Mol Biol Rep (2015) 42:187–199
DOI 10.1007/s11033-014-3758-5

A review on emerging diagnostic assay for viral detection: the case of avian influenza virus

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Received: 28 July 2014 / Accepted: 17 September 2014 / Published online: 23 September 2014

Abstract Biotechnology-based detection systems and sensors are in use for a wide range of applications in biomedicine, including the diagnostics of viral pathogens. In this review, emerging detection systems and their applicability for diagnostics of viruses, exemplified by the case of avian influenza virus, are discussed. In particular, nano-diagnostic assays presently under development or available as prototype and their potentials for sensitive and rapid virus detection are highlighted.

Keywords Nano-diagnostic assay · Biosensors · Diagnostic technique · Avian influenza virus (AIV)

Abbreviations

AIV	Avian influenza virus
HA	Hemagglutinin
NA	Neuraminidase
VI	Virus isolation
ELISA	Enzyme linked immuno-sorbent assay
HI	Haemagglutination-inhibition
RT-PCR	Reverse transcription PCR
LUX-PCR	Light upon extension PCR
RIDA	Rapid isothermal nucleic acid detection assay
LAMP	Loop-mediated isothermal amplification
NASBA	Nucleic acid sequence-based amplification
PLA	Proximity ligation assay
FRET	Förster/fluorescence resonance energy transfer
RCA	Rolling circle amplification
$TCID_{50}$	Median tissue culture infective dose
RP	Reporting probes
SMD	Sulfamethoxydiazine
mAb	Monoclonoal antibody
ITO TFTs	Indiumtin-oxide thin-film transistors
NW-FET	Nanowire field effect transistor
SBP	Silica binding protein
QDs	Quantum dots
AuNPs	Gold nanoparticles
MLD	Magnetically labeled diagnosis
SPR	Surface plasmon resonance
SPF	Specific pathogen-free
DLS	Dynamic light scattering
SELEX	Systematic evolution of ligands by exponential enrichment
SQUIDs	Superconducting quantum interference devices

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Introduction

Biotechnology-based detection systems are presently utilized for numerous applications in biomedicine, including

the detection and diagnostics of viruses. The challenges for effective detection are manifold, as a diagnostic system has to be specific, sensitive, cost-effective and, if possible, suitable for applications in the field, especially in developing countries.

In this review, using avian influenza virus (AIV) as a case study, currently used diagnostic assays are briefly reviewed and emerging principles/systems for detection of viruses are comprehensively discussed. More specifically, emerging principles/systems include molecular recognition and detection systems based on sophisticated amplification or fluorescence techniques, as well as new biosensing principles based on the unique properties of nanoparticles. Among these new detection principles, nanoparticle-based systems are most likely to play a key role in future diagnostics of AIV and other viruses.

Avian influenza virus (AIV)

AIV is the causative agent of major influenza epidemics, associated with millions of deaths and substantial economic losses [1]. AIV is an RNA virus with a genome comprised of 7–8 single-stranded RNA segments, encoding 11 proteins [2]. The RNA segments enclosed within the viral envelope is associated with the nucleoprotein (NP), the main viral structural protein. Moreover, encoded are two nonstructural proteins (NS1 and NS2), and three viral polymerase subunits (PA, PB1, and PB2), which together form the ribonucleoprotein (RNP) complex responsible for RNA replication and transcription. Other major membrane viral proteins include M1, a nuclear RNP export inducer and viral transcription inhibitor, and the ion channel M2. The latter regulates the virus's internal pH, which is essential for uncoating of the virus during the early stages of replication [2–4]. The surface glycoprotein hemagglutinin (HA) plays a central role in interaction with host cells. It binds to the sialic acid moiety of epithelial cell receptors and subsequently facilitates virus entry into host cells. Neuraminidase (NA) shows glycosidase enzyme activity and functions as virion progeny releaser by cleaving sialic acid bound to virion proteins [3].

Based on antigenic differences in nucleo protein and matrix proteins, influenza viruses are classified as type A (infecting a wide range of genera, such as humans, pigs, horses, seals, ferrets, mink, whales, and birds), type B (mostly infects humans, but has also been observed in seals and ferrets), or type C (almost exclusively infects humans, but dogs and pigs are also susceptible) [3–5]. Efficient techniques for rapid and sensitive diagnostics of AIV, based on insight in the molecular structure of the virus, are a key factor for prevention of future influenza pandemics.

Current diagnostic assays

A large number of different methods have been developed for AIV detection and identification. The most traditional laboratory diagnostic methods for viral animal diseases involve virus culturing and isolation (VI), combined with serological or antibody tests. Among other traditionally-used techniques are complement fixation and agar immuno-diffusion [6], micro-neutralization assay, enzyme linked immuno-sorbent assay (ELISA) for antibody or antigen [7, 8], virus neutralization, haemagglutination or haemagglutination-inhibition (HI) [9], as well as viral culture in embryonated eggs or Madin–Darby canine kidney cells [10]. Generally, the traditional laboratory diagnostic methods are laborious, time consuming and the need for high level of bio-safety laboratory and equipments limits the applicability of these assays both in the field and for high-throughput detection of large number of clinical samples [4, 11–14].

In the past two decades, the mainstream techniques applied for pathogen diagnosis have transited from protein and culture-based techniques to nucleic acid testing assays. On such basis, different kinds of molecular detection assays have been developed for detection of AIV including reverse transcription PCR (RT-PCR) [15], real-time PCR and light upon extension PCR (LUX-PCR) [16]. Molecular detection methods in general are sensitive methods, which allow rapid and specific detection of viral nucleic acids, as compared to culture or antigen detection methods. However, the need for specialized equipments, trained personnel and the high costs involved hamper their application for both diagnostics in the field and in poor countries [4, 17].

Due to the above-mentioned disadvantages of the molecular diagnosis techniques, in particular the need for thermal cycling equipment, efforts have been made to circumvent the need for such equipments by developing isothermal techniques. The isothermal amplification techniques used for detection of AIV includes rapid isothermal nucleic acid detection assay (RIDA) [18], Loop-mediated isothermal amplification (LAMP) [19] and nucleic acid sequence-based amplification (NASBA) [20]. Compared with conventional molecular techniques, the isothermal amplification techniques have the advantages of higher speed, affordability and in particular no requirements for thermal cycling equipments. Despite of such clear improvements, isothermal amplification techniques still require complex primer design, denaturing agents, and gel electrophoresis.

Emerging diagnostic assays

Given the overall shortcomings of the traditional and molecular diagnostics, the need to seek more reliable,

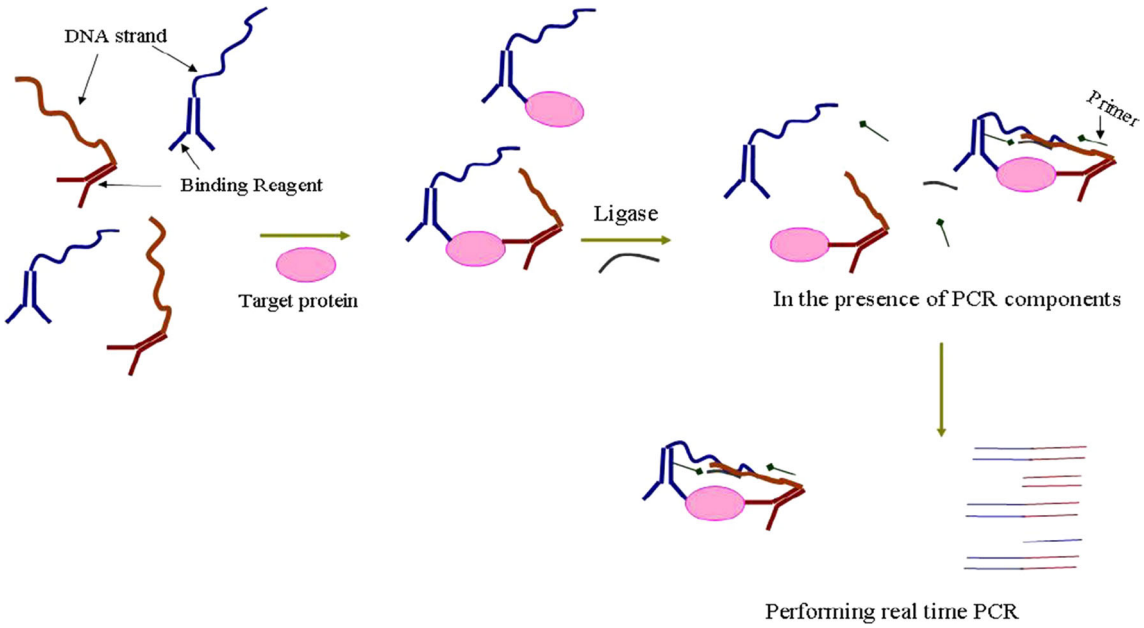

Fig. 1 Proximity Ligation assay. DNA strands are attached to the reagents capable of binding to a target protein. When two or more such binding reagents recognize the same target molecule, the DNA strands are brought into proximity and can be joined by ligation. This novel formed chimeric DNA strand can be amplified in a PCR-like manner or RCA

efficient, and economic procedures is increasingly highlighted. Several of such new systems have already been applied for detection of some RNA viruses already, non for AIV diagnostics though. Some other emerging systems have generated promising prototypes, but have not yet been tested on clinical samples. In this section, we will discuss selected approaches that could potentially meet some of the criteria required by clinical diagnostics, such as the proximity ligation assay (PLA), biosensor-based methods, Förster/fluorescence resonance energy transfer (FRET)-based methods, microarray assays, and in particular nanoparticle-based techniques.

Proximity ligation assay (PLA)

The PLA utilizes nucleic acid ligation and amplification process in order to detect viral proteins [21]. DNA strands are attached to antibodies specific to the target protein. When two of these antibodies recognize the same target molecule, the DNA strands are brought into proximity and can be joined by ligation. This novel formed chimeric DNA strand can be amplified, either as in PCR or by using a rolling circle amplification (RCA) version (Fig. 1) [21]. In fact, PLA is a modified, more sensitive version of ELISA based on recognition of viral or bacterial surface proteins coupled to DNA strands [22]. Recently, this method was successfully applied for AIV detection in chicken specimens [21]. The detection limit of this technique is one or just a few copies of viral particles, far below the detection limit of the capture ELISA used for comparison of protein detection [22].

Microarray-based assays

In microarray technology, a DNA fragment (probe) is functionally attached to a glass or silicone surface and then used to detect a known gene or gene fragment in a (diagnostic) sample [23]. On the other hand, RNA present in the sample is converted into cDNA and labeled with a fluorescent tag using RT-PCR. If the cDNA is complementary to the DNA strand on the chip surface, hybridization occurs and the bound fluorescence-labeled cDNA probe can subsequently be detected by fluorophore excitation with a specific laser wavelength. This method is able to detect thousands of molecular targets simultaneously and can be used to prove phylogenetic relationships between isolates [24]. Microarray assays have been used for detecting and subtyping of all known HA and NA subtypes of AIV [25]. Gall et al. [24] used cloacal swabs from wild and domestic birds to verify the feasibility of the microarray for sensitive and specific detection and characterization of AIV HA gene. The sensitivity of AIV microarray detection method was determined at 94 %, close to values reported for RT-PCR and VI detection systems. The detection limit of microarray technique has been reported at 10 target RNA copies per reaction, 10 times higher than RT-PCR technique [15, 24]. Moreover, it is

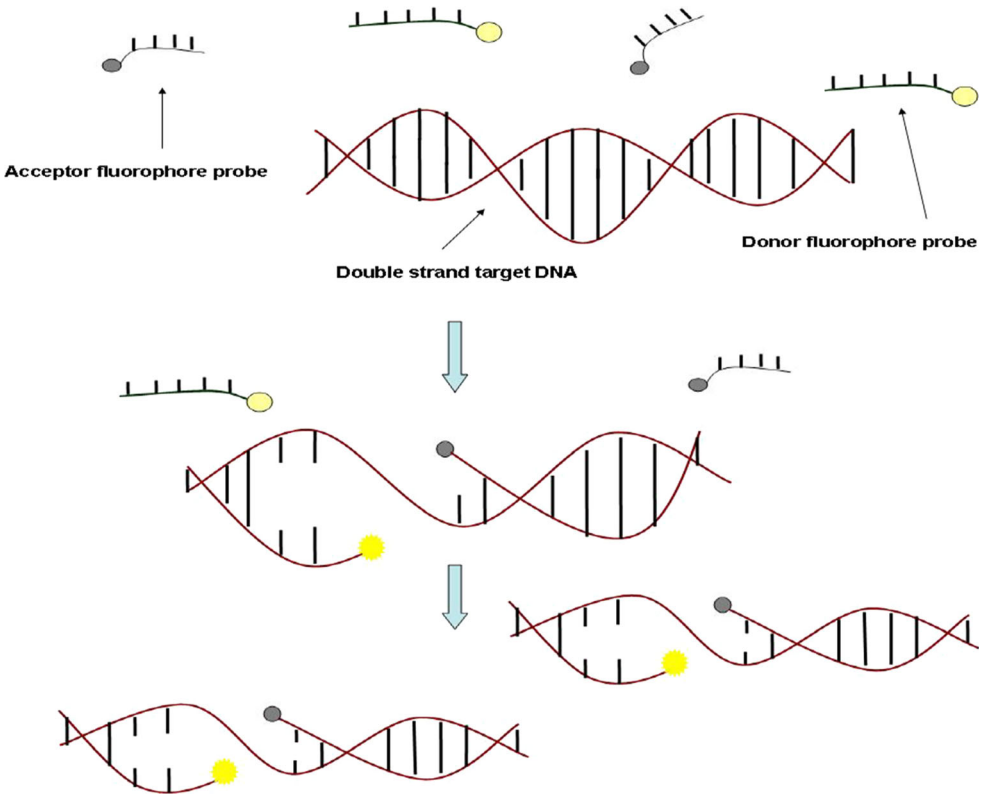

Fig. 2 FRET-based virus detection. Oligonucleotide probes conjugated with two different FRET fluorophores (donor and acceptor) hybridize with the viral DNA. The decreased donor/acceptor distance after hybridization causes increased FRET efficiency and a quenched/decreased donor fluorophore signal

important to quote that this technique can detect thousands of samples swiftly with a minimum cross-contamination between samples. However to date, high costs of the required instruments, trained personnel, and costly individual sample analysis have restricted microarray-based detection to research usage only.

Förster resonance energy transfer-based methods

Förster resonance energy transfer (FRET), also called fluorescence resonance energy transfer, involves energy transfer between two fluorophores. These fluorophores must be chosen to allow overlap of the emission spectrum of the first fluorophore (donor) with the excitation spectrum of the second fluorophore (acceptor) [26]. In case of a large distance between donor and acceptor fluorophore (>10 nm), the light absorbed by the donor fluorophore is directly emitted, so emission of the donor is observed. But, if the fluorophores approach each other close enough (typical distance of 2–10 nm), the absorbed light energy is transferred to the acceptor and consequently the acceptor emission increases. Distance changes between donor and acceptor can thus be detected by monitoring either the decrease of the donor or the increase of the acceptor emission intensity [26].

Götz et al. [27] developed a real-time PCR by introducing FRET hybridization probes for detection of AIV H5 gene in specimens reaching a sensitivity of fewer than ten genome copies. In their investigation, two different fluorophore-labeled oligonucleotide probes, donor fluorophore probe and acceptor fluorophore probe, hybridize with their complementary target DNA during the PCR cycle. Upon incorporation of the FRET probes into the target DNA, the distance between the fluorophores decreases, thus FRET occurs and the intensity of the donor emission decreases (Fig. 2) [27]. To allow simultaneous analysis of different AIV types in a single reaction, as an alternative to melting temperature analysis [28], two FRET pairs with different excitation and emission spectra can also be used [29].

Poddar [30] used a similar FRET-based method for the detection of influenza virus type A and B in the cultured clinical sample. This author used three sets of probes each targeting a type or subtype of the AIV gene segments (i.e. NA, HA and matrix). Based on the target used, the sensitivity of this FRET-based assay was found equal to tenfolds higher (0.001–0.1 median tissue culture infective dose ($TCID_{50}$)) as compared to that of agarose gel electrophoresis detection method [30]. In fact, such FRET method can be applied to many molecular systems, provided that a suitable FRET pair can be produced [30].

Overall, FRET is a rapid method with high sensitivity, and is capable of detecting very small quantities of virus materials. However, there are several limitations to this

method, including the external illumination requirement to initiate the energy transfer, background noise from direct excitation of the acceptor, photobleaching, probe construction, and processing software requirement. Last but not least, this method requires highly trained personnel and costly procedures, hampering its usage as standard clinical detection method.

Rapid isothermal nucleic acid detection assay-lateral-flow (RIDA-LF)

Gao et al. [18] designed a lateral-flow (LF) immunoassay that could be used in combination with RIDA for rapid detection of HA gene of H5N1 influenza virus. The developed RIDA-LF assay uses chemically labeled probes covalently linked to magnetic microbeads, used to separate cleaved probes from the reaction solution. The cleaved probes are then detected with an LF immunoassay. In RIDA-LF reporting probes (RP) labeled with biotin at their 3′ end and sulfamethoxydiazine (SMD) at their 5′ end, are used. These probes, containing a specific binding site for a restriction enzyme, are immobilized on streptavidin-labeled magnetic beads.

Then, the RP-conjugated magnetic beads are mixed with target RNA (or DNA) in suspension and upon hybridization, RIDA reaction generates cleaved RPs. The SMD-labeled fragments are therefore released from beads into the solution, and are subsequently separated from the magnetic beads by applying a magnetic force. At the second stage, an LF immunoassay is used to detect the SMD released in the solution. In this LF immunoassay, an anti-SMD monoclonoal antibody (mAb) conjugated with colloidal gold is attached to the conjugate pad. Thus, in presence of target RNA (or DNA) and consequent SMD release into the solution, SMD would bind to this mAb, which would competitively block its binding to the SMD antigen (BSA-SMD) located on the test line on the nitrocellulose membrane.

Following the flow of liquid, the SMD–mAb (colloidal gold) complex can be captured by a secondary antibody on the control line. Therefore, formation of a single line at the position of the control line would indicate a positive result. On the contrary, if there was no target RNA and consequently no SMD was released in the solution from the RIDA reaction, the mAb (colloidal gold) complex would bind SMD–BSA on the test line forming the test line. The excess amount of mAb would cross the test line and be captured on the control line. Thus, the formation of both the test and control line is an indication of a negative result (Fig. 3) [18].

Overall and in comparison with the methods discussed so far i.e. PLA, Microarray-based assays, and FRET-based methods, RIDA-LF seems to be more promising to be used at clinical diagnostics scale since it does not require any special equipment for the detection of the target ribonucleic acid and is thus less dependent on instrumentation.

Biosensors

Biosensors used for biological applications consist of a recognition agent (probe) specific to its target, e.g. nucleic acids, antibodies or enzymes, connected to a functional unit generating an output signal [4, 17]. For instance, in case of a DNA-based biosensor, the probe is a single stranded DNA, immobilized on a gold microchip via an affinity linkage such as biotin/streptavidin and is functionally connected to a chemical or electrical electrode. If the target DNA binds to the probe, double stranded DNA is formed, resulting in a voltage change that signals target detection. DNA biosensors can be highly sensitive and capable of detecting DNA concentrations as low as 0.5 nM [31] while no PCR instrumentation is required.

Biosensor immunoassays

In a biosensor immunoassay, interaction of the target protein with an immobilized antibody facilitates binding of a fluorophore-labeled second antibody to the target, which in turn generates the fluorescent readout signal proportional to the amount of antigen present in the sample (Fig. 4). In case of an interferometric biosensor immunoassay, detection is not mediated by a labeled antibody, but instead changes in refractive index upon antigen binding are directly observed on a planar optical waveguide [32] (Fig. 5). Interferometric biosensor immunoassays can potentially be used for point-of-care viral diagnostics, utilizing direct and label-free detection of viruses [33]. As an example, Xu et al. [34] described an interferometric biosensor immunoassay for direct label-free detection of poultry AIV by immobilizing antigen-specific antibodies to the HA protein on the waveguide surface. Upon binding of the antigen, the surface waveguide signal changes, leading to target detection with detection limits as low as 0.0005 hemagglutination mL^{-1} (Fig. 5) [34].

Recently Guo et al. [35] applied indiumtin-oxide thin-film transistors (ITO TFTs) to detect label free H5N1 virus as well. More specifically, the ITO TFTs was fabricated on a glass substrate and the specific anti-H5N1 antibodies were covalently attached on the ITO channel. In the presence of H5N1, the targets were captured by immobilized anti-H5N1 antibodies and subsequently the electronic properties of the ITO TFT was affected leading to a significant shift in the resultant threshold voltage. The detection limit of this assay was reported at 0.8×10^{-10} gmL^{-1} [35]. In a different recent investigation, Ahn et al. [36] utilized nanowire field effect transistor (NW-FET) to detect avian influenza antibodies (anti-AI). More specifically, avian influenza antigens

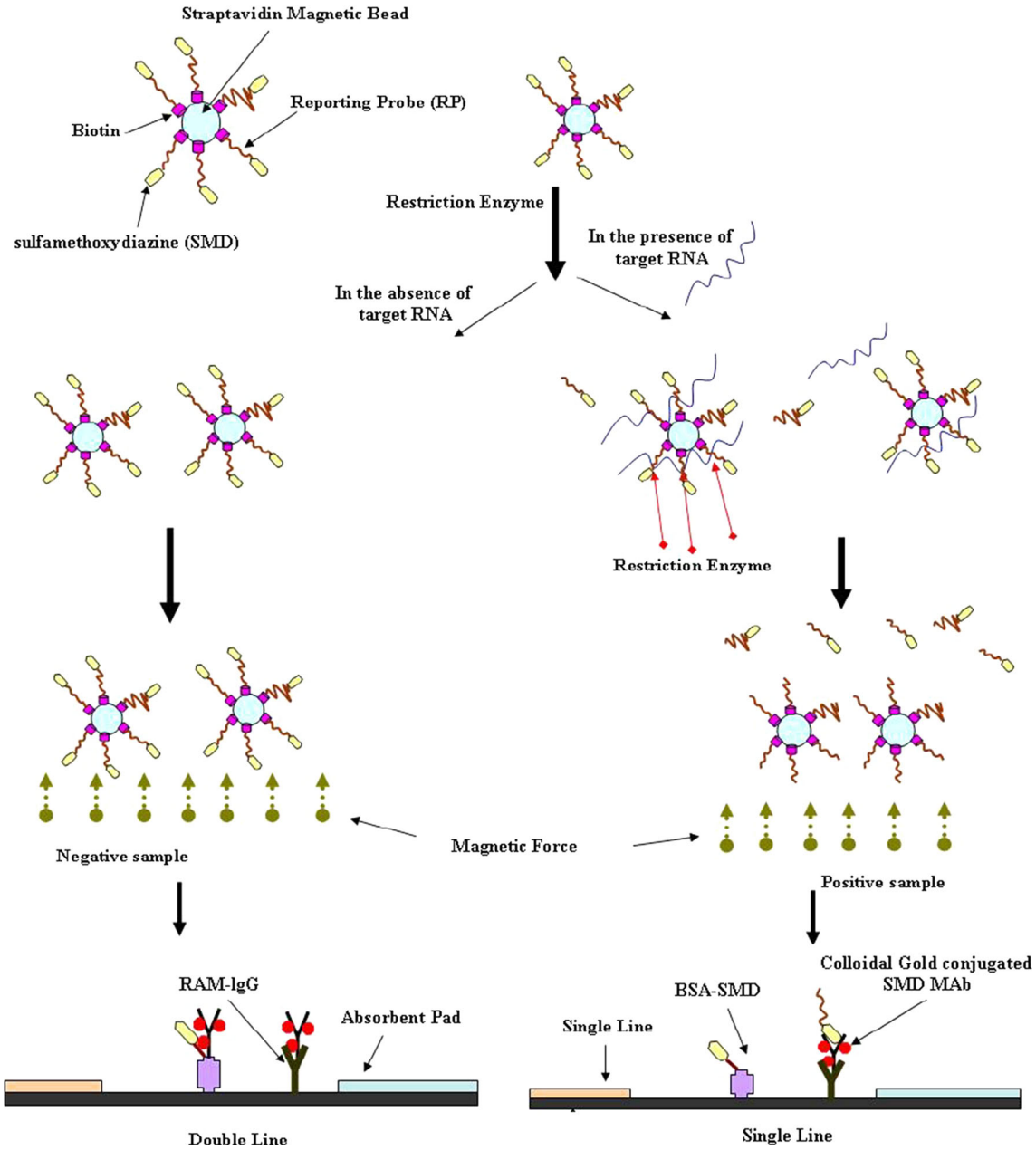

Fig. 3 Rapid isothermal nucleic acid detection assay-lateral-flow (RIDA-LF). This combined assay is a two-step technique in which first Reporting Probes (RP), labeled with biotin at 3′ end and sulfamethoxydiazine (SMD) at 5′ end, are cleaved after hybridization to the target by a restriction enzyme. and are immobilized on streptividin-labeled magnetic beads. The SMD-labeled fragments are separated from the biotin labeled 3′ end by binding of streptavidin-labled magnetic beads by applying a magnetic force. In the LF immunoassay, presence of the SMD labeled fragment is detected by binding to a colloidal gold-conjugated anti-SMD mAb, competitively blocking binding of this mAb to the SMD antigen (BSA-SMD) located on the test line on the nitrocellulose membrane. Following the flow of liquid, the SMD-mAb (colloidal gold) complex can be captured by a secondary antibody on the control line

(AIa) were immobilized on the silica nanowire surface by using a silica binding protein (SBP). In presence of the anti-AI, it binds to the formerly immobilized SBP/AIa on the nanowire surface. Following the binding of the negatively charged anti-AI to the SBP/AIa, the electron transfer over the nanowire surface is repulsed and the electron concentration is decreased, subsequently, the current through the nanowire changes significantly leading to detection. While, no considerable response arises when there is not any anti-AI in the solution injected onto the device [36].

In general, immunoassay-based biosensors can be suitable for detection of viruses, at low concentrations and in complex biological matrixes such as whole blood, serum and other biological fluids [37]. Moreover, the biosensor-

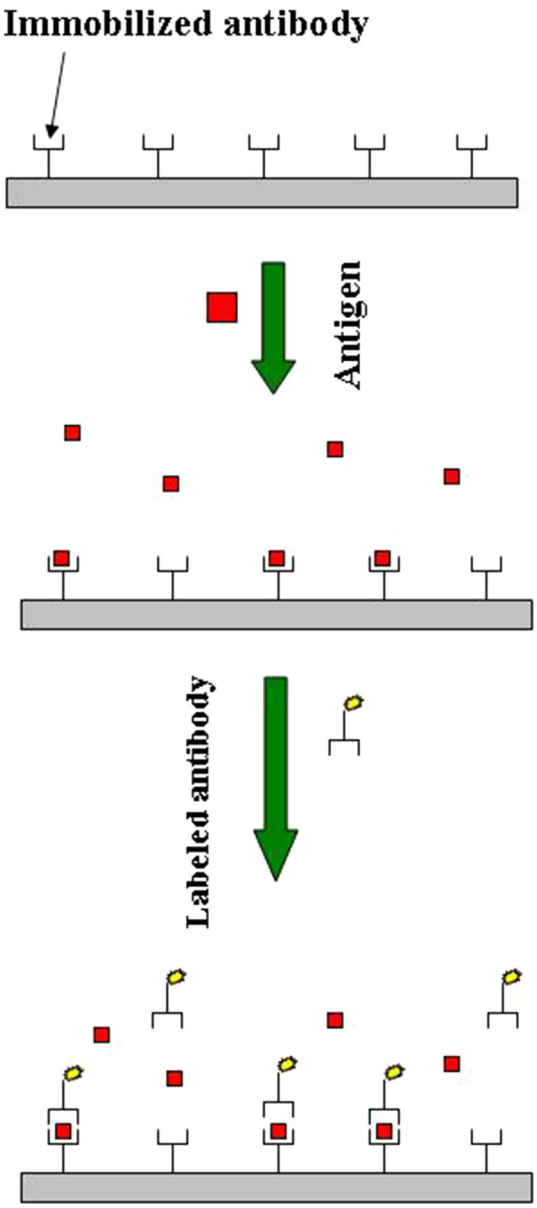

Fig. 4 Biosensor immunoassay using secondary antibody for detection. The target is detected with an immobilized immunological receptor (immobilized antibody) and a labeled antibody, which generates measurable output signals. The interaction of the target protein with the immobilized antibody facilitates the binding of the labeled antibody to the target, which in turn generates the fluorescent readout

based diagnosis approach is potentially suitable for rapid detection with high sensitivity, and in particular amenable for lab-on-chip detection, allowing analysis of multiple samples in parallel on the same chip [38].

Biomachine-based biosensors

In a special type of DNA-based biosensor reported by York et al. [39], the output signal is produced by a biological

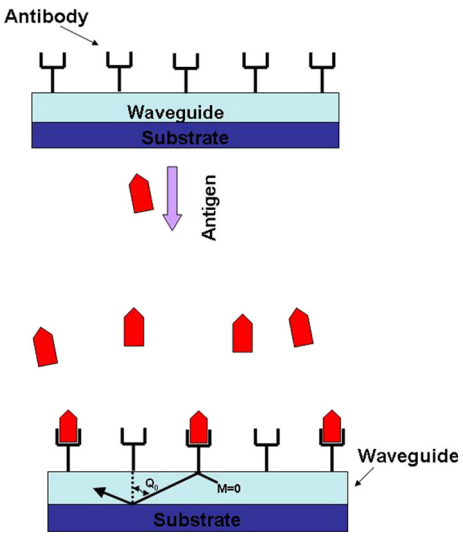

Fig. 5 Immunoassay with label-free detection. Antigen-specific antibodies are immobilized on an optical waveguide surface. Binding of the antigen changes the surface properties of the waveguide, producing an optical read-out

nanomachine. In this system, first the target DNA is recognized by two biotinylated DNA probes, generating a di-biotinylated double strand DNA. In the second step, this double stranded DNA functionally connects gold nanorods to the rotary motor protein F1-ATPase (Fig. 6). Rotation of the nanorods is finally observed by dark-field microscopy [39]. The developed assay does not require thermal cycling since it is performed at room temperature. The detection limit reported for this technique was at 1 zeptomole (600 DNA molecules) [39]. This molecular machine-based detection system is at prototype stage and currently restricted to research settings, but provides a proof-of-concept for a diagnostic assay that is theoretically capable of detecting single RNA or DNA molecules, clearly exceeding the detection limit of currently used assays.

As mentioned above for DNA-based biosensors, biomachines can also be incorporated in biosensor immunoassays. For instance in an investigation by Liu et al., AIV-specific antibodies were connected to the nano-machine protein F_0F_1-ATPase, which generated the output signal. AIV binding caused changes in the flux of proton coupled to this enzyme's activity, leading to intensity changes of a pH-sensitive fluorescence dye [40]. One year later in a related approach, a fluorescent nanocrystal (quantum dot; QDs) was used in conjunction with F_0F_1-ATPase for detection of AIV [41], as detailed later in Sect. 4.6.1. The function of biological machines, such as F_0F_1-ATPase, can respond to a broad range of signals [42–44], making machine-based biosensors systems potentially versatile detection instruments. However, at present these assays are restricted to prototypes in laboratory settings.

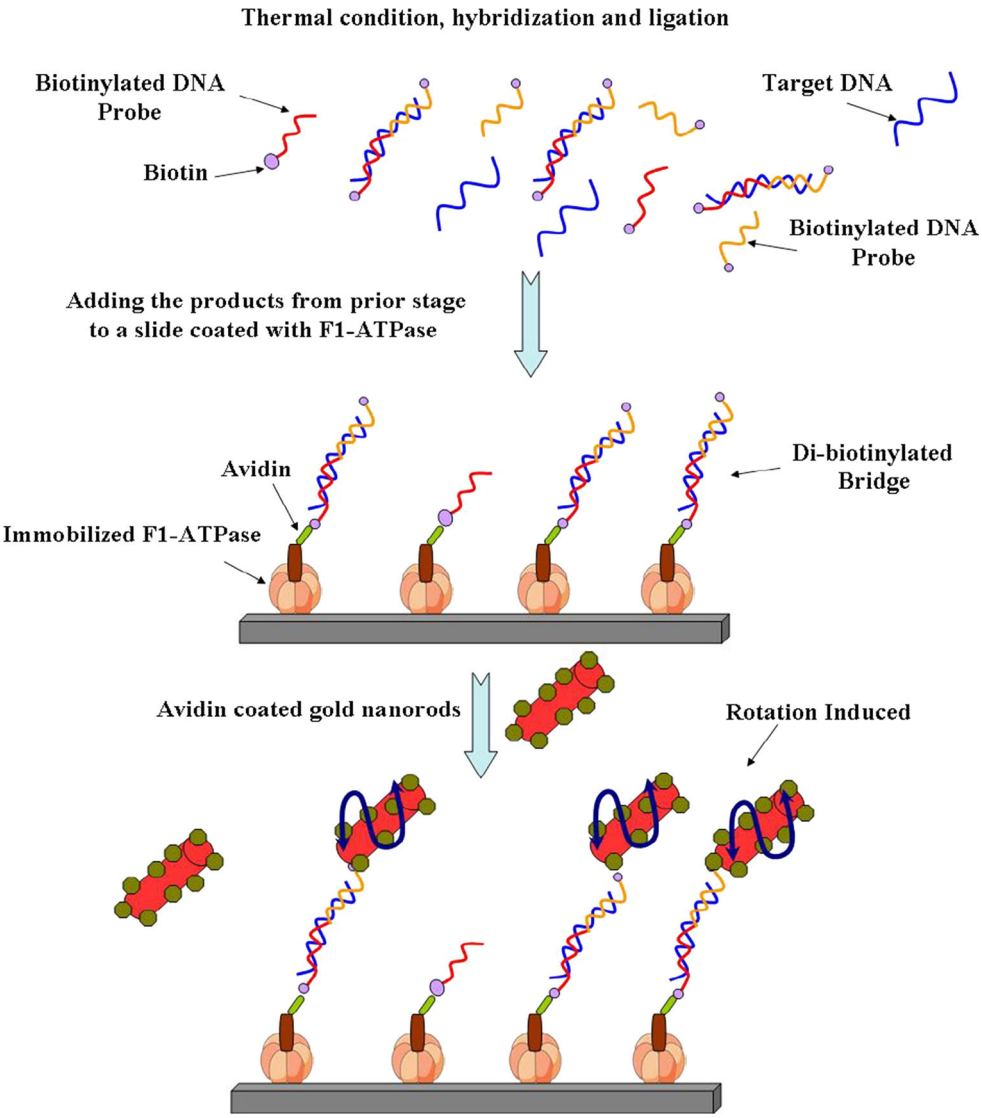

Fig. 6 Biomachine-based sensor. The target DNA hybridizes to two biotinylated probes and the formed di-biotinylated DNA then links gold nanorods to the F_1-ATPase protein motor. Movement of the nanorods, observed microscopically, provides the output signal

Nanoparticle-based detection assays

Nanoparticles have emerged as a new frontier in the field of medical biotechnology. The term 'nanoparticle' refers to a molecular systems, or compound with nanometer-scale dimensions, ranging from approximately 5–100 nm in size [33]. At this size scale, the physical and chemical properties of the particles are significantly different from those of macro-scale materials, e.g., the electrical conductivity, chemical reactivity, or surface area can be considerably enhanced. Nanoparticle-based techniques have been introduced in biological and medical sciences in a broad variety of applications, such as drug and gene delivery [45], separation or purification of biological molecules, cells and viruses [46] as well as detection of pathogens [47]. Moreover, nanoparticle technology includes usage of a broad variety of materials, such as gold nanoparticles (AuNPs) [48], magnetic nanoparticles [49], QDs, carbon-nanotubes [50], mesoporous silica nanoparticles [51] and bio-nanoparticles such as virus-like particles and aptamers [52, 53].

It should be noted that various kinds of nanoparticles have been synthesized and used in clinical detection approaches, and that in the following sections, only a number of nanoparticle-based detection assays including QDs, magnetic nanoparticles, and AuNPs as three prominent types of inorganic nanoparticles with exceptional properties and/or especially high potential for diagnostics are discussed.

Quantum dots

QDs are nanocrystalline semiconducting fluorophores with a typical diameter of about 2–20 nm, commonly composed of

elements from group II, III, V or VI [54]. QDs have a number of advantages as compared to conventional fluorescence dyes, e.g. highly increased stability against photobleaching, high quantum yield, a broad excitation spectrum, extended fluorescence lifetime and a narrow emission [55]. These particles display size-tunable emission, meaning that the emission wavelength can be predicted based on the QD's size. Multiple QDs of different size can be simultaneously excited by a single light source, significantly aiding their application in multiplexing assays.

QDs have also been applied for the detection of AIV (H9) based on an antibody-antigen reaction [41]. In this system, AIV binding to F_0F_1-ATPase via an antibody/biotin-streptavidin linkage causes changes in the activity of this enzyme. As F_0F_1-ATPase is an enzyme that transports protons across a bio-membrane, activity changes induced by AIV binding leads to a local pH change. This pH change can be detected by pH-sensitive QD fluorescence [41]. No performance parameters were reported for this proof-of-concept method. The narrow fluorescence emission spectrum of QDs compared to the organic dyes may facilitate simultaneous virus detection using different QDs.

Magnetic nanoparticles

Magnetic nanoparticles consist of different types of iron oxides and, depending on the material stoichiometries, sustain different magnetic properties. A broad range of surface modifications is available, facilitating functionalization with a recognition agent, such as DNA, antibodies or virus proteins. A striking advantage of magnetic particles is the possibility to remove the functionalized particles after binding to the target from the test assay by an external magnet. Subsequently, the target can be detected using labeled antibodies or other target-specific reagents. This approach can significantly decrease the turnaround time and may also improve recovery of precious analytes.

Using magnetic nanoparticles, the magnetically labeled diagnosis (MLD) technique allows for target detection by measuring variations in the magnetic properties of bio-functionalized magnetic nanoparticles. In this technique, antibodies immobilized onto the surface of magnetic nanoparticles detect a specific bio-molecule, leading to particle aggregation and concurrent magnetic signal variation (Fig. 7) [56]. These larger/aggregated magnetic nanoparticles respond much less to the external magnetic fields than the original individual magnetic nanoparticles. Additionally, the mean diameter of magnetic nanoparticles before and after incubation with samples changes from 50 to 200 nm, or larger, depending on the concentration of to-be-detected molecule, as revealed by dynamic light scattering technology. MLD was proven as a suitable assay for AIV detection, with a detection limit of 5 $pgmL^{-1}$.

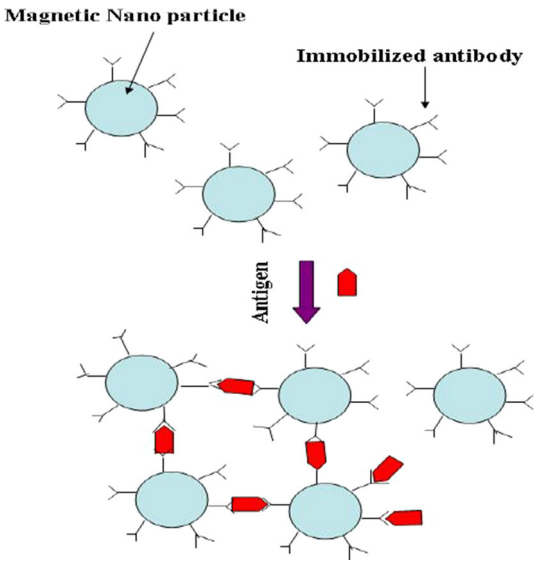

Fig. 7 Magnetically labeled diagnosis (MLD) technique. Target (antigens) binding to antibodies immobilized on the surface of magnetic nanoparticles leads to particle aggregation and concurrent changes of magnetic signal variation. The changes in magnetic signals are detected by superconducting quantum interference devices (SQUIDs). Moreover, the mean diameters of magnetic nanoparticles before and after incubation with samples are detected by using dynamic light scattering technology

However, there is still a need for improved MLD techniques with higher sensitivities [56].

Gold nanoparticles

AuNPs, with dimensions ranging from 1 to 250 nm, are commonly used in various biological applications owing to their relatively simple chemical synthesis and the possibility for surface modifications with different small molecules or bio-polymers, such as peptides, proteins, DNA and antibodies [57]. AuNPs display a special phenomenon referred to as surface plasmon resonance (SPR), which is responsible for the AuNPs' intense red color. Binding of the target causes particle aggregation and leads to color change of the AuNPs colloidal solution from red to blue, making simple detection possible.

Driskell et al. [58] utilized antibody labeled-AuNPs to detect influenza A virus. In their study, aggregation of the AuNPs was induced upon the addition of the target virus and the aggregation and the mean diameter of the AuNPs were measured by using dynamic light scattering (DLS). The detection limit of this system was estimated at 100 $TCID_{50}$/mL [58].

In a very recent study, Li et al. [59] used gold immunochromatographic strips to detect AIV by using naked eyes. In their study, poultry AIV were propagated in the specific pathogen-free (SPF) chick embryos. On the other

hand, AuNPs were conjugated with monoclonal antibodies and second antibodies were attached in a distinct detection zone on the nitrocellulose membrane. The test-strip consisted of the sample pad, the conjugate release pad, the analytical membrane (containing both test line and control line) and the absorbent pad. The specific capture antibodies for AIV were immobilized in the test line and the goat anti-mouse IgG antibodies were immobilized in the control line. Practically, when negative samples flow through the analytical membrane, the AuNPs are attached to the control line and the test line remains un-captured, hence, a single band is observed. In the case where positive samples flow through analytical membrane, the AuNPs are attached to the control line and the antibodies in the test line are captured by targets and the AuNPs are attached to the target in a sandwich manner. Therefore, two different bands are observed in the strips. The used technique demonstrated 100 folds more sensitivity than commercial test strips with the detection limits of 2^{-12} gmL^{-1} [59].

In a different recent attempt to develop an immunochromatographic strip for rapid detection of H5N1 in poultry, a monoclonal antibody against HA was conjugated with colloidal AuNPs. Both standard antigens and isolated viruses utilized in this study were added to allantoic fluids from SPF embryos. In this assay, dissimilar to the traditional immunochromatographic strips assay, four test lines (T) with a same capture complex of different concentrations were used on the nitrocellulose membrane and the goat anti-mouse IgG was used as control line (C). In the test line, a polyclonal antibody against H5N1 was used. The strips contained sample pad, conjugate pad, four test lines, a control line and absorbent pad. In the detection process, samples flow from the sample pad to the absorbent pad and if the negative sample is absorbed just one red band on the control line is observed but in presence of positive samples, more than two bands are observed [60].

Aptamers for viral diagnostics

Aptamers are artificial, single-stranded DNA or RNA molecules with defined 3-D nanostructures (Fig. 8) [61]. Their unique structures enable them to bind to specific target molecules [60]. Soon after their discovery in the early 1990s, aptamer technology evolved enormously [62, 63]. In diagnostic assays, aptamers have been proposed as a promising alternative to monoclonal antibodies [1]. Specific aptamers have been synthesized against a wide variety of intra- and extracellular targets ranging from small molecules [64], amino acids [65] and peptides [66] to different proteins such as cell membrane proteins [67]. Moreover, aptamers have been introduced to analytical applications not only for detection, but also for drug discovery processes [68] and therapeutics [61].

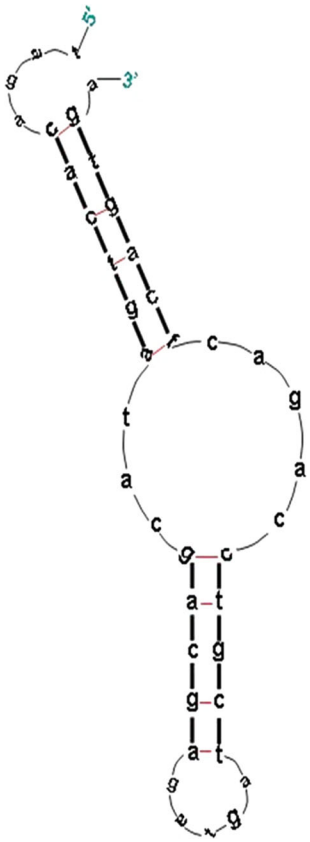

Fig. 8 Aptamer secondary structure

As an example, anti-target aptamers can be synthesized by conjugating them to QDs, followed by subsequent labeling with a fluorophore. Fluorophore molecules (quencher molecules) in this construct (aptamer-QD-quencher oligonucleotide) are close enough to quench the QD fluorescence. In the presence of the target viral protein, the aptamer undergoes a structural change and the quencher is displaced. As a result, the QD surface reinstates the original QD emission (Fig. 9) [69]. In addition, by utilizing various aptamers onto different QDs, successful multiplex detection of numerous targets could be achieved.

Jeon et al. [70] showed that not only RNA aptamers are suitable for AIV detection but also DNA aptamers can be efficiently used against a specific domain of HA from influenza type A virus. Aptamers are capable of discriminating between the HA proteins of influenza A and B viruses by binding specifically to the HA region of the specific strain [70].

Recently, Wongphatcharachai et al. [71] selected high-affinity DNA aptamers against H3N2 by using systematic evolution of ligands by exponential enrichment (SELEX) to detect AIV and demonstrate viral subtype. To show if the aptamer could be used in the detection of live AIV, the dot blot assay was performed. The dot blot assay

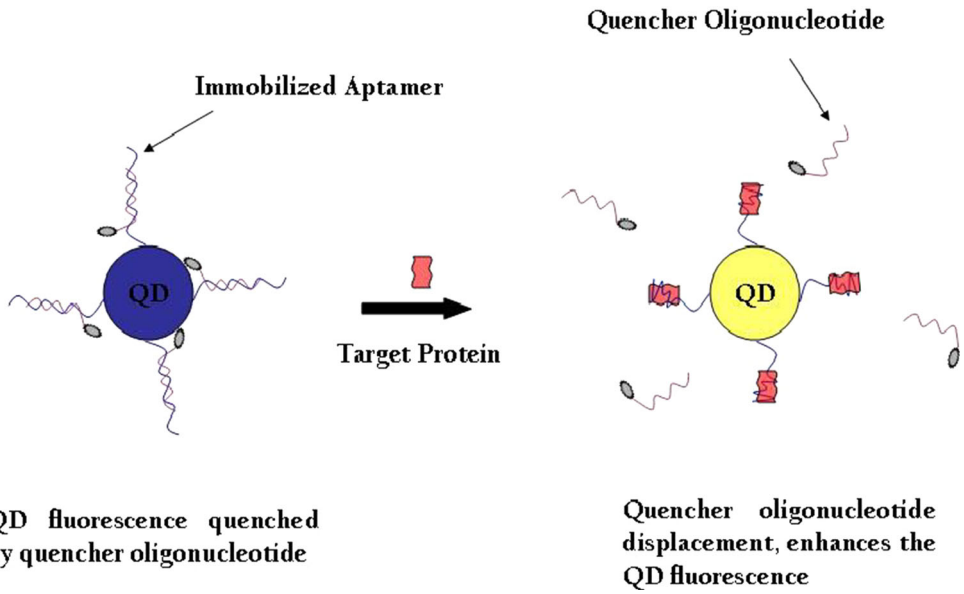

Fig. 9 Schematic picture of an aptamer-based detection assay. Binding of the target protein to an aptamer-immobilized sensor on a QD surface leads to the displacement of the quenching oligonucleotide, thus resulting in an enhanced QD fluorescence as the output signal

demonstrated that the selected aptamers could be utilized to successfully detect and differentiate AIV subtypes. Also recently, Cui et al. [72] constructed a QDs/Aptamer system against the AIV HA to recognize and label the viral particles specifically. To identify AIV, the SDS-PAGE analysis and electron micrograph were used [72]. In the case of QDs/Aptamer attached to the virus, a specific band was observed while this band was not observed where there was not any attachment between the QDs/Aptamer and AIV.

In conclusion, detection based on aptamers has demonstrated high selectivity and specificity, and is believed to be capable of distinguishing closely related virus isoforms. To our knowledge, just a small number of aptamer-based diagnostic assays have been reported for detection of the AIV so far. Overall, the combination of easy chemical synthesis and labeling combined with specificity and sensitivity makes aptamer-based systems exceptionally promising diagnostic assays in the future.

Conclusions and future perspective

Although within the last decades considerable progress has been achieved in development of new assay systems for AIV detection, there is still an urgent demand for more sensitive, simple and rapid detection assays. Among the new and emerging diagnostic systems which could potentially meet the demands of clinical diagnostics, such as simplicity, sensitivity, specificity and cost-effectiveness, nanoparticle-based detection systems seem to play a central role in next generation diagnostic systems. It is worth quoting that further development of diagnostic assays should aim at achieving affordable point of care detection ability to facilitate diagnostics in the field.

Acknowledgments The authors would like to thank Mr. Ahmad Mousavi for his valuable assistance.

Conflict of interests The authors declare that they have no competing interests.

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