single-stranded DNA ssDNA probe was detected even at very low concentration ($\sim$100 pmol/L).

Variable detection sensitivity is due to the fact that ss-oligonucleotides have different electrostatic characteristics than ds-oligonucleotides. This property is the result of ssDNA backbone flexibility when absorbed on Au-nanorods. In contrast, double-stranded DNA (dsDNA) is unable to uncoil itself sufficiently thus the negative charges on the phosphate backbones repulse the nanorods. Hybridization with complementary DNA, i.e., the target, causes dsDNA detachment from the nanorod surface. Detachment results in Au-nanorod aggregation. Increased hybridization of

target HIV oligonucleotides and probe oligonucleotides results in larger damping of the surface plasmon absorption. Following aggregation, HRS changes in response to various factors. These include lost center of symmetry in the nanorods and change of multipoles into strong dipoles, increase single photon resonance enhancement factors of nanorods, increase in size due to aggregation, etc. Hybridization results in significant color change, i.e., a qualitative colorimetric change. The only limitation is slow adsorption of long ssDNA sequences onto AuNPs impedes hybridization [32].

4.1.2 Fluorescence

Fluorescence, i.e., the ability to absorb light at a specific wavelength and emit light of a longer wavelength, is one of the most common optical methods in diagnostic analytics. Fluorescent markers are often used in conjunction with antibodies to specifically tag proteins or cellular components. Fluorescence is widely used in flow cytometry for detection and enumeration of cells. Despite its wide use as a diagnostic tool in the clinical laboratory, this technology is incompatible with a POCT application.

4.1.2.1 Fluorescent nanoparticle-based assay

The use of fluorescent NPs overcomes some of the limitations associated with traditional ELISA and fluorescent-based immunoassays. For example, a fluorescent silver NP (FSNP)-based approach was developed for detection of HIV-1 p24 antigen in clinical specimens [46]. The sandwich immunoassay format demonstrated increased specificity and sensitivity using streptavidin-conjugated FSNPs and biotinylated Ab. Fluorescence intensity was monitored using 401 nm excitation and 716 nm emission in time-resolved mode.

4.1.2.2 Quantum dots

Quantum dots (QD) act as a nanostructure and possess a broad excitation spectra and size-tunable photoluminescence properties having narrow emission bandwidth, exceptional photochemical stability and enhanced quantum yield. Conserved sequences of *gap* gene for HIV-1 and *env* gene for HIV-2 were the two targets used for DNA-based detection [33]. In this study, Alexa fluor 488, Alexa fluor 647 and 605 emission QDs were used as complementary synergistic diagnostic performers in this sensor. A 488 nm laser was used as the excitation source to simultaneously excite 605 QD and Alexa fluor 488. It should be noted that spectral crosstalk between the emission spectra of 605 QD and the absorption spectra of Alexa fluor 647 does occur.

As such, three non-overlapping different emission spectra were visible in the respective channels with the 488 nm laser. HIV-1 target oligonucleotide (*gap* gene) was complementary to both the biotinylated capture probe 1 and the Alexa fluor labeled reporter probe 1. Similarly, HIV-2 target oligonucleotide (*env* gene) was complementary to both the capture probe 2 and Alexa fluor 647 labeled reporter probe 2. The oligonucleotide of HIV-1 bound the aforementioned capture and reporter probe 1 in sandwich hybridization and then the streptavidin-coated QDs via biotin. Thus, only emission for Alexa fluor 488 and QD 605 was detected. When only HIV-2 specific target genes are present, emissions for QD 605 and Alexa fluor 647 were detected. In the presence of both target genes, the Alexa fluor 488-DNA-605 QD-DNA-Alexa fluor 647 complex was formed and the emission spectra for each fluorescent component monitored.

There are other QD (a new class fluorescent NP)-based biosensors targeting other HIV proteins and enzymes of HIV. Due to their ability to regulate biochemical activity of many proteins post-translationally, proteases have been of increasing interest in pathophysiologic states such as cancer [48], AIDS, inflammation and neurodegeneration [41,42]. A fluorescence recovery assay was developed using a fluorescence resonance energy transfer (FRET) quenched acid stable QD-peptide complex [45]. In the presence of HIV protease, the conjugated peptide (which is the quencher of the QD's fluorescent emission) is cleaved resulting in an eightfold increase in QD photoluminescence. Similarly, Biswas and coworkers presented a quantitative QD-based FRET assay using a unique cysteine residue (quencher) for HIV-1 protease in living cells [49,50].

4.1.3 Photonic-crystal

The integration of nanotechnology and optical biosensing provided a novel approach for HIV detection. The nanostructure detection platform uses photonic crystals (PC), a periodic surface structure composed of a low RI polymer coated with a high RI TiO_2 dielectric layer. Alterations in the dielectric permittivity at the transducer-substrate interface provides a rapid sensitive optical detection of biomolecules, cells and viruses [51]. Optical resonance is established at a precise wavelength as a result of a periodic arrangement of dielectric material on a PC sensor causing electromagnetic standing waves to form, which extend into the liquid media in contact with the PC surface. The PC structure, when illuminated with a collimated broadband light source at a normal incidence, reflects only a narrow resonant band of wavelengths with nearly 100% efficiency giving a peak wavelength

value (PWV) and allows all other wavelengths to pass through. The principle of detection results from the shift of the PWV of the reflected resonant spectrum to greater values following target binding.

A sensing platform was developed to detect viral load in whole blood against different subtypes of HIV-1 (A, B and D) [52]. In this device, the surface was immobilized with a control group of unmodified PC, i.e., 3-mercaptopropyltrimethoxysilane

(3-MPS) as a surface activator and modified with N-gamma-maleimidobutyryl-oxysuccinimide ester (GMBS). GMBS provides succinimide on the surface for binding of neutravidin which subsequently allows better immobilization and orientation of the polyclonal antibody (biotinylated anti-gp 120) for increased capture efficiency vs other methods such as physical absorption and chemical binding. An HIV-1 spiked sample was used to test the ability of the device to detect and capture the virus. Analysis of variance (ANOVA) statistical analysis demonstrated that his approach was capable of detecting HIV-1 viral load $>10^5$ copies/mL and was specific for subtypes A, B and D.

4.1.4 Lateral flow

This technique involves a lateral flow (LF) sandwich hybridization strip for qualitative and quantitative detection of HIV-1. This simple, stable, cost-effective and user-friendly device provides rapid analysis with negligible interference due to chromatographic separation. The inclusion of surface-enhanced Raman spectroscopy (SERS) resolves problems such as low sensitivity. The use of AuNPs further enhance the SERS effect due to homogeneous aggregation [53–55], excellent biocompatibility with other biomolecules such as protein [56], DNA [57,58] and antibodies [59–61]. Instead of oligonucleotide-modified AuNPs, a Raman malachite green isothiocyanate (MGITC)-based reporter and detection oligonucleotide functionalized AuNP detection were used. In this system, Raman intensity is used for quantitation and optical signal for qualitative analysis.

The LF strip conjugate pad contained the DNA probe conjugated AuNPs designed for target DNA. Streptavidin-biotinylated control DNA (complementary to a region of the detection DNA) and streptavidin-biotinylated capture DNA (complementary to a region of the target DNA) acted as control and test lines, respectively. Following application, target DNA migrated via capillary action and subsequent hybridization to the DNA probe on the surface of MGITC-AuNPs immobilized on the conjugate pad. The resultant MGITC-AuNPs-detection DNA-target DNA

complex further migrated to the test line where it bound capture DNA. Excess detection DNA conjugated AuNPs migrated to the control line and bound probe DNA. It should be noted that the two red lines appearing on the test and control lines correspond to two independent hybridization events. In the absence of target HIV-1 specific DNA, only the second hybridization event occurs generating a red color on the control line thus providing a qualitative measurement HIV-1. Quantitation can be assessed from red band intensity on the test line by a Raman instrument [29] (Fig. 4).

4.1.5 Microfluidics

On-chip HIV capture and imaging methods have been developed using QDs. Anti-gp 120 antibody immobilized on a microfluidic chip was used

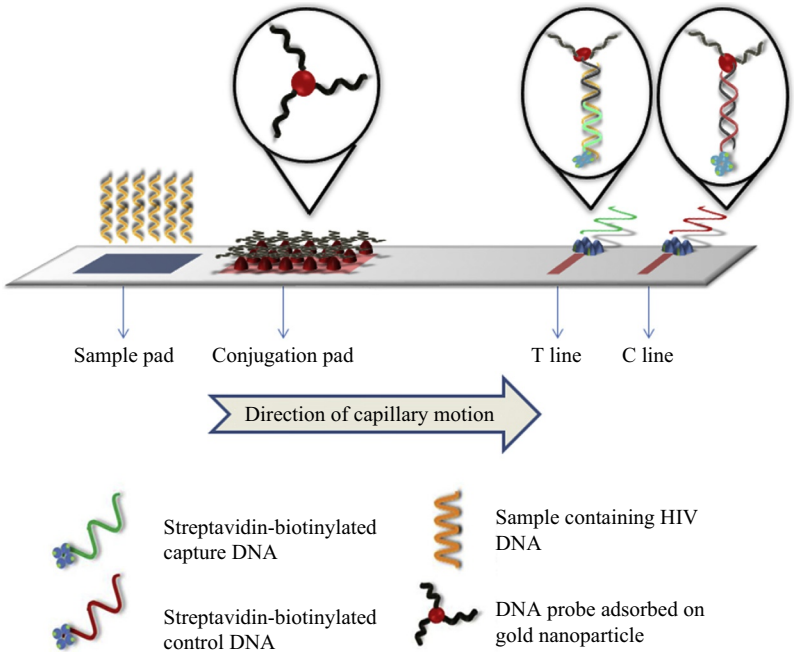

Fig. 4 Lateral flow device. As the sample flows through the device by capillary motion it might encounter both control DNA and capture DNA immobilized on the C line and T line, respectively. Visible red color will develop in T and C line when the sample has the target oligonucleotide complementary to a segment of the capture DNA (HIV DNA). No color will be observed in the T line if sample does not contain the target DNA to bind with the capture DNA, as the detection DNA shows complementarity only to the control DNA thus causing color development only on the C line, which reach desired limit of detection.

to capture the whole HIV particles from finger stick whole blood (~10 μL) [62]. Because they are exceptionally bright due to high extinction coefficient and quantum yield, QDs can overcome photobleaching and specific excitation ranges when conventional virus membrane staining dyes and nucleophilic dyes are used. Nanometer-sized semiconductor particles have been widely used for virus imaging under high magnification because of their unique size-dependent characteristics including photostability, narrow and more symmetric emission spectra and multicolor light emission properties [63].

Another microfluidic device was designed by using polymethylmethacrylate (PMMA) on glass to perform HIV capture and imaging directly from whole blood of patient. PMMA double-sided adhesive (PMMA-DSA) film was attached to a glass slide (pretreated with oxygen plasma) to form a microfluidic channel. Immobilized biotinylated antibody was then incubated with N-gamma-maleimidobutyryl-oxysuccinimide ester (GMBS) and neutravidin. NPs used were QD 525 (red) streptavidin conjugate coupled with biotinylated anti-gp120 antibody and QD 655 (green) streptavidin conjugate coupled with biotinylated Concanavalin A (ConA) lectin. These antibodies were targeted against the key viral proteins, i.e., gp120 glycoprotein on the outer surface of the HIV envelope and mannose residues expressed on the gp120 glycoprotein, respectively. False-positivity was avoided by using lectin vs HIV-1 gp120 antibody. In the microscopic images, co-localized (yellow) signals were generated by probes (red and green) to HIV gp120 glycoprotein and its mannose oligosaccharides. Increased brightness of QDs helps to image dual-stained HIV using low magnification (10×) optical imaging systems.

Critical issues such as the amount of time required for optimal binding and capture efficiency for quantitative purposes is needed relative to the gold standard, i.e., real-time polymerase chain reaction (RT-PCR). Despite these challenges, this rapid (< 10 min), handheld, inexpensive and disposable approach remains a viable option for HIV detection and monitoring [34].

4.2 Micromechanical

4.2.1 Cantilever

In this technology, target molecule binding is sensed mechanically via changes in deflection or dynamic frequency resonance of a micrometer-sized suspended microcantilever beam. Binding to the cantilever can occur via electrostatic repulsion/attraction, hydration/entropic effect and steric interaction. Subsequent cantilever deflection can be measured using a

reflecting laser beam or electrical readout [64]. In antigen-antibody reactions, the cantilever beam shows increased strain energy and reduced reaction free energy. This enables microcantilevers to detect targets at low concentration (pmol/L) with high resolution (nm) [65].

HIV antigen-specific (mainly surface antigen) antibodies have been used to activate the cantilever surface. For example, a cantilever-based platform fabrication was developed to detect HIV-1 envelope glycoprotein (*env* gene) gp120 [66,67]. Researchers modified the cantilever surface by immobilizing monoclonal anti-A32 antibodies (primary antibody) via self-assembled monolayers. Subsequent molecular interactions were obtained via capture of gp-120 protein and the secondary antibody (monoclonal anti-17b antibodies). Cumulative deflections were measured after each surface modification. Using this approach, the limit of detection (LOD) was ~400 ng/mL in vitro and 0.2–100 ng/mL in vivo. Because cells generally express very low gp120 on their surface, this strategy has proven effective for differentiating and capturing free circulating virus. As such, cantilever-based sensors appear useful for small deflection molecular interaction.

Modifications to traditional immunoassay systems targeting HIV capsid antigen p24 include nuclease-linked fluorescence oligonucleotide assay [68], digital bead-based ELISA [69], and ELISA combined with thio-NAD cycling [70]. Although these techniques managed to achieve the desired LOD, they remained a multi-step technology in order to obtain the necessary signal amplification. The nano-mechanical sandwich assay comprises two reaction steps. In the first reaction, the capture antibody-functionalized silicon microcantilevers were incubated with human serum using a traditional 96-well microtiter plate. In the second reaction, the detection antibody functionalized 100 nm AuNPs bind the captured p24 antigen on the cantilever surface. The AuNPs act both a plasmonic and mass label. "Mass increase," followed by capture of NPs resulted in increased mechanical resonance detected via a piezoelectric actuator attached to the chip-base with a LOD of 1 virion/10 mL plasma (10^{-17} g/mL). This approach resulted in improved detection of traditional immunoassays fivefold and nucleic acid amplification tests (NAAT) twofold.

4.2.2 Piezoelectric crystal (QCM)

The principle on which piezoelectric biosensors operate is the change in mass via biomolecular, i.e., antigen-antibody, interaction. Quartz crystal, a widely applied piezoelectric biosensor, transduces mass change into altered resonance frequency. Quartz crystal microbalance (QCM) is capable of

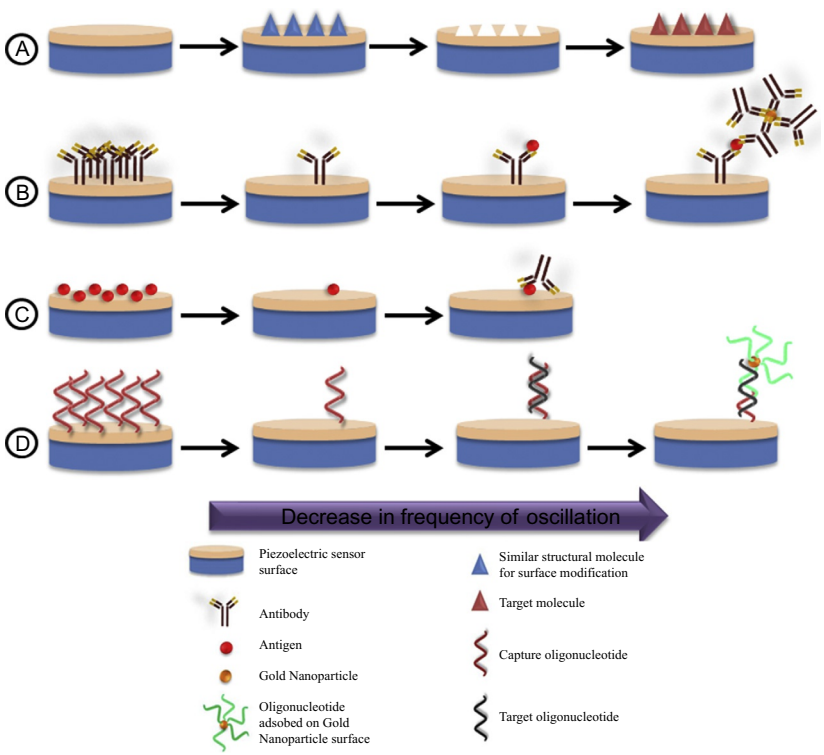

Fig. 5 Principle of piezoelectric biosensor for detection of antigen (B), antibody (C) or oligonucleotide (D). The surface is modified using a replica molecule for accurate detection (A). Often conjugates of nanoparticle and antibody or oligonucleotides are used to amplify the signal by decreasing the oscillation of the piezoelectric crystal (B and D).

direct detection of analytes, i.e., bacterial cells, and eliminates the need for labeled secondary antibodies. In general, detection devices based on piezoelectric materials that generate surface acoustic waves (SAW) are capable of relatively simple, robust and rapid real-time measurements [71] (Fig. 5).

Bionanosensors (BNS) based on antigen-antibody interaction to detect HIV-1 and -2 have been developed [71]. The thickness shear mode (TSM) resonator-based immunosensor directly measures HIV surface glycoprotein gp120 in human plasma. Sensor wave deflection was used to assess in response to binding of gp120 binding to polyclonal anti-HIV-1 antibody coated on the TSM surface.

Sequential binding of antibody, antibody/blocker and antibody/blocker in the presence of gp120 consistently resulted in increased BNS frequency response (5–8000 Hz). Sensor sensitivity was assessed using serial dilutions of

gp-120, HIV-1 virion with or without gp-120, and HIV-1 seropositive plasma. BNS signal intensity and HIV-1 load positively correlated between 0.14 and 1.6×10^6 viral particles/mL with a predicted LOD of 6.5×10^4 viral particles/mL [72].

A prototype biosensor based on antibody-functionalized (specific to HIV-1 and -2) piezoelectric materials has been developed [71]. This study used a modified lithium tantalite ($LiTaO_3$)-based acoustic wave biosensor to differentiate HIV-1 and -2 serotypes in human blood. This biosensor was functionalized with surface glycoprotein gp24 and gp39 antibodies specific for HIV-1 and -2, respectively. HIV detection was expressed as phase (mass) shift recorded with LabVIEW software. A dose-dependent linear increase, with phase shift values, was detected for both HIV-1 and HIV-2 in the tissue culture infective dose 50 ($TCID_{50}$) ranges. The LOD for HIV-1 and -2 was 12 and 87 $TCID_{50}$, respectively (Table 3). Similar results were obtained with the phase shift and $TCID_{50}$ when tested in 50% human plasma. Increased phase shift in the presence of both HIV-1 and -2 was likely due to added mass.

4.3 Electrochemical

In this approach, the coupling of a specific antibody with an electrode transducer converts a binding event into an electrical signal. A number of electrochemical biosensors [73] have been developed for detection of biologic and chemical entities [74]. Amperometric, impedimetric, conductimetric and potentiometric transducers have been used in electrochemical biosensors for HIV detection (Table 4).

Table 3 Mechanical transducers as HIV detection platform.

Biosensor	Biomarker target	Limit of detection (LOD)	References
Gold microcantilever	gp-120 (for HIV-1)	400 ng/mL (in vitro)	[64]
Silicone microcantilever	HIV-1 p24 antigen	10^{-17} g/mL	[31]
Piezoelectric sensor (QCM)	gp120 (for HIV-1)	6.5×10^4 virus particles/mL	[72]
Piezoelectric sensor (QCM)	gp24 (for HIV-1) gp39 (for HIV-2)	12 $TCID_{50}$s (for HIV-1) 87 $TCID_{50}$s (for HIV-2)	[71]

Table 4 Electrochemical transducers as HIV detection platform.

Biosensor	Nanoparticle	Biomarker target	Limit of detection (LOD)	References
Electrochemical impedance	Electrode pixels	$CD4^+$ T lymphocytes	Single cell level	[75]
Electrochemical magneto actuated	Magnetic particles	$CD4^+$ T lymphocytes	(<200/μL); clinically relevant range for HIV screening	[76]
Electrochemical molecular beacon coupled with Nafion-graphene composite film-modified SPCE	Molecular beacon-CA conjugated	HIV-1 *gag* gene oligonucleotide	5 ng	[37]
	Magnetic nanoparticle (MNP)	HIV DNA	160 pmol/L	[77]
Electrochemical detection of short HIV sequences (SWV and EIS)	Chitosan/Fe_3O_4 nanoparticle	HIV sequences	(as low as 50 pmol/L)	[35]
	Gold nanoparticles (AuNPs)	HIV-1 virus like particle	600 fg/mL to 375 pg/mL	[78]
DPV	Long-range self-assembled DNA nanostructures	(38-base) (HIV)-1 U5 long terminal repeat sequence	5 attomol/L	[79]
EIS and CV hybrid method	Au–Ag nanoparticle composite and polymer polypyrrole (PPy)	HIV DNA	0.5 pmol/L	[80]
CV, SWV, EIS	AuNPs	Reverse transcriptase (RT) of HIV-1	0.8 pg/mL (0.7 fmol/L)	[81]
CV and EIS	Graphene stabilized gold nanoclusters (GR/AuNCs)	Exonuclease III-assisted DNA recycling amplification	30 attomol/L	[38]

SWV	Carbon nanotubes	Specific viral sequence of HIV	0.1 pg/L	[82]
CV, DPV, EIS	Enzyme encapsulated in carbon nanotubes-silica as a matrix and graphene oxide (GO) as a nanocarrier	HIV p24	0.5 pg/mL to 8.5 ng/mL with the detection limit of 0.15 pg/mL	[83]
EIS	Graphene oxide (GO)	HIV DNA oligonucleotide	$3–10^{13}$ mol/L	[39]
CV, DPV	AuNPs-SWCNT	HIV-1 PR		[84]
	AuNPs-MWCNTs and AuNPs-AEP	HIV-1 p24	(0.01–60.00 ng/mL)	[85]
	Thiolated single-walled carbon nanotube (SWCNT)/gold nanoparticle (AuNP)	(HIV-1 PR)	10 pmol/L	[86]
	Silicon dioxide-coated magnetic Fe_3O_4 NPs (MNPs)	HIV p24		[87]
DPV	VSNEA (vertical silicon nanowire electrode array)	RNA	1.513 fmol/L	[36]
	Graphene-based field-effect transistors (FET)	HIV p24 antigen	100 fg/mL	[88]

4.3.1 Micro-pixelated electrode-based detection

$CD4^+$ T-helper lymphocytes are the main HIV harbor and are subsequently destroyed during its replication. As such, peripheral blood $CD4^+$ cell count is highly correlated to HIV disease status. For example, a $CD4^+$ cell count greater than or equal to 500/μL is considered healthy whereas a count below 200/μL is considered infected [89].

Although flow cytometry remains the mainstay of $CD4^+$ enumeration, a variety of biosensors have been developed for this application. These include membrane-based microchips and microfluidic devices. Unfortunately, these approaches are limited with respect to dynamic range and potential complexity of the sample matrix. Counting error increases proportionally with cell number and is further complicated by the sparse number of $CD4^+$ cells, i.e., <20/sample. These limitations, however, may be overcome by the use of electrochemical impedance spectroscopy (EIS). This platform is composed of a densely packed array of pixelated working electrodes (100/row), a reference electrode and a counter electrode on a Si/Si_3N_4 base. Electrodes were connected through an aqueous phase composed on phosphate buffered saline. Two polydimethylsiloxane (PDMS) sheets were placed onto the electrodes. One sheet was patterned with a window to serve as a buffer container. The second sheet served as a cover such that the device could perform accurately for hours without drying.

Working electrode pixels (each $7 \times 7\,\mu m$, i.e., comparable to a single $CD4^+$ diameter 8–10 μm) were chemically modified sequentially with (i) -11-mercaptoundecanoic acid (MUA)/3-mercaptopropanoic acid (MPA), (ii) N-hydroxysuccinimide (NHS)-1-ethyl-3-(3-dimethylaminopropyl) (EDAC), (iii) anti-$CD4^+$ antibody and finally (iv) bovine serum albumin (BSA) to prevent nonspecific binding [75]. Optimization of the resultant impedance (Z) change at 0.1 Hz frequency of the working electrodes was assessed by EIS measurement at each modification step. Impedance change due to capture of $CD4^+$ cells clearly distinguished occupied vs unoccupied electrodes by indicating "on" and "off," respectively. This digitalized approach and ability to count individual $CD4^+$ cells on a single electrode surface may prove useful as a POCT technology.

4.3.2 Electrochemical magneto-actuated biosensor

Peripheral blood $CD4^+$ count below 200/μL is a critical marker in identifying disease progression in HIV infection. In contrast to the macrophage and monocytes, $CD4^+$ T lymphocytes express both CD3 and CD4 surface markers. An electrochemical magneto-actuated sensor has been developed to assess this

difference [76]. This approach uses anti-CD3 antibody-functionalized magnetic particles to separate $CD3^+$ cells then an anti-CD4 antibody-functionalized electrode to separate $CD4^+$ T-helper cells. This technology was successfully employed to small whole blood sample volumes (10 μL).

4.3.3 Nafion-graphene composite film-modified SPCE-based biosensor

Molecular beacons (MB) are dually labeled oligonucleotide probes useful for direct and continuous analyte measurement. This highly sensitive technique is based on a stem-loop structure in the absence of analyte, but opens to permit hybridization when analyte is present.

Using this approach, a MB was designed against the conserved sequences of *gag-1* gene exclusive to HIV-1 to cover almost all subtypes and have no overlap or similarity with the human genome. In this study, carminic acid (CA) was used as the labeling agent because its self-association property (dimer to monomer) to electrochemically demonstrate "active" vs "inactive" status of the MB [90–92].

Graphene, with its novel properties such as high mobility of charge carriers, high surface area and outstanding electrical conductivity, acts as an electrode surface modifying agent in this sensing platform [93–95]. Subsequent work showed that Nafion promoted dispersion of graphene in aqueous solutions to prevent formation of irreversible aggregates and thus provide additional stability to graphene-modified screen-printed carbon electrodes (SPCE) [96–98].

In the absence of HIV-1 *gag* gene oligonucleotide, CA monomers were close enough for self-association into dimers and the MB assumed a stem-loop conformation. Therefore, the electrochemical signal of the CA monomer remained switched off. When the target probe induced a change in MB structure during hybridization, it facilitated the dissociation of the aromatic groups of CA and the signal gets switched on (Fig. 6). The electrochemical signal could be visually observed when the hybridization mixture is poured onto the graphene-Nafion-modified SPCE. In serum, the LOD was 5 ng for HIV-1 with recovery of 88–107% [37].

4.3.4 Vertical silicon nanowire electrode array (VSNEA)

A promising electrochemical biosensor was developed using silicon nanowires (NWs) in combination with artificial peptides to recognize HIV-1 Rev response element (RRE) RNA. NWs overcome the problems associated with nano-integrated devices via controlled particle size and uniform dispersion. In addition, NWs mitigate aggregation-influenced degradation

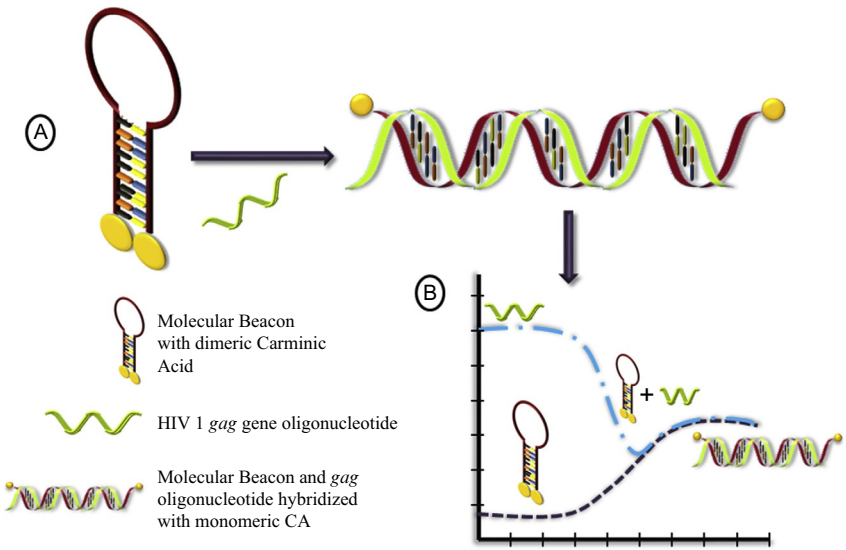

Fig. 6 (A) HIV-1 *gag* gene binds with the complementary molecular beacon and dimeric carminic acid dissociates, becomes monomeric and gives detectable signal; (B) after hybridization change in the differential pulse voltammetry (DPV) signal transduced by the functionalized electrochemical sensor.

associated with high temperature device fabrication, a phenomenon that leads to a loss of sensing strength [99].

In one NW study, artificial peptides were used as capture probes instead of purified viral proteins. Artificial peptides are economical, small in size, synthesis less laborious and more reproducibly controlled. Sensitivity and selectivity are dependent on the density and size of NWs [19]. In this approach, the VSNEA plays the role of an electrode with a partially blocked surface operating under total overlap diffusion conditions [100].

The first step in creating this platform was fabrication of the Au-coated VSNEA [101,102]. Immobilization of peptides on the surface of VSNEA was confirmed by cyclic voltammetry. The unmodified VSNEA produces well-defined redox peaks with an anodic peak potential (Epa) of 0.13 mV (potential range 0.8 to −0.8 V) with 43.05 μA of anodic peak current (Ia). The redox reaction can be attributed to these peaks at the VSNEA surface. In the latter steps of fabrication, peptide (34.32 μA) and RNA (25.46 μA) immobilized on the VSNEA surface resulted in gradually decreased Ia. The peptides and RNA covered the VSNEA surface and interrupted transfer by blocking the current pathway. Therefore, the inhibiting effect of immobilization and functionalization on the redox reaction

$(Fe(CN_6)^{4-} \leftrightarrow Fe(CN_6)^{3-} + e)$ was demonstrated by Ia decrease. A concentration gradient of RRE IIB RNA (1–20 fmol/L) was used to characterize differential pulse voltammetry signals of the RNA functionalized VSNEA. The Ia decrease was proportional to increased RNA concentration. LOD was calculated to be 1.5 fmol/L. VSNEA device performance was found to be superior to other biosensors using nanopore [103], NPs [104] and graphene composite film technology [37]. The nanosize and unique shape of NWs as an electrode contributed to improved sensitivity via enhanced mass transport and fast electron transfer kinetics. Advantages of the vertical one-dimensional NW electrodes include high surface area to volume ratio and total overlap diffusion of ions [36].

4.3.5 Chitosan (CS)/Fe_3O_4

A novel CS/Fe_3O_4-based platform for electrochemical DNA detection of HIV-1 was developed using methylene blue (MB), an intercalating organic dye, as the electroactive indicator of DNA hybridization. Fe_3O_4 is one of the metal-oxide NPs with large surface to volume ratio, high surface reaction activity, high catalytic efficiency and strong adsorption ability. These characteristics are useful for improving biosensor sensitivity and stability [105–107]. In addition, chitosan possesses excellent biocompatibility and can form a stable link with DNA thus strengthening immobilization vs traditional self-assembly monolayer and adsorption. Together, chitosan and Fe_3O_4 enhanced electron transfer resulting in increased sensitivity. The screen-printed electrode (SPE) was functionalized with an organic-inorganic (chitosan/Fe_3O_4) expected to synergistically improve biosensor performance [92].

MB is the hybridization redox marker used in this biosensor. DNA hybridization event between the target HIV oligonucleotide and 25-mer gene expression of modulator 91 (GEM91) is the immunoreaction homolog at the electrochemical interface. Square wave voltammetry and EIS measurements were taken during the hybridization event with a complementary target probe. The current peak induced by MB reduction decreased after GEM91 immobilization onto the SPE. A further decrease in current peak was observed following hybridization thus indicating the presence of the target oligonucleotide. No significant current differences were observed with non-complementary or single base pair mismatch 1m-HIV oligonucleotide. Decreased signal was the result of a true hybridization event not nonspecific adsorption or signal instability. Electron route transfer of electrons was from the electrode surface via Fe^{3+}/Fe^{2+} through hybrid to MB/LB. It is noteworthy that MB has increased affinity to single- rather than double-stranded

DNA. Following hybridization, the target strand significantly hinders MB diffusion to the electrode surface by forming an inert-electron and mass transfer blocking layer thereby decreasing EIS and SWV [35].

4.3.6 AuNPs nanocomposite for label-free detection

This sensor is based on label-free electrochemical detection using AuNPs as the modifying nanocomposites on Tin oxide (TiO_2) working electrodes [78]. Virus-like particles were substituted for actual or virus-spiked samples for validation purposes. Anti-gp-120 antibody fragments were self-assembled onto the thiol-functionalized AuNPs for biorecognition. The generation of an electroactive site of an analyte (protein) from which the electron is transferred remains deeply buried inside the structure. Due to the electrocatalytic properties of AuNPs in directing free electron transfer, the desired site was exposed resulting in a redox reaction with the application of each component. LOD was reported to be 600 fg/mL to 375 pg/mL.

4.3.7 Graphene stabilized gold nanoclusters (GR/AuNCs)

This sensor used graphene stabilized gold nanoclusters (GR/AuNCs) synthesized in a one-step ultrasonic wave process [108,109]. These NPs were applied to modify the glassy carbon electrode (GCE) and provide a novel electrochemical biosensor platform. In this technique, exonuclease III (Exo III) assisted target recycling amplification strategy was employed for the detection of HIV DNA. An aptamer containing a 3′-MB label and a 5′-Cytosine-rich base was used as the capture probe for target DNA detection.

The GCE was first modified with GR/AuNCs followed by the aptamer capture probe. Exo III mediated hydrolysis of duplex DNA resulted in release of 5′ mononucleotides from the 3′ end of DNA and recycled target and Exo III via electrical sensing [110–112]. Target DNA hybridized to the capture probe formed a twisted double helix thus enabling Exo III to digest the free 3′ end of the capture probe and release MB causing decreased redox signal. Exo III incubation time showed a profound effect on the sensor performance and was optimized at 60 min. LOD was 30 pmol/L when HIV DNA spiked human serum was used [38].

4.3.8 Graphene oxide-based sensor

A DNA-based biosensor was developed for HIV-1 detection using electrochemically reduced graphene oxide (ERGO) as the signal amplifier [39]. Briefly, the glassy carbon (GC) electrode was drop-coated with GO and

the ssDNA probe immobilized to it. Hybridization with target DNA resulted in formation of double-stranded complex at the electrode surface resulting in an increased negative charge. The electron transfer resistance (Re) of the $[Fe(CN)_6]^{3-/4-}$ redox couple changed at each step of fabrication and significantly increased after HIV-1 interaction. Electrochemical impedance was inversely proportional to Re in response to target analyte. ERGO efficiently accelerated electron transfer and thus increased sensitivity to minute impedance changes. A linear relationship was found for Re vs logarithm of target DNA in a concentration range of 1.0×10^{-12} to 1.0×10^{-9} mol/L. LOD was reported to be in the nanoscale range (3.0×10^{-13} mol/L) for this sensor.

5. Conclusions

Biosensors can be employed as alternative diagnostic platforms for the detection of HIV infection at nanoscale levels. In this review, we have discussed the role of various biorecognition elements, signal amplifiers and transducers as essential biosensor components. Nano-sized materials, such as NPs (colloidal gold and silver NPs, quantum dots) and micro/nano-sized structures (photonic crystal, micro-pixilated electrodes) can be used for single cell and individual biomolecule (protein, DNA, RNA) detection. Micro-cantilever technology provides a novel nano-platform for biomolecular interactions relevant to HIV detection. In general, NPs substantially improve biosensor performance in general. Other biosensor configurations such size-tunable optical properties of quantum dots further enhance sensitivity. Advances in fabrication, i.e., elimination of labeling step as well as reagent simplification (fluorescent dye, enzymes), have facilitated biosensor design as applicable to POCT-based detection of HIV. The use of novel label-free biosensors and biomarkers enable early detection of HIV prior to seroconversion. Although much progress has been made in these state-of-the-art technologies, it is clear that additional more comprehensive validation is required to move this non-traditional approach as a ultrasensitive diagnostic platform in a POCT setting.

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