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Multiplexing of nanoparticles-based lateral flow immunochromatographic strip: A review

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Introduction

Nanotechnology is one of the research fields that have gained much attention worldwide in the past few decades. Applications of nanotechnology, especially in medical and health care, are increasing and significantly benefiting many people worldwide [1]. The interest in this technology nurtured the development of nanoparticle-based (NPs) biosensors for diagnostic applications. Subsequently, biosensors became very significant tools and applied in everyday life as a point of care, such as a pregnancy test strip, food allergen detector, chronic disease biomarkers, and dangerous pathogen in food and water biomarkers [2].

Biosensors, especially in a paper-based format, have entered daily life considering the paper material used is cheap, simple, abundant, easy to manufacture, compatible with many biochemical relations, and controllable in porosity and capillary force [2, 3]. The most popular among paper-based biosensor devices is the lateral flow immunochromatographic strip (LFICS).

LFICS

As stated, LFICS is a paper-based biosensor device and a modern version of immunoassay (IA) that possesses characteristics required by biosensors, which are rapidity, high sensitivity, good specificity, low limit of detection (LOD), require few sample operation volume, low manufacturing cost, robustness, no complicated equipment, and user-friendly format [2]. Moreover, LFICS can be operated in the field because it can be read without the aid of any optical instrument and utilizes a dry form of reagents incorporated into this device [4, 5]. As a result, LFICS can be widely used as a point-of-care device even in low-income countries because of its low-cost materials and reduced manufacturing cost.

LFICS incorporated with nanomaterials as label has improved its performance in various sample applications compared to previous commercial LFICS for pregnancy tests in the early 1980s [2, 5]. Several nanomaterials that have been used as labels are colloidal gold (AuNPs), silver (AgNPs), carbon (CNPs), selenium (SNPs), quantum dots (QDs), up-converting phosphors (UCPs), dye-doped, and magnetic NPs [2, 5, 6]. These NPs increased the performance of LFICS by qualitative detection and also made it available for semi-quantitative and quantitative detection with the aid of simple reader devices [5].

Principle of LFICS

Typical LFICSs are manufactured in a rectangular shape with a width around 4–6 mm and length up to 6–7 cm. LFICSs are formed from four parts with different materials (Figure 6.1a). Each part has its own function [2]. Most LFICSs are covered with a plastic case to protect the strip.

A sample application is the pad located in the first end of LFICS (Figure 6.1a). The pad is made of cellulose paper or glass fiber, which initiates the flow of sample in liquid form to other parts and pre-treats the sample to move continuously in a smooth and homogenous manner. The sample then flows to the second part called conjugate pad. Labeled bio-conjugate solution (usually antibodies) in dry form is impregnated in this part. The conjugate pad is built with glass fiber or cellulose material. The function of the conjugate pad is to hold the labeled bioconjugate and release it when rehydrated by the sample. Reaction also occurs at this pad when the analyte from

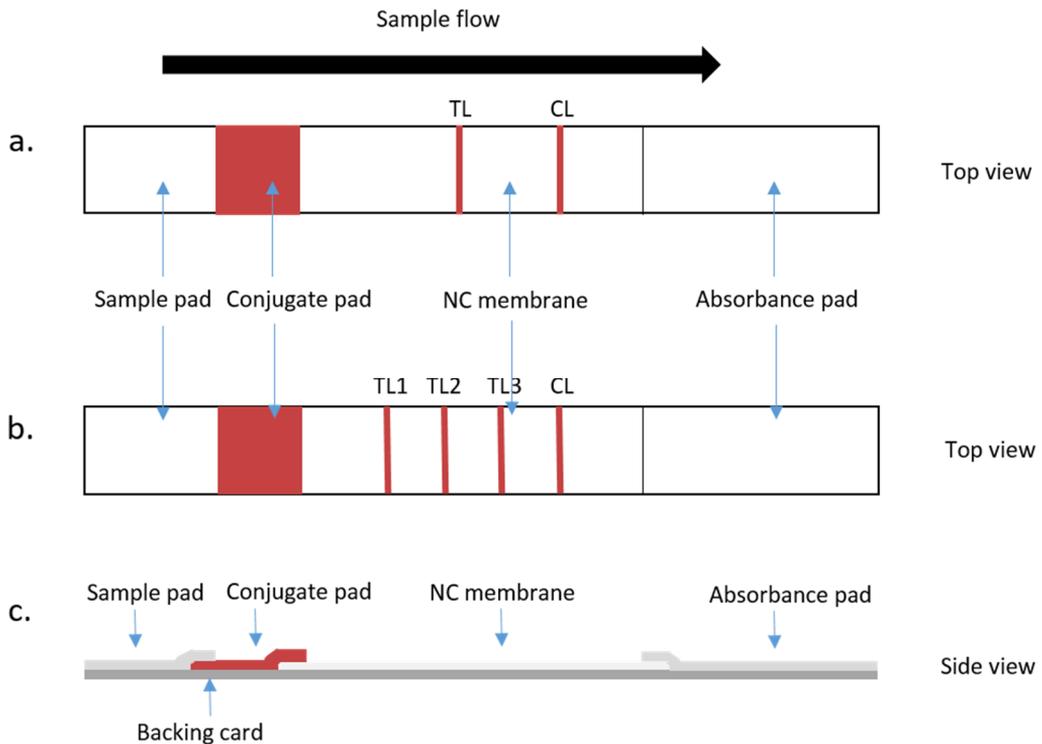
the sample binds with the labeled-antibody conjugate to form an analyte-labeled-antibody complex. This complex then flows into the nitrocellulose membrane (NC), which is made of nitrocellulose and available in different grades. In this NC membrane, test and control lines are drawn with a specific antibody against the analyte and labelled conjugate, respectively. In this part, analyte-labeled-antibody complex binds with the specific test line, whereas excess or labeled-antibody binds with the control line. Then, the conjugate label can be detected either through the naked eye or any reading device based on label use. Finally, the sample flows to the adsorbent pad at the end of LFICS. The adsorbent pad acts as a sink to hold, which maintains the flow rate and prevents the backflow of the sample. The material used is usually the same as the sample pad, which is cellulose [2, 6].

Multiplex LFICS

Multiplex assay is an assay that can simultaneously analyze or measure several analytes (more than one) within a single run or test. Multiple types of analytes can be analyzed at the same time within a single set of conditions, especially when applied in clinical diagnosis. The importance of multiplex detection format is to rapidly analyze (because multiple analytes are analyzed at the same time) at different stages of disease, detect multiple infections at once, reduce test cost (no need to perform the test separately), and examine other inter-dependent situations [6-8]. To overcome these problems, many analytical methods have been developed to detect multiple analytes simultaneously. The methods include Raman scattering [9, 10], fluorescent emission [11-15], spectrally encoded beads [16, 17], electrochemical impedance spectroscopy [18-21], microchip platform [22-24], array test strip [25], immunoprecipitation [26], multiplex enzyme-linked immunosorbent assay [27-29], and LFICS [30]. These methods were used for multiple analyte detection of disease biomarkers, proteins, small molecules, biological thiol molecules, oligonucleotide targets, heavy metals, and mycotoxin simultaneously [31].

Among the aforementioned methods, multiplex LFICS is the best choice due to its many advantages, such as rapidity, low cost, requirement of small amount of sample, simultaneous detection of all suspected parameters, and other benefits of the typical LFICS [32]. Based on these advantages, multiplex LFICS has gained much attention recently and became advanced. Few strategies exist for fabrication of multiplex LFICS. A traditional multiplex LFICS strategy is performed by adding a test line corresponding to the number of analytes to be tested, as shown in Figure 6.1b [33]. Other strategies are performed by varying the label for detection of multiple analytes within the same strip and varying test line numbers for each analyte and architecture tuning of typical LFICS [33, 34].

Thus, this review aims to discuss the recent development of multiplex LFICS based on the use of NPs as a detection label. The discussion includes several strategies used for the development of multiplex LFICS including label used, format, detection system, number of test lines, and architecture tuning from typical LFICS. In addition, multiple test lines for detecting a single analyte are also discussed and considered as multiplex LFICS. This review also discusses the application and performance of developed multiplex LFICS. Finally, some major challenges or limitations of multiplex LFICS are discussed, and then the conclusion and future perspectives are presented.

**FIGURE 6.1**

Schematic representation of various components in (a) typical single detection LFICS, (b) multiplex LFICS, and (c) side view of LFICS

Label, Conjugation, Format and Detection Systems

NPs have several distinctive physical and chemical properties that make them promising synthetic scaffold for the creation of novel chemical and biological detection systems. As NPs size is small it has large surface area. For example, silica-fluorescent NPs conjugated antibody can provide a better fluorescent signal when dispersed at a test line of LFICS than an organic dye because thousands of antibody-conjugated NPs are used to label a single analyte as compared with a single molecule of the organic dye [35]. Thus, NPs conjugated antibodies could improve the sensitivity of LFICS.

In addition, homogeneous in size and monodispersed NPs can improve the sensitivity of LFICS. As example, in the synthesis of AuNPs using seeding-growth method, small seed of AuNPs was first synthesised by citrate reduction method and followed by growth using hydroxyamine as mild reducing agent to form larger AuNPs. This method produces spherical and homogeneous size of monodispersed AuNPs compared to AuNPs synthesised by citrate reduction method. As a result, AuNPs produced using seeding-growth method significantly improved the sensitivity of LFICS [36].

NPs Conjugated Biomolecules

The NPs label is used for conjugation with potential biomolecules usually antibody, aptamer, or nucleic acid (NA) which give a typical interaction to analyte via biomolecule-analyte recognition system. The conjugated label-biomolecule is used to detect the presence of the specific analyte by forming a complex within the test line in LFICS. For example, an antibody has two identical binding sites composed of polypeptide chains loops which are very specific and complementary to a specific region of antigen molecules called epitopes. When epitopes of antigen match with binding site of its specific antibody, binding interaction occurs [37]. This interaction is similar to lock and key mechanism. As label is attached to antibody, the presence of that antigen is recognizable (Figure 6.2).

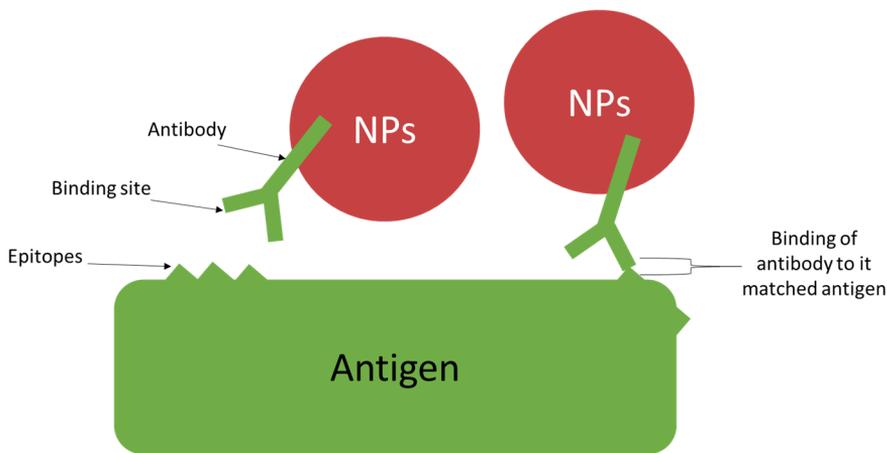


FIGURE 6.2

Schematic representation of interaction between NPs conjugated biomolecule with antigen

There are two main approaches for conjugation of NPs to biomolecules which are non-covalent, and covalent modes. The non-covalent mode can be further divided into ionic interactions, and hydrophobic interactions. The ionic interaction occurs between positively charged group at antibody with negatively charged NPs surface. Hydrophobic part of antibody on other hand can combine with NPs surface via hydrophobic interaction. These two non-covalent methods are commonly used as they are more easier and cheaper. The covalent mode offers more advance in conjugation process. This mode results in stronger bond between NPs and antibody. However, the covalent mode requires more complex method and slightly expensive. The method includes chemisorption via thiol derivatives, dative binding, and mediator linkers or bifunctional linkers such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) chemistry, and adapter molecules: streptavidin and biotin [38].

Various labels have been reported, which can be categorized as colored, luminescent, magnetic, and other NP labels [5]. These NPs must possess special characteristics to be used in the development of multiplex LFICS similar with typical LFICS, which are high sensitivity, low limit of detection (LOD), form stability when conjugated, unchangeable features of biomolecules, and ease of conjugation process [6]. One additional property that NPs must have as a label in multiplex LFICS is the ability to produce a signal corresponding to each analyte to be tested and the ability to differentiate those analytes [6].

Colored NPs

Some NP labels can be detected directly by the naked eye as they produced visual colored signals under visible light. Colored NPs are usually in colloidal form and they can emit certain wavelength from visible light depending on their sizes and types. This phenomenon is called surface plasmon resonance. This type of NPs is suitable for the development of multiplex LFICS and they can be categorized as colored NPs. Colored NPs include colloidal AuNPs, AgNPs, CNPs and SNPs. LFICS that uses these NP labels produces qualitative results that can be reported as presence (positive) or absence (negative) of detected analytes. However, semi-quantitative and quantitative results are available with the aid of strip reader devices. The principle is by measuring the color intensity either in test or control lines and converting it into optical density (OD) [5, 39]. With the advancement of strip reader devices, the sensitivity and specificity of multiplex LFICS can be improved by reducing human error (visual interpretation) [40].

One of the colored NPs that is commonly used as a label in LFICS is colloidal AuNPs. AuNPs have many advantages, such as biomolecule compatibility, time stability, size-tunable, easily synthesized, manipulability, and provides intense red color by using visible light source that can be detected by the naked eye [2, 6]. Based on these properties, AuNPs was selected as a label in the development of multiplex LFICS. The AuNP-based multiplex LFICS was reported by many researchers either using sandwich IA format or competitive format [6].

In the sandwich format, conjugated label-antibody (for each analyte) binds to specific antigens to form an antibody-antigen complex. This complex then binds to a specific antibody to immobilize another antibody against each corresponding analyte and forms sandwich like antigen-antibody complex (Figure 6.3a). The advantages in sandwich format is that the sensitivity can be increased as many analytes can be bound with two anti-analytes (the first analyte is labeled and the second is immobilized in the NC membrane). In addition, the result can be interpreted directly where the presence of a visual red color in the test line or lines represents a positive result (presence of analyte) and the absence of red in the test line or lines represents a negative result (absence of analyte) [6].

Many experiments have been performed by using the sandwich format to develop multiplex LFICS and their performance were evaluated by interpreting the result as qualitative without using a reader. Researchers used multiplex LFICS to detect several types of protein and bacterial species simultaneously [41-46]. Fernández-Sánchez et al. and Lee et al. [47, 48] integrated a reader device to obtain semi-quantitative results. They studied the relationship between OD of each test line and the concentration of corresponding analytes by plotting a calibration curve. Lee et al. [48] calculated the peak intensity ratio of the test to the control line as relative peak intensity and used it as a detection signal; this procedure is performed to remove the effect of fluidity and conjugated solution that influenced the line intensity to accurately measure the assay performance. However, the disadvantage of sandwich IA was that it provided a false negative result because of very high analyte concentrations. Oh et al. [49] combined the sandwich with competitive IA format to detect C-reactive protein by forming signal intensity versus analyte concentration graph to overcome this effect.

The competitive format for multiplex LFICS is the second type of IA. This format is usually applied when the analytes to be tested have low molecular weight. The result is interpreted inversely compare with the sandwich format where the presence of visual red color on the test line or lines represents a negative result (absence of analyte) and the absence of red color on the test line or lines represents a positive result (presence of analyte). The principle of the competitive format is the competition between immobilized analytes in the test line(s) with the present analytes in the

sample toward labeled anti-analyte conjugate that flows to the test line. If the analyte to be detected is present, it will bind with the anti-analyte. As no free labeled anti-analyte conjugate is available for binding with the immobilized analyte, no color is observed in the test line (Figure 6.3b). If the analyte is absent, the labeled anti-analyte conjugate will bind with the immobilized analyte in the test line and color can be observed [6].

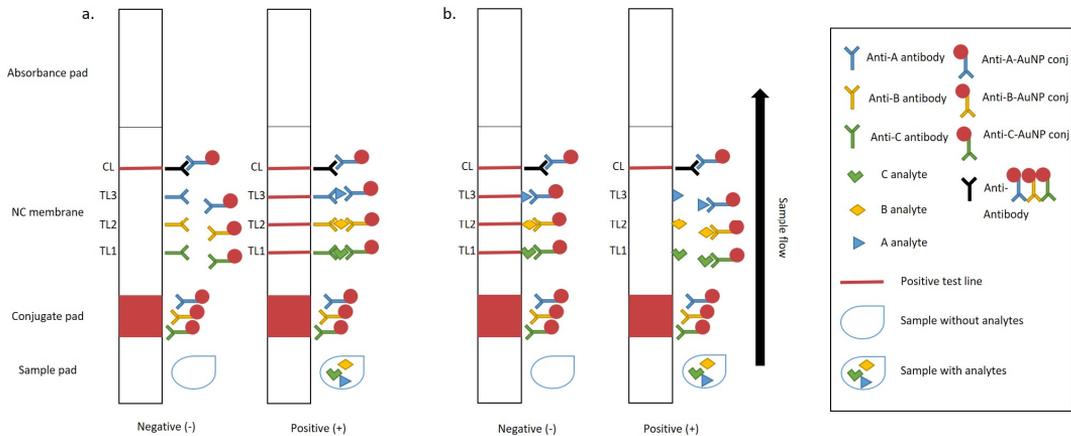


FIGURE 6.3

Schematic representation of different formats for multiplex LFICS with positive and negative results: (a) sandwich format and (b) competitive format

Multiplex LFICS using the competitive format can be also read either through qualitative or semi-quantitative detection. For qualitative detection, results are interpreted visually. For example, Huang et al., Kim et al., Kolosova et al., Li et al., and Wang et al. [30, 50-53] developed multiplex LFICS for simultaneously detecting mycotoxin; Guo et al. [54] for pesticide; Kranthi et al. [55] for insecticide, and Chang et al. [56] for environmental hormone contamination. In some studies, researchers used calibration curves to determine the concentration of each analyte as a quantitative measurement [57] to measure visual LODs [58, 59] and determine half-maximum inhibitory concentration (IC50) [59, 60] in real sample extracts for semi-quantitative measurement. Song et al. [61] developed multiplex LFICS that can be read qualitatively by the naked eye and semi-quantitatively using a strip reader. Moreover, Wang et al. [62] developed visual read-outs (without a reader device) through semi-quantitative multiplex LFICS by using four strips to detect different concentrations of analytes simultaneously. However, Leung et al. [63] developed a special LFICS against “typical” competitive LFICS in which the signal produced is directly proportional to cortisol concentration (analyte) in plasma samples.

Another colored label used in the development of multiplex LFICS is AgNPs, which has special properties where different sizes and shapes of AgNPs produce different optical properties. Considering this characteristic, Yen et al. [64] developed multiplex LFICS to distinguish dengue, yellow fever, and Ebola viruses by visually observing different colors corresponding to each virus (Figure 6.4). Furthermore, CNPs were also used as a colored label, which were more sensitive than AuNPs [65]. In multiplex LFICS, Blažková et al. and Noguera et al. [66, 67] used CNPs to detect few pathogens simultaneously. They combined polymerase chain reaction (PCR) method with LFICS. Mens et al. [68] also developed the same type of multiplex LFICS to detect *Plasmodium* spp. and all

species simultaneously in one test line. All these multiplex LFICSs can be read visually as qualitative or semi-quantitative if needed.

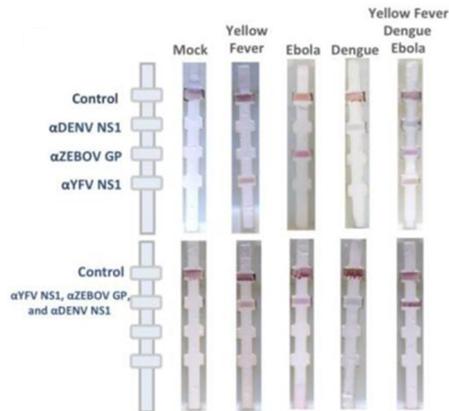


FIGURE 6.4

Multiplex LFICS using silver nanoparticle (AgNPs) reprinted with permission from (Yen C-W, de Puig H, Tam JO, Gomez-Marquez J, Bosch I, Hamad-Schifferli K, et al. Multicolored silver nanoparticles for multiplexed disease diagnostics: distinguishing dengue, yellow fever, and Ebola viruses. *Lab on a Chip*. 2015;15(7):1638-41). Copyright (2015) Royal Society of Chemistry

Luminescent NPs

Several NPs require a reader device (indirect detection). Some examples of luminescent NPs are QDs, UCPs, and dye-doped NPs. The results of luminescent NP-based LFICS are quantitative as the signal is quantitatively read by a special reader. This type of LFICS is more sensitive than colored NPs-based LFICS although it needs additional equipment (reader device) that increases manufacturing cost [2]. In the development of multiplex LFICS, this type of NPs is valuable because it produces a special quantum-sized effect where different particle sizes emit spectrum at different wavelengths when excited [34].

QDs are the reported labels used in multiplex LFICS due to their capability of simultaneous quantification of multiple analytes. In addition, QDs have the capability to produce strong luminescence, photostability, broad absorption, narrow and symmetric photoluminescence spectra, and size-tunable emission [5]. Similar to the colored NPs-based LFICS, QD-based LFICS also have two formats: sandwich and competitive. For the sandwich format, Wu et al. [69] developed multiplex LFICS to simultaneously detect influenza A virus subtype H5 and H9 via QD-based IA in separate test lines. In the presence of analytes, the sandwich complex formed in the test line produced a bright fluorescent band when excited using 365 nm ultraviolet (UV) light sources. The result was read as quantitative where the intensity of fluorescence was detected using the reading device corresponding to the virus concentration. In other studies, Wang et al. [70] used one test line for simultaneous quantitative detection of tumor markers because multicolor QDs can be detected separately using reader devices. Taranova et al. [71] developed multiplex LFICS based on the competitive format. They used “traffic light” format where each test line produced different colors that can be easily identified by the naked eye (qualitative). The fluorescence intensity of the lines could also be detected through quantitative analysis.

Another class of luminescent fluorescent is UCPs with a unique property where it can emit high energy either visible or UV light when irradiated with low-energy infrared light. Corstjens et al. developed two quantitative multiplex LFICS using the sandwich format to simultaneously detect cellular and humoral immune responses against leprosy [8] and humoral immune response against human immunodeficiency virus (HIV), hepatitis C virus (HCV), and tuberculosis (TB) [72]. A 10-combined LFICS for detecting different antibodies against *Yersinia pestis* with the use of single sample pad was also developed using UCPs as a label [73].

Other NPs

Several other NPs used as a label for multiplex LFICS is a fluorescent probe. Xu et al. [74] used fluorescent probe-based LFICS for multiplex NA detection. They combined PCR with LFICS. Worsley et al. [33] used fluorescent microsphere as a label. They used optical discrete fluorescent or Raman spectra peak signal as spectral separation of mixed conjugates within a single line in a multiplex assay to simultaneously detect wound biomarkers. In another study, Carter and Cary [75] used dyed microspheres as a label to detect NA; their approach was based on miniaturized lateral flow device.

Multiplex LFICS Design

Another existing strategy to realize simultaneous detection of multiple analytes is by varying LFICS design. This strategy includes the design of several test lines, miniaturized design version of LFICS, bi-specific labelled-biorecognition design (commonly detects several analytes per conjugate), and architecture turning in formulating multiplex design prototypes [5]. Variation of test line strategies can be divided by multiple test lines for each analyte and a single test line to detect multiple analytes.

Multiple Test Lines

Multiple Analytes

Multiple test lines for multiple analytes is a common design for the development of multiplex LFICS. In this design, additional test lines are included for each additional analyte to be tested (Figure 6.1b and Figure 6.3). Sun et al. [76] developed multiplex for simultaneously detecting two types of mycotoxin, ochratoxin A (OTA), and zearalenone (ZEN) in cereals. They used competitive format (Figure 6.3b) where OTA-BSA and ZEN-BSA conjugates were separately immobilized at different test lines (TL1 and TL2, respectively) on NC membrane. AuNPs, as the label, were conjugated to anti-BSA and anti-ZEN monoclonal antibody (mAb). If the sample contained OTA, it binds to AuNP anti-OTA mAb. Thus, no free site of AuNP anti-OTA mAb binds with OTA-BSA in TL1 and result in the absence of color is observed at TL1 (positive). If ZEN is present in the sample, no color is observed at TL2. However, if OTA and ZEN are present in the sample, they will bind with AuNP-mAb conjugate and no free site AuNP-mAb conjugate binds with the test lines, which result in the absence of visual color is detected in the two test lines. For the sandwich format (Figure 6.3a), the

result is interpreted inversely as explained in the preceding parts. Most of the literature explained earlier in the colored NPs section used this design (multiple test lines for multiple analytes).

Single Analyte

In this design, several test lines are printed on an NC membrane to detect a single analyte. The purpose of this additional test line is to increase the sensitivity of typical LFICS through visual semi-quantitative measurement. A typical semi-quantitative format needs a reader device to convert the color intensity of the label that is proportional to the amount of detected analyte. The main drawback of this design is the requirement of a reader device. As a result, some researchers developed device-free semi-quantitative detection of analyte, which is called a barcode-style LFICS, to determine the level of disease [77, 78], hazard compound [79], mycotoxin [80, 81] and analyte percentage in whole samples [82]. In this strategy, each test line represents a fix detection value or cut-off value of the analyte. When an analyte at a certain concentration flows on LFICS, it binds with the respective cut-off line. In other words, the number of lines on the test strip is directly proportional to the analyte concentration. The preceding principle is only applied in the sandwich format [77-79]. For the competitive format, increasing the analyte concentration causes the test lines to disappear gradually, which indicates that the disappearance of each test line represents a threshold level of analyte concentration [80, 81].

Ang et al. [82] developed barcode-style LFICS in a slightly different manner; they used dual detection (two different AuNPs size conjugated to mAb) to measure HbA1c and total Hb. This design provides a valuable percentage of HbA1c values for improved diabetes care onsite. This strip comprises five lines that contain three test lines to detect different HbA1c concentrations; one Hb line and one control line. The ratio of HbA1c to total Hb was calculated as percentage HbA1c.

Ang et al. [83] developed a different strategy by detecting a single analyte with multiple-line LFICS. They combined the PCR method with LFICS. The purpose of this strategy is not for visual semi-quantitative detection but for additional detection of positive control (two control lines). This LFICS has three lines: one for detecting the targeted analyte, and the other two for detecting positive control; one for validating PCR method and the other for validating LFICS.

Single Test Line

Multiple Analytes

In this design, multiple analytes are detected simultaneously within a single test line without the need for an additional test line for each analyte to be tested. Lin et al. [84] developed multiplex LFICS to detect immunoglobulin G (IgG) antibodies in *Treponema pallidum* by using two recombinant antigens as a labeled-antigen conjugate (TPN17 and TPN47). This multiplex LFICS detects IgG-specific samples with TPN17 and/or TPN47 by binding to capture immobilized antibodies on a single test line. Labeled-antibody conjugate can also be designed to detect several analytes in addition to separate conjugate (bi-specific antibody). For example, Li et al. [85] used a mAb against fumonisin B1 (FB1) with high cross-reactivity with fumonisin B2 (FB2) and fumonisin B3 (FB3) as a conjugate probe. In their study, LFICS can rapidly detect total fumonisin (FBs) (FB1, FB2, and FB3) in a single test line. Zhang et al. and Grimaldi et al. [86, 87] also used a similar strategy. However, this strategy has drawbacks; different analytes cannot be distinguished and the concentration of each specific analyte cannot be determined. To address this problem, Worsley et

al. [33] used fluorescent microspheres as a label as discussed in the previous section for quantitatively detecting two analytes (interleukin 6 and tumor necrosis factor alpha) as chronic wound biomarkers in a single test line. The difference in optical discrete emission characteristics of the label made this strategy possible.

LOGIC System

The idea for the LOGIC system is based on electronic logic gates of the transistor-based computational device. This concept is then applied as an analogy for the molecular logic gate where a set of molecular computation is used to generate “outputs” from chemical or physical “inputs” for the development of intelligent diagnostics [88]. The LOGIC systems can be AND, OR, NOR, XOR, or INHIBIT (INH) depending on design purpose.

Chen et al. [88] developed LOGIC-based LFICS using AND and OR operation for detection of thrombin and ATP. In this system, split/integrated aptamers were used as a molecular recognition element using AuNPs as tracer. Thrombin and ATP were used as inputs in this system, whereas the outputs could be directly visualized by a red color on the test line. For output interpretation, when the red color was observed, the output = 1, whereas if no color was observed in the test line, the output = 0. Furthermore, in the AND LOGIC system, only the presence of inputs, thrombin, and ATP (1, 1) provides an output of “1”. In the absence of both inputs (0, 0), or in the presence of either input (1, 0; 0, 1), the output is “0” and no color is observed on the test line. In the OR LOGIC system, the presence of both (1, 1) or either one input (1, 0; 0, 1) provides an output of “1”. The absence of both inputs (0, 0) provides an output of “0” (Figure 6.5a-b).

Huang et al. also [89] reported the use of LOGIC systems for OR and INH operation. They detected carcinoembryonic antigen (CEA) protein and c-DNA based (as inputs) on target-induced conformational changes of hairpin oligonucleotide (HO) using AuNPs as detector. The output was similar with the findings of Chen et al. [88] where “1” reflects the color that appeared, whereas “0” indicates that no color appeared on the test line. Furthermore, the OR operation is similar to the aforementioned operation. For the INH operation, the presence of the only specific input (CEA) provides an output of “1”. Thus, the presence of another input (CEA-HO) (0, 1) and the presence and absence of both input (1, 1), (0, 0) provides an output of “0”.

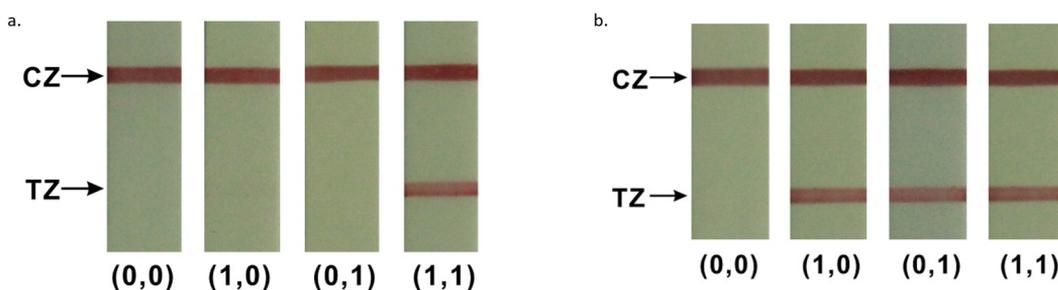


FIGURE 6.5

LOGIC based multiplex LFICS: (a) AND LOGIC system and (b) OR LOGIC system reprinted (adapted) with permission from (Chen J, Fang Z, Lie P, Zeng L. Computational lateral flow biosensor for proteins and small molecules: a new class of strip logic gates. *Analytical chemistry*. 2012;84(15):6321-5). Copyright (2012) American Chemical Society

Multiple Dots

Advances in technology led to the development of DNA microarrays, which represent a sophisticated high-throughput system. DNA microarrays may be combined with the dry-reagent-based format, LFICS. Multiple test spots are drawn on the NC membrane of LFICS for visual detection. Furthermore, this type of design can be used for genotyping several mutations/polymorphisms in parallel. The principle of the test is simple. The dot appears when anti-biotin AuNPs conjugate binds to a specific biotinylated-PCR product that hybridizes with specific capture oligonucleotide probes pipetted on the NC membrane [90, 91].

The above mentioned design was reported by several researchers as an example to detect a mutation in *HBA1* and *HBA2* gene of alpha thalassaemia [92], genotyping of 15 beta-globin gene (*HBB*) mutations [93] and mutation of *ATP7B* gene in Wilson disease [90]. The multiple-dot design involves three steps. First, target DNA sequence is amplified by PCR. Second, an allele discrimination step is used through a primer extension reaction that produce biotin incorporated allele-specific product. Finally, a visual detection of the product through an anti-biotin AuNPs label conjugate result is detected by observing a red spot on LFICS (Figure 6.6). Furthermore, this design can be applied to determine the concentration of four types of drug abuse [94].

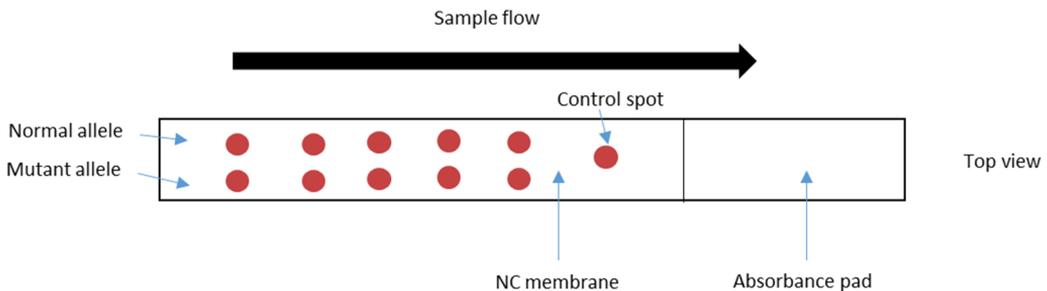


FIGURE 6.6
NA-LFICS: LFICS detection and interpretation of the genotyping result

Architecture Tuning

Aside from using internal modification strategies as discussed previously, LFICS multiplexing can be conducted by formulating external modification where the architecture of the strip itself can vary. Furthermore, it can either be by designing various test-strip shapes or combining several test strips into one. Fu et al. [95] presented the capability of 2D paper networks with multiple inlets per outlet for multi-analyte detection. They evaluated this method for controlling fluid transport through the geometry of network and dissolvable barriers, which provide higher sensitivity. Moreover, they concluded that the arrival time of multiple reagents to the detector region was faster when using this design (three inlets along one common outlet), as demonstrated in Figure 6.7a.

In addition, Fenton et al. [96] demonstrated 2D shaping paper- and NC-based lateral flow devices for multi-analyte detection. They shaped the paper/NC into a star, candelabra, and other structures using a computer-controlled knife as illustrated in Figure 6.7b. The shaped device was then spotted with multiple bioassay reagents to produce multiplex LFICS. To avoid contamination and minimize evaporation, laminar composites were fabricated where NC media was between vinyl and polyester plastic films in the final version. This fabricated method was claimed to be novel, low cost, and

suitable for resource-poor areas, prototype development, high-volume manufacturing, and reducing operator error.

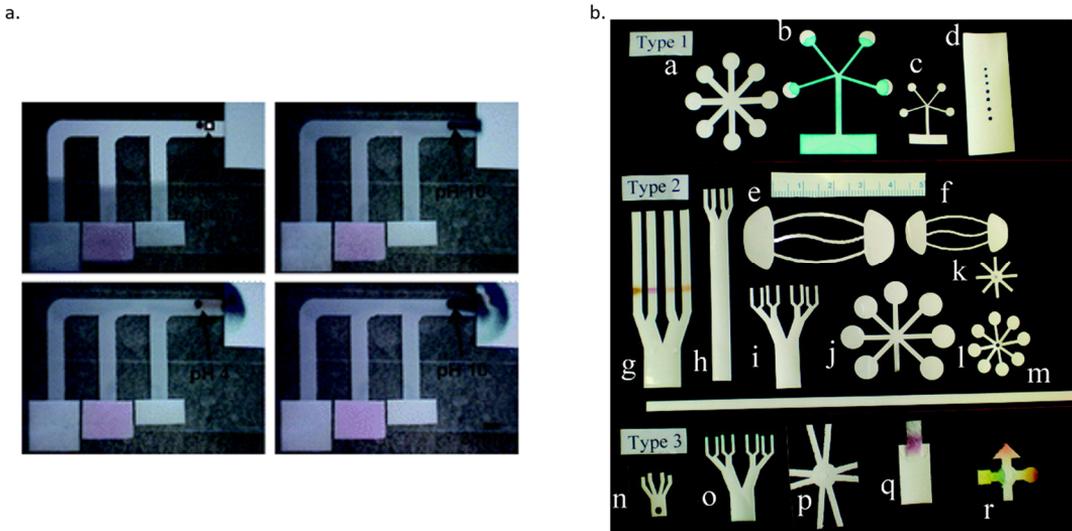


FIGURE 6.7

Multiplex LFICS design: (a) Three inlets along one common outlet design reprinted with permission from (Fu E, Lutz B, Kauffman P, Yager P. Controlled reagent transport in disposable 2D paper networks. *Lab on a Chip*. 2010;10(7):918-20). Copyright (2010) Royal Society of Chemistry. (b) The star, candelabra and other structures shaped using a computer-controlled knife are reprinted (adapted) with permission from (Fenton EM, Mascarenas MR, López GP, Sibbett SS. Multiplex Lateral-Flow Test Strips Fabricated by Two-Dimensional Shaping. *ACS Applied Materials & Interfaces*. 2009;1(1):124-9). Copyright (2009) American Chemical Society

Hong et al. [73] used UCPs as a label to develop 10-channel LFICS. In this method, they combined 10 strips of typical design (one test line and one control line) with separated conjugate pad. The 10 strips were then assembled symmetrically into the disc type plastic cartridge with shared sample pad and different result-scanning window. This design allows the sample to synchronously and uniformly distribute for simultaneously detecting 10 targets using a single sample. Aside from the aforementioned process, a special reader device was designed to implement linear and rotary motions for realizing the scanning detection of 10 strips on the disc. This reader device captures the signals of 10 strips consecutively within 2 min.

Li et al. [97] designed and optimized several prototypes for multiplex detection of whole-cell antigens of *Pseudomonas aeruginosa* and *Staphylococcus aureus* simultaneously. The optimized design prototypes included fork, peacesign, and triangular centerpiece. Among these designs, the triangular centerpiece was chosen because it provided more effective LFICS performance than other prototypes. In addition, they designed and fabricated a compact portable device to convert the color intensity of AuNPs on the test line into a quantitative result by means of voltage reading. Bacterial concentration in the detected sample is proportional to the voltage generated when using this device.

A number of interesting designs in the development of multiplex LFICS is obtained by using lateral flow microarrays. Carter and Cary [75] developed a miniaturized lateral flow device that uses hybridization-mediated target capture. In this design, several capture oligonucleotides were immobilized on lateral flow, whereas detection oligonucleotides conjugated with dyed

microspheres as the label was injected into the conjugate release pad. Positive and negative hybridization control was printed on the NC membrane. The NC membrane was then cut into 3 mm-wide strips, assembled with conjugate pad, and then covered with custom design plastic housing. This device offers the simplicity of LFICS-based platforms and sensitivity for detecting and discriminating closely related minority sequences of microbial signatures in complex NA mixtures within 2 min.

Conjugate Specificity

The conjugated probe can be designed either as bi-specific, which can detect simultaneously several analytes or monospecific to separately detect one specific analyte. Guo et al. [54] reported the comparison of developed multiplex LFICS using bi-specific monoclonal antibody (BsMcAb) conjugate against both triazophos and carbofuran with separate monospecific McAb (MsMcAb) conjugate against each triazophos and carbofuran. They discovered that by using MsMcAb as a separate conjugate with AuNPs provide higher sensitivities for both pesticides rather than using BsMcAb when visually detected by the naked eye. Fernández-Sánchez et al. and Yanget et al. [44, 47] developed multiplex LFICS using bi-specific McAb to simultaneously detect free and total prostate specific antigen; and serotypes O, A and Asia 1 of foot-and-mouth disease virus. However, Lee et al. [48] used antigen (protein A) conjugated with AuNPs that have bi-specificity toward AIDS, hep C and hep A antibodies in serum. They used indirect IA method where antibody was detected in serum compared to direct IA method, which detected antigen in specimens.

Applications

Nucleic Acid LFICS (NA-LFICS)

Multiplex LFICS can be applied to detect various types of analytes in various fields, including health, medicine, and forensics. One of the applications detect nucleic acid product derived from the PCR method. Furthermore, it is a combination method where the PCR method was first used to amplify the target DNA of specific species or genes, which are later combined with LFICS as a detector for such targets. The PCR method is widely known for having high specificity and sensitivity, but the target DNA detection requires an additional laborious and hazardous method (gel electrophoresis). Thus, when combined with a simple detection method, such as LFICS, a perfect combination could be obtained.

In the beginning, Oku et al. [98] explained the basic principle of NA-LFICS for simultaneous detection of multi-analyte antigens and/or antibodies (HBsAg and TP) in the specimen. This device uses colloidal AuNP-labelled antibodies and oligonucleotide-labeled antibodies as labels that are fixed in the conjugate pad. Complimentary oligonucleotide-labeled proteins are immobilized on an NC membrane. When the target is present in the specimen, it forms a complex with AuNP-antibody and oligonucleotide-antibody. This complex continues to flow until it reaches a place where the immobilized complementary oligonucleotide is placed. Then, DNA-DNA interaction occurs when specific matches are available and the result can be visualized through an appearance of red band on the specific test line.

Subsequently, NA-LFICS was applied for simultaneous and differentiated virus and bacterial DNA in

specimens. For virus detection, Dineva et al. [99] reported the use of NA-LFICS to detect and differentiate HBV DNA, HCV RNA, and HIV-1 RNA, whereas Kuo et al. [100] reported the same for HPV 16 and HPV 18. Horng et al., Soo et al., and Zulkiply et al. [101-103] developed NA-LFICS for multiplex bacterial detection, whereas He et al. [104] detected mutation sites of transglutaminase 1 gene in autosomal recessive congenital ichthyosis using hairpin oligonucleotide (HO) as molecular recognition probe.

All the first amplified targeted genes use the PCR method. Two sets of primer pairs were designed to amplify longer DNA fragment (external primer) and shorter internal DNA fragment. This primer was then labeled with biotin and either digoxigenin (Dig) or fluorescein (F) at 5'-terminus. Two steps of PCR occurred with the first step amplified longer DNA fragment and then the amplified product was used in second PCR to amplify a shorter DNA fragment. Amplified PCR products contained biotin in one end for both targets and either F or Dig corresponds to each target. In LFICS, anti-Dig and anti-F were immobilized on the NC membrane forming test lines 1 and 2 and the control line. Thus, corresponding species could be observed when the red color was observed at the corresponding test lines.

NA-LFICS can have an additional control line for PCR control, namely, internal amplification control (IAC) to validate the PCR process. The false negative result may occur due to DNA degradation or PCR inhibitors; to prevent it, IAC must be included [105]. Ang et al. and Wong et al. [106, 107] integrated IAC in their NA-LFICS for detection of cholera toxin gene (*ctxA*) and *Cordyceps* species, respectively. Nurul Najian et al. [108] reported the use of IAC to validate NA-LFICS. They simultaneously detected targeted gene (pathogenic *Leptospira*) and IAC product. However, they amplified the targeted gene using a loop-mediated isothermal amplification (LAMP) method that offered more advantages such as quick processing time and no thermocycling equipment compared to the conventional PCR method.

Large and Small Analytes

As discussed, multiplex LFICS works effectively to detect the various types of analytes, including large and small ones. The larger analytes include protein, a bacterial cell, and a toxin. Multiplex LFICS that was used to detect larger analytes mostly used the sandwich format. However, smaller analytes such as aptamer, mycotoxin, pesticides, toxic pollutant, and heavy metals used the competitive IA method as format. Due to a smaller size, these analytes require conjugation with another protein such as bovine serum albumin (BSA) before it can be immobilized on the NC membrane as a competitor for the analytes to be detected [109].

Applications of multiplex LFICS include food safety, clinical disease biomarkers, and environmental health. In food safety, multiplex LFICS is used to detect food-borne pathogens and their toxins. Most researchers developed multiplex LFICS to simultaneously detect mycotoxins in food items including ZEN, OTA, FB1, deoxynivalenol (DON), and aflatoxin B₁ (AFB₁) [30, 50-53, 57, 60, 61]. Le et al. [58] developed multiplex LFICS for simultaneous detection of cyromazine (CA) and melamine (MA) in animal origin foods whereas Zhang et al. [59] developed multiplex LFICS for simultaneous detection of clenbuterol (CLE) and ractopamine (RAC) in swine urine. Several pathogenic bacteria that caused food-borne disease were detected simultaneously, such as *Escherichia coli* (*E. coli*) O157:H7, *Shigella boydii*, several types of shiga-toxin-producing *E. coli*, *Salmonella enterica* serovars Typhimurium, and Enteritidis and *Vibrio cholera* serogroups O1 and O139 [42, 43, 45, 110]. Furthermore, multiplex LFICS was applied to clinical disease. Several viruses have been detected simultaneously through multiplex LFICS such as AIDS, hepatitis C, hepatitis A, influenza A/H1N1

virus, and foot-and-mouth disease [44, 46, 48]. Disease biomarker can be detected, such as free and total prostate specific antigen in serum [47] and wound healing [33]. Moreover, multiplex LFICS can be used to detect genetic defects, such as detecting drug-induced inhibition of mitochondrial DNA replication and mtDNA-encoded protein synthesis [111] and multi-allele genotyping of mutations [90, 92, 93].

In environmental health, several types of toxin, heavy metals, and pesticides frequently contaminate the environment and may be harmful to human health. As a result, multiplex LFICS that is simple, rapid, and sensitive to simultaneous detection of several heavy metals (Hg(II), Cd(II), and Pb(II)) [109]; pesticides (imidacloprid, chlorpyrifos-methyl, and isocarbophos) [62] and insecticide (pyrethroid and endosulfan) [55] have been developed. Pesticide residues such as carbofuran and triazophos [54], synthetic contraceptive levonorgestrel and glucocorticoid methylprednisolone residues [56] and mixture of several chemicals (microcystin-leucine-arginine, Pb(II), chloramphenicol, testosterone and chlorothalonil) [31] can simultaneously be detected in water samples to ensure water quality and safety.

Multiplex LFICS Performance

The performance of multiplex LFICS is evaluated by sensitivity or LOD toward analytes, and specificity which is the ability of LFICS to correctly identify or recognize specific analytes to be tested. The performance of LFICS generally depends on the label used as conjugate. LFICS based on QDs as label gave LOD for AFB₁ as low as 0.42 pg/mL whereas using AuNPs, LOD just 250 pg/mL [30, 112]. Moreover, multiplex LFICS using CNPs as a label is able to detect *E. coli* with LOD between 10⁴ – 10⁵ colony forming units (CFU)/mL compared to AuNPs as label only 10⁶ CFU/mL [43, 66]. The reason why AuNPs probe showed lower sensitivity (higher LOD) compared to other NPs probes is that 20–30 nm spherical AuNPs exhibits insufficient signal intensity [113, 114]. Linares et al. [115] proved that different conjugate probe gave variable LOD where AuNPs probe produced the poorest results. However, in the specificity aspect, no cross-reaction occurs between them, thereby indicating that the specificity of AuNP-based multiplex LFICS is good.

Even though other NP labels show higher sensitivity compared to AuNP, the latter are still the most chosen bioconjugate due to its properties such as biocompatibility, ease of synthesis, and stability with time [2]. To improve the detection limit of AuNP-based multiplex LFICS, researchers have developed LFICS with modified AuNPs. The modification of AuNPs is also known as an enhancement strategy. The strategy can be through the modification of the AuNP surface by chemical method or by altering physical properties. The chemical enhancement method could be performed by adding additional NPs, which are AgNPs to AuNPs. Silver ions in silver staining solution was catalyzed by AuNPs to form metal silver and deposited on the AuNPs surface. Deposited Ag on AuNP allowed the enlargement of particle dimensions of ordinary AuNPs. The black coloration result by Ag deposition provides better contrast to the test line against a white background of NC membrane. Yu et al. [116] developed a multiplex LFICS for FB₁ detection and DON in maize samples using silver staining as enhancement strategy to increase the sensitivity of the test. They found that LOD for FB₁ and DON by silver staining was 2 ng/mL and 40 ng/mL compared to 5.23 ng/mL [57] and 100 ng/mL [52], respectively. Another strategy is by adding a second AuNPs conjugate with a larger size than primary AuNPs conjugate for analyte detection. Furthermore, this approach is also known as a dual AuNP-based signal enhancement. Zhu et al. [117] used this strategy to enhance the sensitivity of multiplex LFICS for detection of cardiac troponin I (hs-cTnI) and myoglobin. The sensitivity of enhanced multiplex LFICS was 9 ng/mL for hs-

cTnI and 10 $\mu\text{g/mL}$ for myoglobin. On the other hand, the specificity of these two-enhanced multiplex LFICS remained high.

The sensitivity and specificity of the reported multiplex LFICS were high and in good agreement with those obtained through conventional and reference standard methods, such as ELISA, GC/MS spectrometer, HPLC, and culture methods [43, 52, 61]. These indicate that the developed multiplex LFICS was accurate, reliable, reproducible, stable, and comparable with the conventional and standard method. Table 6.1 summarizes the performance of multiplex LFICS using a variety of formats and strategies.

TABLE 6.1

Overview of label, format, and design of multiplex LFICS

Label	Format	Readout	Design	Sensitivity/LOD	Assay time (min)	Samples tested / analytes	Ref.
AgNPs	Sandwich	Qualitative	Multiple TL, multiple analytes	YFV NS1, ZEBOV GP & DENV NS1: 150 ng/mL	Not reported	Dengue, yellow fever, and Ebola viruses	[64]
AuNPs	Competitive	Qualitative	Multiple TL, multiple analytes	AFB1: 0.25 ng/mL; OTA: 0.5 ng/mL; ZEA: 1 ng/mL	20	Agro-food	[30]
AuNPs	Competitive	Qualitative	Multiple TL, multiple analytes	DON: 50 ng/mL; ZEA: 1 ng/mL	15	Grains	[51]
AuNPs	Competitive	Qualitative	Multiple TL, multiple analytes	LNG and MP: 10 ng/mL	10	Water	[56]
AuNPs	Competitive	Qualitative	Single TL, multiple analytes	FBs mixture: 2.5 ng/mL	10	Maize	[85]
AuNPs	Competitive	Qualitative	Bi-specific mAb conjugate	Carbofuran: 32 $\mu\text{g/L}$ & triazophos: 4 $\mu\text{g/L}$	8 – 10	Water sample	[54]
AuNPs	Competitive	Quantitative	Multiple TL, multiple analytes	ZEN: 0.35 ng/mL; FB1: 5.23 ng/mL	30	Corn and Wheat	[57]
AuNPs	Competitive	Semi-quantitative	Multiple TL, multiple analytes	Imidacloprid: 50 $\mu\text{g/L}$; chlorpyrifos-methyl: 100 $\mu\text{g/L}$; isocarbophos: 100 $\mu\text{g/L}$	7	Chinese cabbage and soil	[62]
AuNPs	Competitive	Visual semi-quantitative	Barcode	AFB1: 0.06 ng/mL; AFB2: 0.25 ng/mL; AFG1: 0.125 and AFG2: 0.25 ng/mL	15	Toxic or harmful substances in food	[80]
AuNPs	Sandwich	Qualitative	Multiple TL, multiple analytes	<i>S. boydii</i> and <i>E. coli</i> O157:H7: 106 CFU/mL	5-10	Bread, milk and jelly	[43]
AuNPs	Sandwich	Qualitative	Multiple TL, multiple analytes	serotype O: 17 viral particles (VP); serotype A: 3400 VP; serotype Asia 1: 7200	15	Foot-and-mouth diseases virus	[44]
AuNPs	Sandwich	Qualitative	LOGIC	Thrombin: 10 nM & ATP: 10 μM	10	Human serum for thrombin and ATP	[88]

AuNPs	Sandwich	Qualitative	LOGIC	CEA: 200 ng/mL & c-DNA: 1 μ M	10	CEA and c-DNA in 10% human serum	[89]
AuNPs	Sandwich	Qualitative	Multi-dots	100 fmol/ μ L of amplicon	Total time: <2 h	Wilson disease related <i>ATP7B</i> gene mutation	[90]
AuNPs	Sandwich	Qualitative	Multi-dots	100 fmol/ μ L of amplicon	Total time: <2 h	Alpha thalassaemia for <i>HBA1</i> and <i>HBA2</i> gene mutation	[92]
AuNPs	Sandwich	Qualitative	NA-LFICS	HBV DNA: 50 IU/mL; HCV RNA: 125 IU/mL; HIV-1 RNA: 500 IU/mL	15	Plasma patients	[99]
AuNPs	Sandwich	Qualitative	NA-LFICS	100 copies of HPV viral DNA	10	HPV 16 and 18	[100]
AuNPs	Sandwich	Qualitative	NA-LFICS	Synthetic target DNA: 0.3 ng & pure genomic DNA: 1 μ g	20 – 25	Cholera toxin gene (<i>ctxA</i>)	[106]
AuNPs	Sandwich	Qualitative	NA-LFICS (m-LAMP)	Pathogenic <i>Leptospira</i> : 3.95×10^{-1} genomic equivalent/mL	30 (for m-LAMP only)	Pathogenic <i>Leptospira</i>	[108]
AuNPs	Sandwich	Qualitative & quantitative	“Peace-sign” design immune disc	<i>P. aeruginosa</i> & <i>S. aureus</i> : 500 – 5000 CFU/mL	10	Infectious disease	[97]
AuNPs	Sandwich	Semi-quantitative	Multiple TL, multiple analytes	100% sensitivity compared to ELISA	30	AIDS, hepatitis C, and hepatitis A	[48]
AuNPs	Sandwich	Semi-quantitative & quantitative	Bi-specific mAb conjugate	f-PSA & t-PSA: 1 μ g/L	<20	Male serum	[47]
AuNPs	Sandwich	Visual semi-quantitative	Barcode (multiple TL single analyte)	Gliadin: 10 μ g/mL	30	Gliadin in foods	[79]
AuNPs	Sandwich	Visual semi-quantitative	Barcode	Prostate acid phosphatase (PAP): 0.25 ng/mL	30	Serum PAP	[77]
CNPs	Sandwich	Qualitative & Semi-quantitative	Multiple TL, multiple analytes	<i>vt1</i> , <i>vt2</i> , <i>eae</i> , <i>ehxA</i> and 16S: 10^4 – 10^5 CFU/mL or 0.1 – 0.9 ng/ μ L DNA	Total time: <1 h	Shiga toxin-producing <i>E. coli</i>	[66]
Fluorescent	Sandwich	Semi-quantitative	NA-LFICS	HPV type 6, 11, and 16: 10 copies plasmid DNA/ μ L; HPV type 18: 100 copies plasmid DNA/ μ L	30	Common HPV types	[74]
Fluorescent microspheres	Sandwich	Quantitative	Single TL, multiple analytes	IL6: 7.15 pg/mL; TNF α 10.7 pg/mL	Not reported	Plasma	[33]

QD	Competitive	Quantitative	Multiple TL, multiple analytes	Ofloxacin: 0.3 ng/mL; chloramphenicol: 0.12 ng/mL; streptomycin: 0.2 ng/mL	10	Milk	[71]
QD	Sandwich	Quantitative	Multiple TL, multiple analytes	H5: 0.016 hemagglutinating units (HAU); H9: 0.25 HAU	15	Influenza A virus subtypes H5 and H9	[69]
QD	Sandwich	Quantitative	Single TL, multiple analytes	Alpha fetoprotein (AFP): 3 ng/mL and CEA: 2 ng/mL	15	Tumor marker in human serum	[70]
UCP	Sandwich	Quantitative	10-channel in disc	YPO3827: 18.20 µg/mL; YPO2131: 7.67 µg/mL; LcrV: 0.97 µg/mL; YPO1089: 0.41 µg/mL; YPO1303: 2.75 µg/mL; YopD: 97.11 µg/mL; YPO1435: 3.90 µg/mL; YPO1613: 30.36 µg/mL; F1: 0.37 µg/mL & YPO2118: 3.39 µg/mL	15 for incubation; 2 for reading	Serum for profile antibodies against <i>Yersinia pestis</i>	[73]

Major Challenges, Limitations, and Commercialization Potential of Multiplex LFICS

A few challenges and limitations in the development of multiplex LFICS are still present. The limitations can be in terms of sensitivity, specificity, reproducibility, ease of result to be interpreted, manufacturing process, and cost to the end users. The multiplexing of LFICS becomes tedious during the manufacturing process due to the need for special design or additional bio-conjugate probe to ensure availability for multiple detections of analytes. However, manufacturing cost can be reduced if a similar material and design can be used in conventional LFICS. Furthermore, bio-recognition such as antibodies can be designed to be bi-specific toward several analytes, thereby reducing the production cost of several conjugates of each antibody toward different targeted analytes.

Moreover, the lack of sensitivity observed in multiplex LFICS is compared to single-detection LFICS especially when using AuNP as a label [43, 118]. This may be due to the competition of several conjugates toward specific analytes within a single aliquot of sample. This situation can limit the chance of the conjugate label to bind with an analyte and decrease the intensity of color on the test line. To overcome this issue, careful optimization of conjugate amount per analyte is the key point for reducing the competition. The use of other labels such as luminescent NPs or magnetic NPs can improve sensitivity. However, the use of NPs may require additional equipment and synthesis of such NPs. However, enhancement strategies can be applied in this situation if AuNPs are required to be used as a label in multiplex LFICS. As discussed, the enhancement of AuNPs produced a

significant increase in the sensitivity of multiplex LFICS. In addition, AuNP-based multiplex LFICS is reproducible and has a simple result interpretation.

Most multiplex LFICS developers intend to commercialize their products. In this situation, several aspects require careful examination. To bring the product into the market, detailed optimization assay steps, device materials, and the result readout must be presented. Due to the high growth rate of commercial LFICS in the market, the developers must carefully optimize their device to produce acceptable performance. If not, then the product cannot compete with commercially available LFICS. Furthermore, multiplex ELISA can be a major competitor for multiplex LFICS if the end user needs a better performance without considering the price and assay time [6, 119].

Conclusion and Future Perspectives

The LFICS has been used for a long time as a portable device that detects a wide variety of analytes with the advantages of simplicity, affordability, speed, and promising acceptable performance to the end users. Moreover, with the recent advancement in nanotechnology, several types of NPs have been successfully synthesized and used in the LFICS device as detector labels. A modification of materials used in LFICS and its component, such as the sample pad, conjugate pad, NC membrane, and absorbent pad could strongly increase the performance and advantages of LFICS. However, the only single-detection capability offered by LFICS concerned many researchers to develop novel multiplex LFICS with the capability to simultaneously detect several analytes.

Several novel design, format, and modification of the typical LFICS have been successfully developed to simultaneously detect multiple analytes. An additional test line corresponding to additional analytes to be detected is mostly applied in multiplex LFICS. Analytes can be simultaneously detected and differentiated by visual appearance of corresponding test lines. Furthermore, when the line is used to detect the corresponding analyte, a dot design can be used if too many analytes require simultaneous detection because the dot is smaller and can save space in LFICS. In other ways, each typical LFICS combination detecting different analytes into one sample pad is possible. However, this design may require increased sample volume. Moreover, a single test line, which offers more space saving in LFICS to detect multiple analytes, is possible when using multicolor NPs than AuNPs. Furthermore, the incorporation of bi-specific bioconjugate, which can detect many analytes using single bioconjugate, may reduce manufacturing cost. The performance of multiplex LFICS in any design is good when compared with the gold standard method. Furthermore, improvement can be seen when enhancement strategies are implemented in AuNP-based multiplex LFICS.

Multiplex LFICS have been used in many fields to detect a variety of analytes such as in clinical disease, food safety, environmental safety, and in forensics for detection of drugs of abuse. In the future, the research and development of multiplex LFICS for commercialization is possible. Making multiplex LFICS friendlier to the end user is necessary if these tools intend to continuously become the diagnostic tool of choice. A simplified design such as the miniaturization microarray of LFICS can be further improved because it offers the simplest design, which detects several analytes simultaneously within a single strip.

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