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Rapid Test

Advances in Design, Format and Diagnostic Applications

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RAPID TEST - ADVANCES IN DESIGN, FORMAT AND DIAGNOSTIC APPLICATIONS

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Notice

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Meet the editor

Laura Anfossi received her M.S. degree in Chemistry with full marks and honors and her Ph.D. degree in Biochemical Sciences at the University of Torino, Italy, in 1997 and 2001, respectively. Since 2015 she has been an associate professor of Analytical Chemistry in the Department of Chemistry (University of Torino). She has published more than 85 papers in peer-reviewed international journals, four book chapters and one patent. She is a member of the editorial board of the journals *Toxins* and *Food and Agricultural Immunology*. She has been involved in national and international competitive projects and has been responsible for joint projects with companies that brought to the market several immunodiagnostic kits. Her main research interest is the development of innovative immunodiagnostic methods.

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Chapter 1

Introductory Chapter: Rapid Test - Advances in Design, Formats, and Detection Strategies

Laura Anfossi, Cristina Giovannoli and Claudio Baggiani

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1. Rapid tests

The term "rapid test" is used to indicate a series of analytical methods that generally share common features, such as being easy to operate, quick, and cheap, and above all enable on-site application. Among several variants, the most explicative synonym of such kind of analytical methods is represented by the locution "point-of-care test" ("point-of-need test" to extend to fields of applications other than medicine), which highlights the key feature of rapid tests, i.e., the ability to provide a response to an analytical demand exactly where the demand is posed. The point-of-need testing approach bases on simplifying and shortening the analytical process by cutting most of the steps required by traditional, laboratory-based analysis (**Figure 1**). This strategy allows for multiple benefits to be reached, besides saving time and money, namely, (i) enabling timely decisions and intervention, (ii) encompassing issues associated to sample transportation and storage, (iii) enlarging the access to control especially in low-resource settings, and (iv) allowing for multiple measures over time, thus increasing the efficiency of monitoring programs.

The story of rapid tests originates in the medical field where they were developed for providing portable diagnostic tools that can be operated directly by physicians or even patients themselves. In the clinical context, a timely response means superior benefits for the patient, who immediately access the treatment, and for the society as a whole. In fact, the point-of-care testing strategy enables efficiently controlling the spreading of infectious diseases and also generally reducing healthcare costs.

The first rapid test dates back to 1962, when a new method to measure glucose in blood was developed [1]. The next milestone was reached in 1976 with the approval of three home pregnancy tests by the US Food and Drug administration (FDA). These tests were placed on

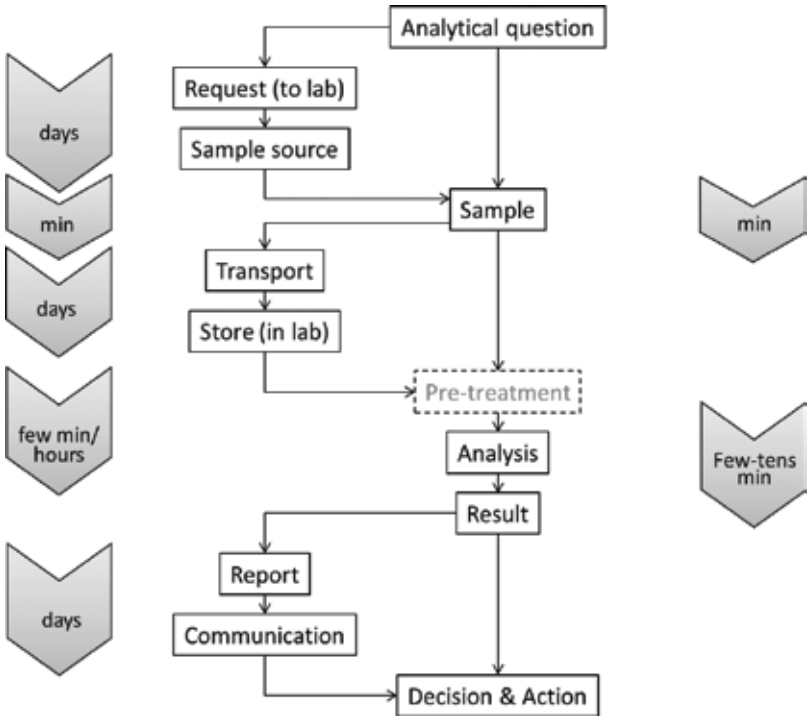

Figure 1. Schematic workflow for laboratory-based (left) and for point-of-need testing (right). The time delay within the analytical question and the decision (action) consequent to the obtainment of the response (result) falls from several days to few minutes.

the market the following year, and since then, sales have been continuously growing. The huge success of home clinical testing boosted the research in the field that, at least initially, was completely driven by industrial interests.

In the late 1990s, a scenario mutated under the influence of two new incentives: on the one hand, point-of-need testing started to attract the interest of scientists and academics, which meant a rapid evolution of technologies, materials, and analytical strategies, and on the other hand, new fields of applications were opened. The transition is clearly perceivable when considering the trends of scientific publications describing the development and applications of rapid tests plotted by time and by subject area (**Figure 2**). The picture is partial, as the number of techniques exploited for point-of-need testing and, in parallel, the ways used to indicate them are growing. However, the general trend is still increasing and is expected to further expand in the next decade.

According to the prevalent use of medicine, rapid test definition has been furnished by the World Health Organization (WHO) as follows: "rapid tests are diagnostic tools designed for use where a preliminary screening test result is required" [3]. Paradoxically, the rapidity is just one (and not the primary) of a lists of features that contribute to define this kind of analytical methods (**Table 1**). In particular, simplicity, portability, and inexpensiveness, together with

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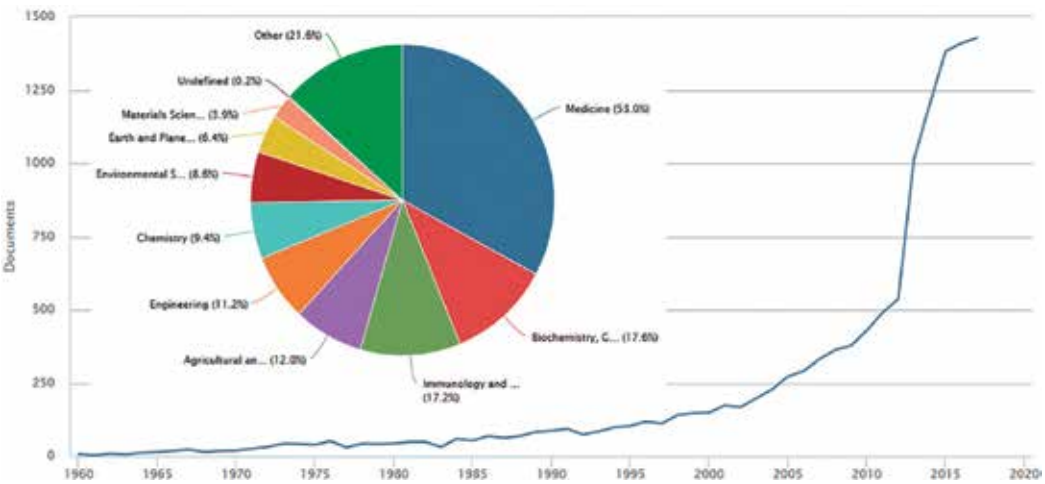

Figure 2. Trends of scientific documents sorted by year and by subject area. Search conducted by keywords "rapid test" or "lateral flow." (Source: Scopus [2]).

Requirement	Technology	Intended for
• Easy to use • Quick (10 min–2 h) • Require little or no additional equipment • Economical • Possibility to store at room temperature for extended period of time	• Agglutination • Immuno-dot • Immunochromatographic • Immuno-filtration	• Use with individual or a limited number of samples • Use in resource-poor settings • Enabling timely treatment interventions • Emergency testing

Table 1. Characteristics of rapid tests (for infectious disease diagnosis) according to the WHO [3].

speediness, are all unavoidable requirements. Techniques and technologies able to meet these requirements are various and numerous.

Although biosensors can be considered as rapid tests based on the abovementioned definition (as they are designed for enabling fast and in situ analysis), usually are not included in the category of point-of-care testing. Principally, this is due to the fact that the ideal rapid test does not require any equipment, while biosensors include a transducer device by definition. However, the boundary is uncertain and destined to be overcome by advances in both fields: rapid tests are increasingly more sophisticated, while biosensors are turning to operational simplification.

At the state-of-the art, most rapid tests are based on the lateral flow, flow-through, and paper-based microfluidic assays. A lateral flow assay (LFA), also known as immunochromatographic assay or strip test, relies on the use of a porous membrane that enables the movement of samples and reagents through the capillary force. Flowing along the membrane, the sample encounters first a labeled recognition element (the probe), which is pulled along the membrane by the sample itself and, then, some capturing (bio)reagents that are anchored onto the membrane in well-defined zones called "lines." The result of the sample migration is the formation of complexes (involving the analyte and the probe) in correspondence of the lines, which

provides a signal related to the analyte presence/amount (**Figure 3A**). The most popular LFA uses antibodies as capturing reagents and as recognition elements, while colored nanoparticles (typically gold nanoparticles) are employed as labels. Additional membranes can be included in the stand-alone device in order to mitigate the interference of the sample composition and heterogeneity (sample pad), hold the dried probe for long-term stability (probe pad), and help sample and probe flowing to reduce background signal (adsorbent pad) [4, 5].

Flow-through assays are also based on a series of layered membranes (**Figure 3B**). The core of the device is represented by the reactive membrane where capturing reagents are fixedly bound. The sample is passed through the reactive membrane by gravity or by applying a positive (negative) pressure. Subsequently, the labeled recognition element is fluxed to reveal the formation of the complexes between the analyte and the capturing reagent. A typical flow-through assay employs antibodies as capturing reagents and recognition elements, again. Different from LFAs, labels are usually enzymes that enable signal amplification and, therefore, increased sensitivity. However, the use of enzymes requires an additional step to introduce the enzyme substrate, and often rinse steps are also required to avoid sample interference on the enzymatic activity and to decrease background signals. Additional membranes, such as the sample and adsorbent membranes, are included in the device. The role is parallel to those above described for the corresponding parts of LFAs [6].

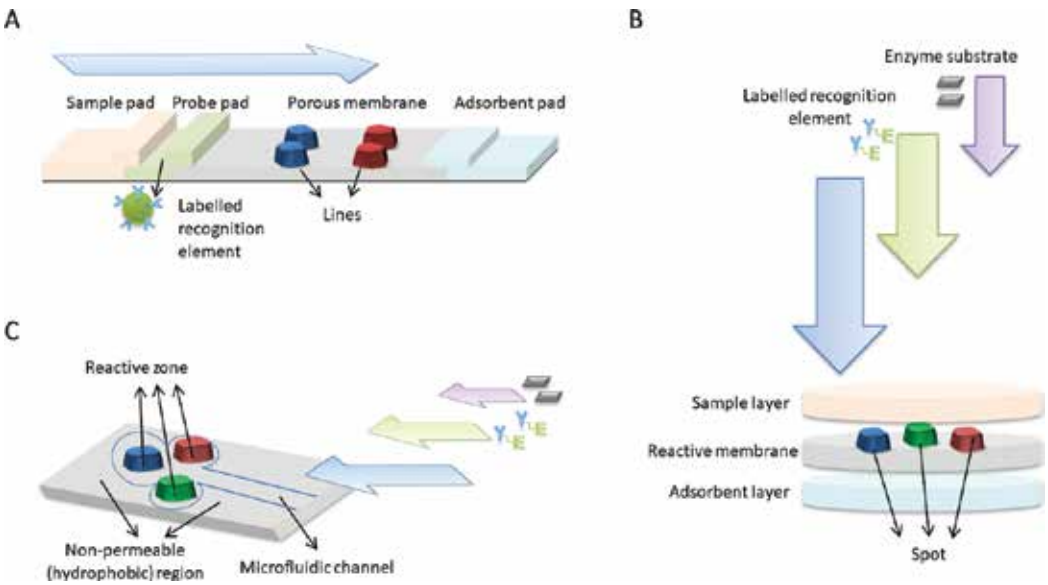

Figure 3. Schematic picture of generic lateral flow (A), flow-through (B), and paper-based (C) rapid tests. In the LFA architecture, the sample flows and pulls the labeled recognition element along the porous membrane. Capturing reagents are anchored onto the membrane to form lines. The color produced by accumulation of the label (usually colored nanoparticles) at the lines is indicative of the presence (amount) of the analyte. Flow-through and paper-based microfluidic assays use enzymatic labels that are added to the device following the sample. The analyte modulates the binding of the labeled recognition element to the capturing reagent immobilized onto the reactive membrane as spots or the reaction zones. Upon the further addition of the enzyme substrate, the color develops and indicates the presence (amount) of the analyte. Different capturing reagents disposed as spots in array format (or in different reactive zones) allow for multiple detection.

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Paper-based microfluidic assays have been introduced more recently. In fact, the lateral flow and the flow-through assays can be also regarded as "paper-based" assays, since the porous/reactive membrane is made up of cellulosic materials [7, 8]. However, the paper-based microfluidic assay definition strictly applies to devices that use filter papers (of various grades) and a single cellulose-based material for playing the role of the sample, reactive and adsorbent material. This is obtained by folding a single sheet of paper so that paper-based devices are described as origami devices. The paper surface is modified with hydrophobic materials (typically wax) to fabricate hydrophilic microfluidic channels that convey the sample toward reactive zones where capturing reagents are immobilized. The principle of the assay relies on capillary forces as in the LFAs, while detection is accomplished by the enzyme-mediated production of the signal. Therefore, as for flow-through assays, several sequential steps to apply the sample, the probe, and the enzyme substrate are required [7, 8]. Usually, the device furnished an electrochemical output and requires to be coupled to microelectrodes. In such a way, paper-based microfluidic assays can be regarded as a bridge between equipment-free rapid tests and biosensors.

Flow-through and paper-based microfluidic assays are also available in array form, where multiple capturing reagents are immobilized on the same support and eventually multiple probes are used to allow the simultaneous detection of several analytes in one sample, therefore reaching high-throughput analysis.

Independently on the assay architecture, the detection of rapid tests relies upon the generation of a colored output that allows for the visual inspection and does not require any reading system. In this way, the result is partially subjective and usually provides a bare yes/no response, according to the level of the analyte being above or below a cutoff value.

2. Advances in "point-of-need" testing

Nowadays, rapid tests are widely applied as an efficient screening method for conducting on-site analysis for several applications, such as food, forensic, and environmental analysis.

Still, the medical field represents the largest for a number of available systems and a variety of applications. The availability of specific and sensitive diagnostic tools that can be operated almost everywhere and by no-trained operators is strongly appreciated in low-resource settings and is regarded as a feasible way for improving access to care.

In this field, some lines of future developments can be traced.

Provided that rapid tests are intended for self-testing and also for frequent testing, the preferential specimen should be repeatedly and noninvasively collected. Moreover, noninvasive collection enables sampling children and non-collaborative patients (i.e., for testing abuse drugs, sport doping, adherence to therapy, etc.). Therefore, diagnostic devices capable of working with specimens other than blood (i.e., urine, saliva, hair, and feces) are expected to expand. Parallel to the evolution of the materials and device architecture required to achieve this task, also a robust confirmation of the clinical relevance of detecting a biomarker in an unconventional specimen is needed.

Furthermore, we are entering the era of personalized medicine that is giving new impulses to the development of diagnostic tests enabling each patient to watch over himself/herself.

Beside clinical diagnostics, rapid tests are expanding to several other fields of applications. The role played by these analytical tools in veterinary, forensic investigations, and food safety controls is worth mentioning. Application to veterinary diagnostics is the natural continuation of the human diagnostics and takes advantage especially of the development toward exploitation of unconventional biological matrices (e.g., saliva, hair) that can be collected easily from animals by the veterinarian.

The same specimens are also useful in forensic investigation as they represent samples that can be collected from non-collaborative subjects. Other aspects that are of utmost relevance for the forensic use of rapid tests are their on-site usability and speediness of response that allows for implementing actions of controls at the street level and timely intervening in dangerous situations.

Food safety controls represent a vast area of applications for rapid tests, mainly because of international regulations that are becoming more and more severe about the quality of food (i.e., the need to assess the absence of contaminants, to discover frauds, to trace production and transformation chain, to ascertain freshness, to confirm origin, etc.) and because of the globalization of trades. Key features of rapid tests that especially meet requirements in ascertain food safety are cheapness and rapidity. The first feature enables self-testing carried out by producers, transformers, and sellers, thus assuring the safety across the whole food chain. The second feature is of paramount relevance when dealing with perishable goods. It should be noticed in this context that the designing of rapid tests for food analysis implies facing a number of diverse sample typologies, each deserving an appropriate study of materials and reagents.

Synchronous to the extension of applications and thanks to the increasing scientific activity on the subject, the research on rapid tests is being drawn from the urgency of immediate marketability. Therefore, new and most efficient probes to generate the signal have been proposed, such as those based on fluorescence, chemiluminescence, and fluorescence quenching detection and on magnetic nanoparticles. Several signal enhancement strategies have been described for improving sensitivity. Besides, artificial recognition elements with increased stability and robustness compared to antibodies have been incorporated in rapid test devices. In particular, aptamer-based LFAs are the new frontiers in this area.

Finally, test design and device architecture are becoming more flexible to adapt to novel demands, such as the hyphenation with PCR amplification to detect molecular markers and the increase of multiplexing capacity.

Conflict of interest

Authors declare no conflict of interest.

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Chapter 2

Ways to Reach Lower Detection Limits of Lateral Flow Immunoassays

Anatoly V. Zherdev and Boris B. Dzantiev

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Abstract

This chapter considers factors influencing sensitivity of lateral flow immunoassay and modern developments that are focused on reaching lower detection limits. The existing variety of proposed approaches is classified in accordance with the "big five rules" for these assays, including proper sample, receptor, interaction, response, and output. The solutions for rapid extraction of target analytes and preventing negative influence of extractants are considered. Role to antibodies affinity and specificity is characterized. Potential of alternate bioreceptor molecules is discussed. Immunoreactants' compositions, concentrations, and locations on the test strip are characterized as factors determining assay parameters. The existing variety of labels is compared in terms of their optical and alternate registration. Tools to modulate a sequence of analytical reactions and to form aggregates of the detected labels are considered. The discussed approaches are illustrated through developments of test strips for detection of mycotoxins, veterinary drugs, and other analytes.

Keywords: immunochromatography, test strips, nonequilibrium interactions, increasing assay sensitivity, nano-sized labels, signal amplification, digital optical measurements

1. Introduction

The history of lateral flow immunoassay (LFIA, immunochromatography) began in the 1980s. The first solved task was to transfer pregnancy tests from a specialized laboratory directly to the point of sample collection [1]. The test strip developed for this purpose fully complied with the requirements for nonlaboratory diagnostics, and its basic principles remain to this day.

The overall design of the immunochromatographic test strip is shown in **Figure 1**. It is a composite of several membranes of different structures and porosities, fixed on a support. The bundling of the test strip can vary, so it makes sense to consider its design based on what analytical tasks are being performed on its different sites.

A. Typically, the lower portion of the test strip contains a **sample pad**. It ensures the absorption of sample components, in which the presence of the target analyte is checked.

B. The following is a section with immunoreagents that are washed out during the analysis and move upward along with the components of the sample. As a rule, a **conjugate pad** forms this zone. It contains a conjugate of antibodies against the target analyte with a nanodispersed label—particles of colored latex, colloidal gold, and so on.

The next two sections are located on the main **working membrane** of the test strip.

C. First, there is a zone along which the movement of the absorbed components of the sample and the washed immunoreagents continues. During this movement, immune reactions occur, and specific intermolecular complexes are formed.

D. Next, a mixture of reacted and unreacted molecules enters the **binding area** with immobilized immunoreagents. Depending on whether the target analyte was present in the sample and in what amount, binding of labeled immune complexes occurs in certain areas (in the traditional case, with the formation of narrow colored lines). Usually, additional reagents are located here to control the functionality of the test system.

E. The upper part of the test strip with the **final pad**, usually structurally similar to the sample pad, ensures the further movement of the reaction mixture under the action of capillary forces and the washing of unreacted components from the underlying areas. These processes allow the label's binding to be evaluated correctly.

Membrane components of the test strip are fixed on a **plastic support** and partially overlap with each other for effective fluid movement.

Depending on the tasks to be performed, additional reagents can be used in the test strip, and some of the membranes can be added, combined, or eliminated. However, at the same time, the general design and principle of conducting analytical interactions during the movement of reagents along membranes is preserved.

Simplification of the analysis is achieved by refraining from additional processing and incubation enhancing the signal as well as by visual (device-free) evaluation of the results. Because of this, traditional LFIA, meeting the needs of practice in simplicity and speed, is generally considered inferior to alternative immunoassays (such as ELISA) in sensitivity.

At first, this restriction was not critical. Test systems made it possible to control target compounds in diagnostically demanded concentration ranges, which was enough for their mass application. The implementation of standard LFIA protocols for the detection of new compounds was viewed as an exclusively technological task for manufacturing companies, uninteresting in the scientific sense. In this regard, the number of publications on LFIA in the late 1990s to early 2000s was relatively small. It was believed that the all main methodological problems of LFIA had already been solved.

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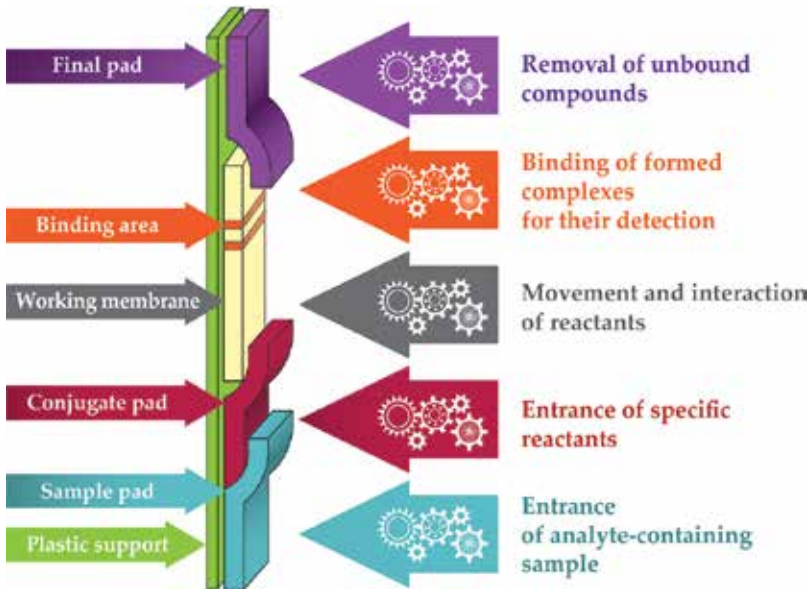

Figure 1. Components of the immunochromatographic test strip and their functions.

2. "Big Five Demands" for new solutions in LFIA

However, the application of LFIA did not stop at the control of the formed row of objects. This method actively developed (especially in the last decade) and covered an increasing number of analytes. What were the reasons for this?

- As applied to medicine: the general trend toward the diagnosis at the site of the requirement (point-of-care (POC)): (i) the use of tests for quick decisions outside the clinic; (ii) the provision of mass screening tests by rapid and inexpensive diagnostic tools; and (iii) providing the doctor with information for decision-making during the time of communication with the patient without transporting samples to the laboratory.
- With regard to other areas of application: interest in promptly obtaining information about mass consumption products, for example, about the quality of raw materials coming to food enterprises and the end products being sent to the trading network.
- Biosafety control is extremely important in modern society. The conclusion about the presence of a toxin should be given promptly and directly at the testing site.

Taking into account this expansion of controlled analytes and types of tested samples, tasks were frequently encountered for which highly sensitive detection was required but not provided by traditional analytic formats.

During the last decade, the development of LFIA modifications has been intensified, allowing highly sensitive analysis, while maintaining the basic merits of the analysis—the rapidity, ease of implementation and interpretation of the results. These developments are systematized in

a number of recent reviews that characterize the general trends in the development of LFIA, its application in different practical spheres, and the most successful methodological decisions [2–8].

On the one hand, this progress is accompanied by the expansion of the assortment of commercial tests and the more active application of LFIA for solving a variety of practical problems. On the other hand, a significant part of new developments remains at the level of single publications and approbation using the example of a single analyte, without realistic assessment of their advantages and limitations. From such isolated examples, it remains unclear how much gain in sensitivity will be achieved if we apply the proposed approach to the new analytes and what conditions must be used for this. A simple demonstration of the minimum detectable concentrations in traditional and modified LFIA leaves open the question of how correctly all the conditions for the analyses were selected, including the concentration and composition of the immunoreagents. It is also unclear which of the approaches for reducing sensitivity can be combined and whether this combination leads to a multiplication of results improvements achieved for each of these approaches individually.

Of course, general theoretical arguments are not enough to answer these questions. Further studies of many research teams are needed. However, it is important to evaluate new developments with the use of a grounded concept to understand (i) what changes are introduced into the traditional LFIA protocol and for what purpose; (ii) by what criteria are the new LFIA protocols assessed and compared with existing ones. Such ordering is the subject of this review. We did not attempt to form a limited list of developments that are most widely represented in recent publications. Our goal was to create a general classification within which different existing and future developments can be characterized.

The structure of the immunochromatographic test system considered (**Figure 1**) allows us to identify groups of problems that should be solved to ensure high-sensitivity, as well as other practically significant characteristics of the analysis (productivity, selectivity, etc.).

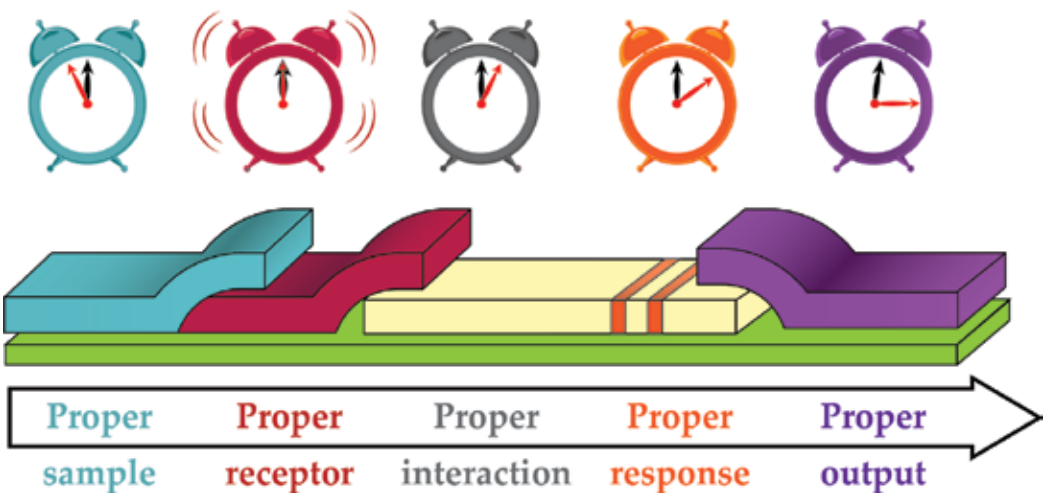

Figure 2. Compounds of immunochromatographic test and "big five demands" associated with them.

We matched each element of the test system and the reagent or process used at this element. Therefore, the choice of the most appropriate (proper) actions during the analysis includes

A. Choice of the sample preparation method—**proper sample**. A procedure should be chosen whereby a liquid containing the target analyte will be obtained from the initial liquid, semisolid, or solid matrix and used for contact with the immunochromatographic test strip.

B. The choice of receptor molecules used to selectively bind the target analyte—**proper receptor**. It is necessary to establish which antibodies and their derivatives or alternative compounds will interact with the target analyte during the analysis, and what their derivatives will be and by what methods they should be obtained for inclusion in the test system.

C. Choice of the conditions for interaction of reagents during the analysis—**proper interaction**. Optimum quantities (concentrations) of reagents, duration of interactions leading to the formation of detectable complexes, and conditions (composition of the medium) for carrying out these interactions should be chosen.

D. Choice of the registered response of the test system—**proper response**. It is necessary to decide with the help of which label the complexes formed during the analysis will be detected and their quantities will be estimated. Which way of registration will be applied? What additional reagents and actions will be used to increase the response of the test system?

E. Choice of the procedure for processing the measurement results—**proper output**. It should be suggested how the detected signal will be transformed into a decision about the presence and content of the target analyte and how the most informative results will be obtained.

These five groups of requirements ("big five demands", **Figure 2**) make it possible to simply and uniquely classify the methodical solutions proposed for the improvement of the LFIA protocols.

This review will be based on our results (from the Laboratory of Immunobiochemistry in the A.N. Bach Institute of Biochemistry of the Federal Centre of Biotechnology of the Russian Academy of Sciences, Moscow, Russia) and on examples from the literature that will be ordered and characterized in accordance with this classification.

3. Proper sample for LFIA

Some types of liquid samples, characterized by the LFIA method, do not require sample preparation: urine, blood serum, natural and drinking water, milk and juices. Their analysis can be initiated by contacting the test strip with the sample as is. To accelerate the movement of the fluid (blood serum and milk), the sample can be diluted immediately before analysis [9]. However, in most cases, the analysis should be preceded by sample preparation.

The main difficulty of sample preparation is the need for a short period to destroy the matrix structures that interfere with the analyte molecules contained in it to interact with antibodies.

Actions that separate matrix components that interfere with analysis, or to destroy these components, are also reasonable. Such complex types of matrices may be tested as tissues of organisms, food and agricultural products, soil, and so on. Sample preparation is extremely important to easily detect the target compounds in these matrices.

The requirements for sample preparation were studied in detail with respect to other analytical methods—liquid and gas chromatography, enzyme immunoassay, and so on. However, the accumulated research results cannot be transferred to LFIA without further development. The main advantage of LFIA—rapidity—cannot be lost because of the long (lasting several hours) extractions recommended in many chromatographic techniques. Work with samples cannot begin from complex procedures that require expensive equipment.

An additional feature of sample preparation for LFIA is that many analytes are extracted efficiently only with organic solvents and water-organic mixtures, but not with aqueous-salt solutions. (Such situations are usually associated with the hydrophobicity of the compounds and their surroundings in the samples.) However, these solvents inactivate antibodies; it means that the extract cannot be directly used as is as a sample for LFIA. As a result, the extracts are either significantly diluted (which is accompanied by a loss in sensitivity), or by means of additional steps, the analyte is transferred to another medium.

The complexity described above determines the tasks that should be solved for effective sample preparation—see their summation in **Figure 3**. In **Figure 3** and the following ones, we depict

- **Strategic tasks** that require complex and risky time-consuming and labor-intensive work in the framework of research projects and
- **Tactical tasks**, which can be solved by varying several known parameters when finalizing the final product.

With respect to proper samples, the success of the developments offered directly by test system manufacturers should be noted. Alexeter Technologies (United States) uses special adhesives placed at the beginning of the test strip, which allow one to collect target molecules of the analyte from a large surface area by simple contact. In many cases, portable homogenizers and low-speed centrifuges are proposed for completing the analytical laboratory. In the case of the 4MycoSensor test systems (Unisensor, Belgium), mycotoxins are extracted from the ground grain in a special Mycobuffer on a shaker for 3 min (5 min for corn). Similar solutions are offered by other manufacturers. A special aqueous two-phase system for the concentration of protein analytes, containing polyethylene glycol, potassium phosphate, and phosphate-buffered saline, was used by Chiu et al. [10]. With its help, a 100-fold reduction in the detection limit was achieved. Concentration of samples combined with dialysis was used by Tang et al. [11] on the examples of myoglobin detection (fourfold signal growth) and nucleic acid of HIV (10-fold growth). Mosley et al. [12], using the examples of *Chlamydia trachomatis* and human immunoglobulin M analyses, showed that the formation of an aqueous two-phase system on the test strip by applying a PEG-potassium phosphate and UCON-50-HB-5100-potassium phosphate obtained a 10-fold reduction in detection limits. In Jue et al. [13], micellar two-phase systems were used for this purpose, which reduced the detection limit of bacteriophage M13 by a factor of 10. An original solution based on

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concentrating the analytes in an electric field was proposed by Kim et al. [14]. Using a conventional 9 V battery and commercial tests for choriogonadotropin, they acquired a 25-fold concentration of the target compound.

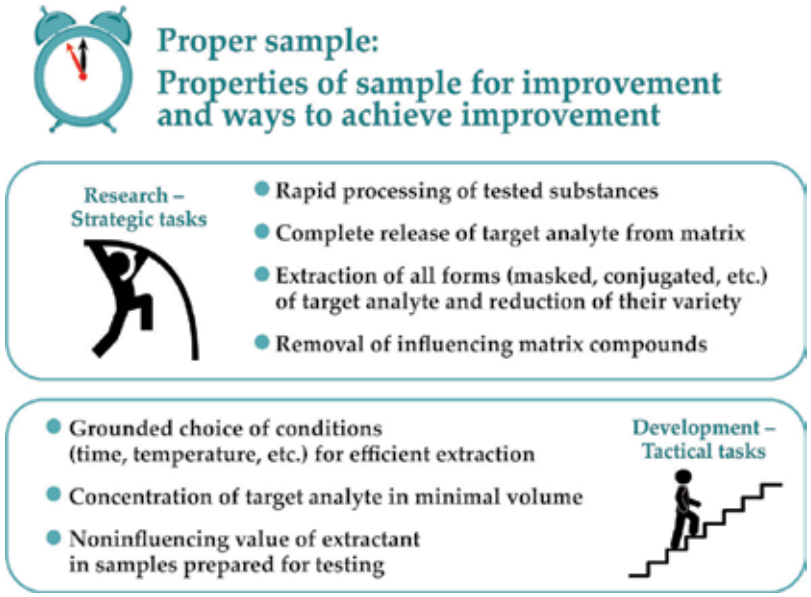

Figure 3. Main research and development tasks to obtain proper samples for LFIA.

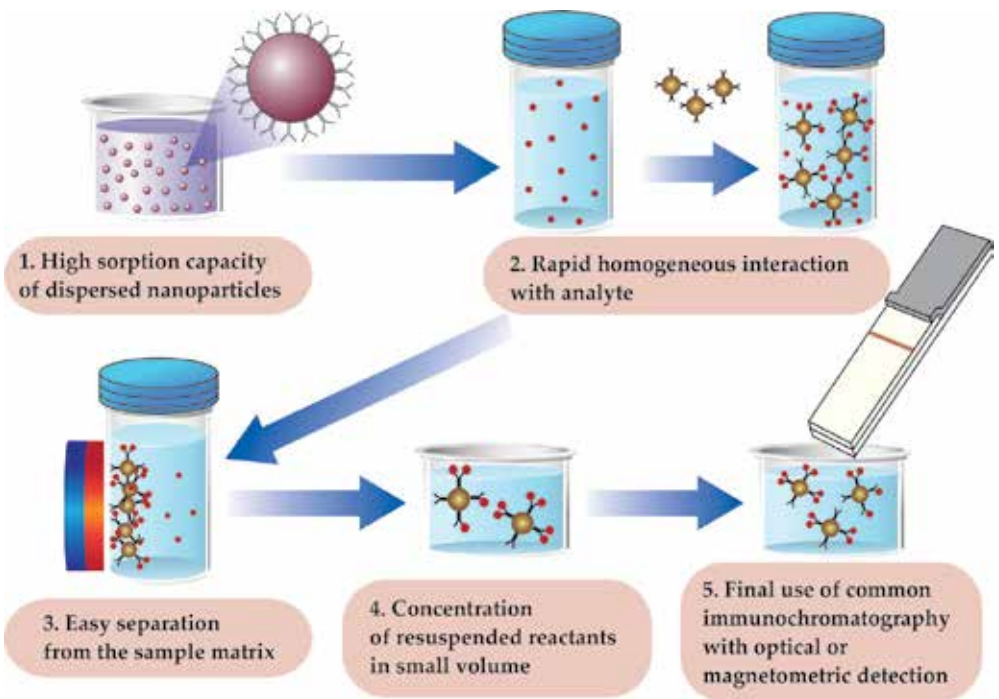

Figure 4. Advantages of magnetic immunosorbents application in LFIA.

Efficient approaches for sample preparation are pseudo-homogeneous analytical techniques, where a dispersed carrier with immobilized receptor molecules is added to a large volume of tested samples. This carrier quickly and efficiently, without diffusion restrictions, captures the analyte from the entire volume of the sample, and then the carrier is separated from the solution rapidly. Note that when the separated carrier is then redissolved in a small volume, the analyte is not only concentrated but also cleared from the organic solvent, thus excluding the influence of this solvent on LFIA. Antibodies, immobilized on a carrier, are often more stable to the denaturing influence of organic substances than free antibodies. According to the data of Urusov et al. [15], when working with magnetic immunosorbents, the content of methanol in the test sample can be increased from 10 to 30%.

The use of particles of iron oxide and other carriers with magnetic properties is extremely promising for immunochromatography because of the simple and rapid separation of the carrier by contact with a permanent magnet. The principle of such an analysis is shown in **Figure 4**, and approaches to the production of magnetic immunosorbents are systematized in the review [16].

Liu et al. [17] showed that the combination of magnetic concentration and immunochromatography yields a 25–50-fold gain in the detection limit of aflatoxin M1 in milk compared to the variants in which magnetic or gold nanoparticles are used as conventional labels. A 40-fold gain in the detection limit was demonstrated by Lu et al. [18] upon the detection of *Listeria monocytogenes*. In Petrakova et al. [19], using the examples of zearalenone and T-2 toxin, the authors showed that magnetic nanoparticles can be used as directly detectable optical markers. Razo et al. [20] combined the use of magnetic immunosorbents to bind analytes, potato virus X, and functionalized gold nanoparticles, which, thanks to the biotin-streptavidin reaction, provide the formation of aggregates of two kinds of nanoparticles. This analysis was 32 times more sensitive than the nonenhanced one. As a whole, the described gains in sensitivity with the use of magnetic immunosorbents did not exceed two orders of magnitude. A greater concentration requires a significant increase in the consumption of immunoreagents and/or time for binding the analyte.

Concentration can also be achieved if LFIA is preceded by a stage with a transverse flow of large volumes of samples through a small volume of a membrane with antibodies or other binding reagents applied to it (immunofiltration). Such analyses usually complete the detection of binding results directly in the filtration zone [21, 22]. Note that the use of LFIA for control of toxicants in solid foods is associated with a certain restriction. To correctly determine the content of the unevenly distributed analyte, several samples of large volumes are selected from different parts of the tested object and combined for subsequent extraction [23, 24]. However, the small volume of liquid absorbed by the test strip allows only a small part of the analyte molecules present in the extract to be taken into account (even with magnetic concentration). Immunofiltration concentration will overcome this limitation and come close to obtaining the proper samples for highly sensitive analyses.

4. Proper receptor for LFIA

The basic requirements for antibodies used in LFIA are related to their affinity and selectivity. However, the topic of which characteristics of antibodies provide the most sensitive analysis

requires additional clarification. Immune reactions during immunochromatography are carried out in the kinetic regime. Therefore, it is unimportant whether the detectable complexes will dissociate for hours or days. Their number is determined primarily by the kinetic constants of the association, which for receptors that are the same in structure and antigens that are similar in size vary within a limited range. In the case of competitive LFIA, the dependence of the number of complexes formed on the analyte concentration in the sample is determined primarily by the affinity of antibodies to the free analyte. Effective binding to a competitor modified by the analyte will interfere with the highly sensitive detection of the free analyte in the sample. In other words, the binding of antibodies to the analyte-protein conjugate should be somewhat worse than with the native analyte. The influence of the characteristics of immunoreagents on the sensitivity of analysis is considered in detail in works devoted to the mathematical modeling of LFIA [25–30].

Given the above limitations, the affinity of antibodies is an important characteristic that affects their analytical use. However, the possibility of natural production of antibodies with more and higher binding to the analyte is limited. This is because an increase in the half-life of an antigen complex with B-cellular receptors greater than the endocytosis time of the complex is not supported by the selection of the corresponding B-cellular receptor lines [31]. The cases of "infinite affinity" of antibodies are rare exceptions for the analytes that form covalent bonds after the immune interaction [32]. An additional way to increase affinity is the genetic modification (directed design) of the active center of antibodies. The use of these methods in routine development is still very limited, despite confirmations of their effectiveness [33].

As far as specificity is concerned, an important problem is which series of structurally close compounds should be detected using this antibody to solve practical problems. Two kinds of situations are possible: (i) it is necessary to recognize a single compound possessing biological activity, in contrast to its analogs and metabolites and (ii) information is required on the total content in the sample of a significant number of homologous compounds. For the second, class-specific assay, it is desirable that affinity of antibodies to homologous compounds correlate with their biological activities, but this is not always possible. In some cases, regulatory documents establish maximum residue levels (MRLs) on the basis of the sum of concentrations of structurally similar toxicants, without correction factors, taking into account their biological activity. Therefore, class-specific analysis usually requires the detection of the maximum number of compounds of this class with at least 10–15% cross-reactivity with respect to the maximum [8].

Additional practically important characteristics of antibodies are the values of their stability under storage and in the course of the assay. The stability may be effectively enhanced by chemical modification of antibodies as well as by addition of protective agents that are common for drying of different immunoreactants.

The strategic tasks for improving receptors for immunoassays are summarized in **Figure 5**. However, in the final development of test systems, commercially available antibodies are usually used, and there is no possibility of directed production of new, improved antibodies. This is the reason for the interest in the use of receptor compounds of a different nature as a substitute for traditional immunoglobulins.

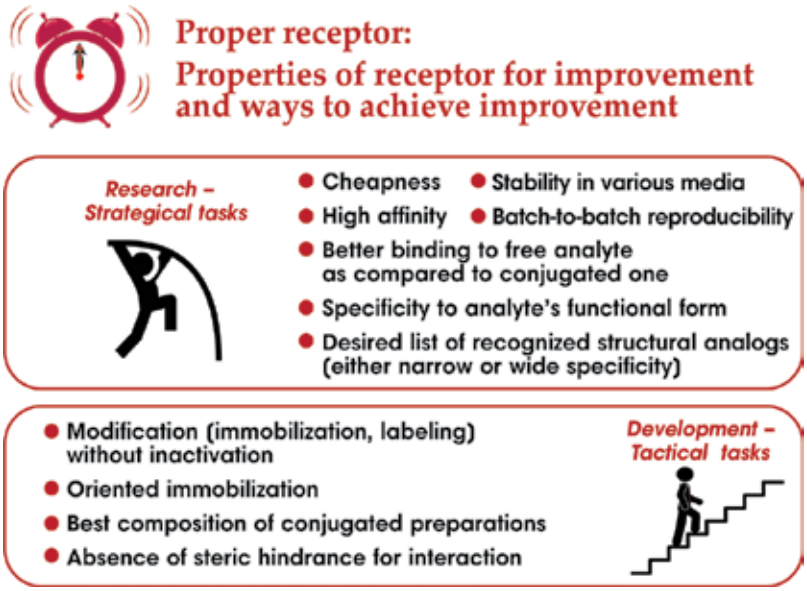

Figure 5. Main research and development tasks to obtain proper receptors for LFIA.

Thus, the single-domain antibodies produced by *Tylopoda* and sharks are characterized by significantly greater stability under different conditions [34]. Interest is caused by so-called protein scaffolds of a non-antibody nature that also combine conservative basic structure with hypervariable segments providing receptor functions [35]. However, their analytic application is a matter of the future. The development of test systems based on aptamers—receptor oligonucleotides, selected from random libraries—is being actively pursued. Aptamers are significantly cheaper and more stable reagents in comparison with antibodies, and their properties are well reproducible. Limitations in affinity typical of many of the known aptamers are overcome by improving the selection procedures and subsequent directed design which is a much simpler process than for antibodies [35]. The possibilities of using aptamers in membrane test systems are shown in a number of works and summarized in recent reviews by Jauset-Rubio et al. [36], Chen et al. [37], and Dhiman et al. [38]. For developments on the use of nanobodies, see Tang et al. [39], who presented LFIA for the simultaneous determination of aflatoxin B1 and zearalenone.

Requirements for proper receptors also include its effectiveness after immobilization on a membrane or on the surface of a marker nanoparticle label. Physical adsorption and random covalent coupling may be accompanied by significant loss of antibody reactivity. Therefore, systems are needed in which the oriented immobilization of antibodies is realized through the chemical conjugation of IgG in areas remote from the active center, or by their indirect binding with a preformed antibody-binding layer. In the role of this layer, staphylococcal protein A, streptococcal protein G, or (strept)avidin (reactive with biotinylated antibodies) can act. Approaches to the oriented immobilization of antibodies are systematized in a number of recent reviews [40–43].

Filbrun et al. proposed a procedure for chemical modification of the lysine residues of antibodies before conjugation with gold nanoparticles and showed that it provided conjugates

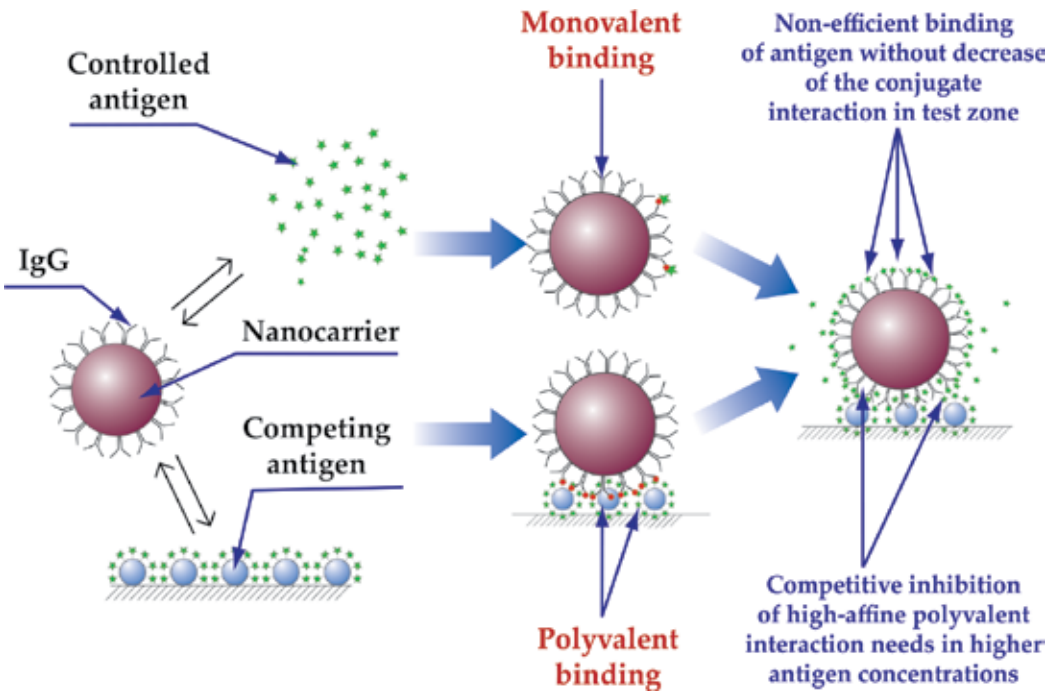

Figure 6. Limitations in the use of common antibody-nanoparticle conjugates in competitive LFIA.

that were stable over a wide pH range [44]. Bauer et al. [45] developed a technique for the preparation and use of antibody conjugates modified with histidine-rich peptides (called "capture and release" antibody reagents). These preparations are affine to metal surfaces and magnetic particles and so may release immobilized antibodies when necessary. The benefits of oriented binding of antibodies to magnetic nanoparticles through modification of antibodies' carbohydrate components were shown by Puertas et al. using the example of LFIA for choriogonadotropin [46]. A comparison of methods of immobilization for receptors in bacteriophage-based LFIA is given in the works of Kim et al. [47, 48]. In particular, article [48] discussed the use of in vivo-biotinylated peptide for oriented immobilization of receptor molecules on a test strip.

The composition of conjugates of antibodies with nanoparticles also plays an important role. Although the increase in valencies for immune interaction is accompanied by an increase in conjugates' affinity [49], structural changes of antibodies or steric restriction of their availability to interact with antigens may occur in parallel. With adsorption immobilization of antibodies (i.e. the widespread approach for LFIA purposes), their excessive loading causes the formation of additional layers, the molecules in which can dissociate during the analysis, and preventing the formation of a detectable labeled complex. Additional complications are associated with the use of antibody-nanoparticle conjugates having high surface density in competitive LFIA (**Figure 6**). Such conjugates can form high-affine polyvalent complexes in the analytical zone, which impede competitive interaction with the monovalent analyte from the sample. Further, the resulting complexes contain a significant number of unreacted antibodies and can bind analyte molecules without weakening the detected signal [50]. Therefore, the composition of the conjugate should be selected in relation to the features of each

analytical system as well as other variable parameters—see the list of tactical tasks in **Figure 5**. Describing the development of LFIA for aflatoxin M1 [51], Anfossi et al. found that the lowering the ratio between antibodies and gold nanoparticles caused improvement in the assay sensitivity. The proposed change was to decrease amount of antibodies used for immobilization twice as compared with saturating conditions and by that way to lower the limit of detection, too, almost twice with a minimal weakening of the staining.

5. Proper interaction for LFIA

Because LFIA is a fast analysis, all the processes that should be performed during the time of reagents' movement along the test strip and proper conditions for the interaction of these reagents are necessary (**Figure 7**).

- **First**, the interacting molecules should be in a state corresponding to their high reactivity in a medium without blocking and inactivating components.
- **Second**, the reagents should be included in the stream in accordance with the order and quantities that will ensure a highly sensitive detection of the analyte.
- **Third**, the movement of the reagents should be accompanied by their effective mixing with minimal nonspecific binding to membranes.
- **Fourth,** the location of the immobilized reagents on the test strip and the rate of movement of the soluble reagents (both given parameters depend on the composition of the reaction medium) should allow the time for the formation of detectable complexes to be increased.

These general requirements remain little studied. Studies of the localization of reagents and immune complexes in a 3D membrane structure are limited [52, 53]. A significant variation in reaction media causes problems with mobility and nonspecific sorption of reagents on commercial membranes, the structure and coating of which are established by manufacturers. The developer can only compare several membranes and select reagents that affect the release of dried components and the speed of the flow. An example of such recommendations is provided by Lee et al. [54]. The contribution of fast nonspecific processes of formation of the so-called "protein corona" on the surface of gold nanoparticles to the effectiveness of immune interactions in LFIA is described in a recent paper by de Plug et al. [55]. Choi et al. [56] characterized the effects of temperature and humidity on the analytical characteristics of test systems and somewhat unexpectedly found that the transition to room temperature, conditioned by the requirements of point-of-care diagnostics, may be accompanied by a deterioration in sensitivity. In their work, the analysis at 37–40°C and relative humidity beyond 60% was three times more sensitive. Posthuma-Trumpie et al. [57] focused on the effects of the composition of solutions used in the manufacture of test systems on the analysis parameters. Interesting opportunities for further development are provided by the use of so-called nanomotors for enhanced reagent mixing, which has so far been described only for other types of immunoassays [58, 59].

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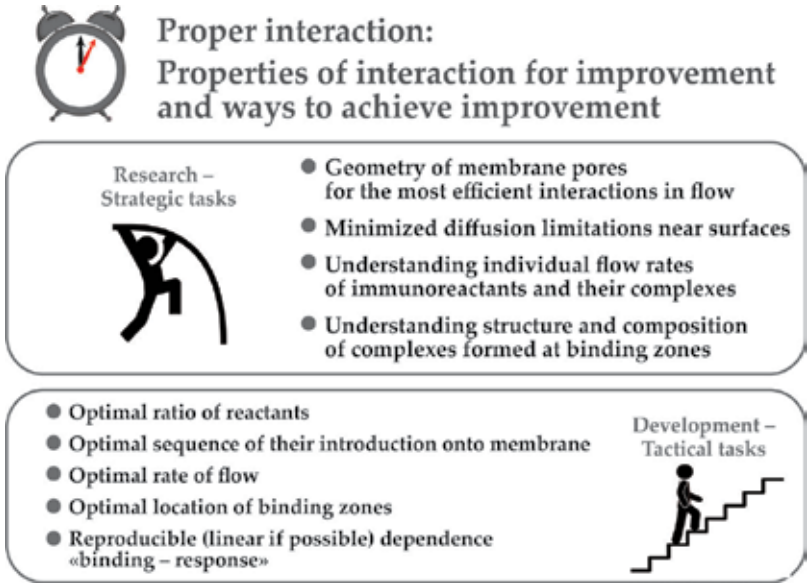

Figure 7. Main research and development tasks to obtain proper interaction for LFIA.

More accessible tools are the choice of concentrations of reagents applied to the test strip and their locations. By varying these parameters, it is possible to provide extremely sensitive detection or to select the threshold of discrimination between positive and negative samples (cut-off level) that meets the regulatory requirements for the maximum permissible level of contamination. A number of works have been published with analyses of the individual effects of these parameters on the analytical characteristics [60, 61] and with the application of multiparametric optimization procedures [62]. Hsieh et al. [52] described a general scheme for the consideration of various factors in the course of LFIA optimization.

In Zvereva et al. [63], the possibility to change the cut-off level by varying the composition of the hapten-protein and the antibody-(gold nanoparticles) conjugates is considered. Using an example of competitive LFIA of chloramphenicol, it was shown that by reducing the load of immunoreagents on carriers, it was possible to shift the detection limit by two orders of magnitude. For sandwich analysis, Liu et al. [64] showed theoretically and experimentally the optimality of the antibody: the nanoparticle ratio was equal to 30:1, but the universality of these recommendations requires further study.

Fu et al. proposed the use of a two-dimensional paper network to control the sequence of interactions in LFIA and, using the example of choriogonadotropin, showed the gain achieved in sensitivity [65]. Similar problems were solved in Rivas et al. [66] using wax-printed pillars as delay barriers (three-fold gain for human IgG detection) and Choi et al. [67] by incorporating agarose into the test strip to achieve flow control (10-fold gain for detecting dengue viral RNA). A sponge shunt was applied by Tang et al. [68] to reduce the fluid flow rate during LFIA (10-fold signal enhancement in nucleic acid testing of Hepatitis B virus). Liu et al. [69] considered the use of a pencil made from polyethylene glycols for the application of reagents

to control the rate of their subsequent release. Shin et al. [70] developed a rotary device for this purpose, the rotation of which makes it possible to initiate a reaction and then sequentially introduce into the system the necessary reagents. The volume of reagents introduced into the system during analysis can be controlled by the vertical flow immunoassay method proposed by Oh et al. [71] and successfully implemented by them for the detection of C-reactive protein. For the same antigen, Rey et al. [72] described an approach to managing the kinetics of interactions that allowed exclusion of the so-called hook effect (falsely low results for very high concentrations of the analyte). The existing variety of approaches to controlling the order of interaction of reagents in test systems is summarized in Jeong et al. [73].

The position of the binding zone influences the degree of equilibrium reached for the reactions occurring during the flow of reactants along the test strip. Moving these zones along the test strip, we can adjust the assay sensitivity. Theoretical aspects of this approach were considered by Ragavendar et al. [74]. However, despite successful overlapping of monotests in multitests with a sequential arrangement of binding zones [75, 76], general practical recommendations for ensuring a highly sensitive detection of all analytes have not yet been formulated.

Because synchronous movement in the flow of antigen, antibody, and immune complex molecules is difficult to provide, an alternative is to start the analysis with a quick (several minutes) preincubation of the analyte molecules in the sample with the free or labeled antibodies that are specific to analyte. A number of commercial systems operate on this principle, such as tests for antibiotic control in food produced by Bioo Scientific, United States, and Nankai Biotech, China. Developing this idea, it is possible to implement universal test strips without compounds specific for a concrete analyte. The combination of such test strips with specific reagents added during the incubation stage with the sample allows adaptation of the consumption of test strips to the tasks being solved. Such strips are manufactured by D-r Fuke, Germany, for the detection of immunoglobulin E against various allergens: a complex of immobilized streptavidin, a biotinylated allergen from a preincubation mixture, specific immunoglobulins E, and colloidal gold-labeled anti-species antibodies is detected in the analytic zone of these tests.

The problem of the polyvalence of antibody-nanoparticle conjugates in competitive LFIA noted in the previous section can be solved by replacing the conjugate of analyte-specific antibodies with gold nanoparticles by a combination of native specific antibodies and labels conjugated with anti-species antibodies. It gives possibility to vary the content of antigen-binding sites and the marker independently and therefore combine the high-sensitivity of competitive immunodetection (requiring a low content of specific antibodies) and the intensity of the detected signal (achieved with a high label content). This principle was implemented in our developments in the immunodetection of mycotoxins and demonstrated gains in sensitivity from one to three orders of magnitude [50, 77, 78].

Note that the implementation of competitive analysis in LFIA involves another problem. Visual out-of-laboratory diagnostics makes it possible to distinguish only assay results consisting of the presence or absence of a colored line in the analytical zone. For a visible disappearance of color, the sample must contain a sufficient number of analyte molecules to block all binding sites for labeled specific antibodies (**Figure 8**). In this respect, analysis formats with a direct dependence of the detected signal on the analyte content are preferred. For these

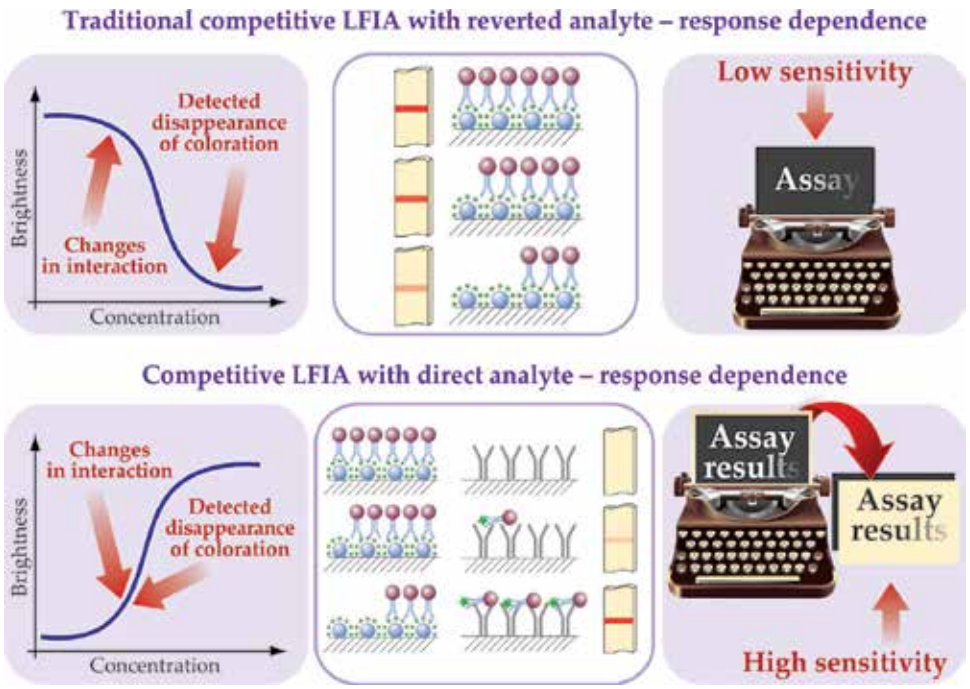

Figure 8. Limitations of competitive immunoassay and one of the ways to overcome them.

formats already small concentrations of the analyte ensure the coloration of the analytical zone in contrast to the absence of color in the absence of the analyte (see **Figure 8**).

However, the implementation of such an analysis for low molecular monovalent antigens is not an easy task. Its solutions for various types of immunoassay are summarized in the reviews of Fan and He [79] and Liu et al. [80]. Unfortunately, many of these approaches, such as idiometric assay [81] and immunoassay using anti-metatype antibodies [82] require the production of antibodies not simply against the target analyte but against more complex antigenic structures, which limits their widespread use. A more universal idea is to use quenching of fluorescence caused approaching between donor and acceptor in the binding zone of the test strip. Such pairs can be two kinds of nanoparticles attached to different immunoreagents. Thus, Shi et al. [83] successfully used for this purpose quantum dots and gold nanoparticles in the analysis of ractopamine, Anfossi et al. [84]—quantum dots and gold or silver nanoparticles in the analysis of fumonisin, and Jiang et al. [85]—ruthenium-doped silicon nanoparticles and silver nanoparticles in the analysis of ochratoxin A. Another perspective approach is open sandwich immunoassay (OSI). The given assay is based on the association of the separated VH and VL chains of the antibody and reinforcement of this association after addition of the target antigen [86]. This approach with the use of so named Quenchbodies is implemented in different versions, mainly with fluorescent detection [87, 88], and it seems promising for LFIA.

In our works, two types of immunoassay for low molecular compounds with direct analyte-signal dependence are described. They do not require special reagents. In Urusov et al. [89],

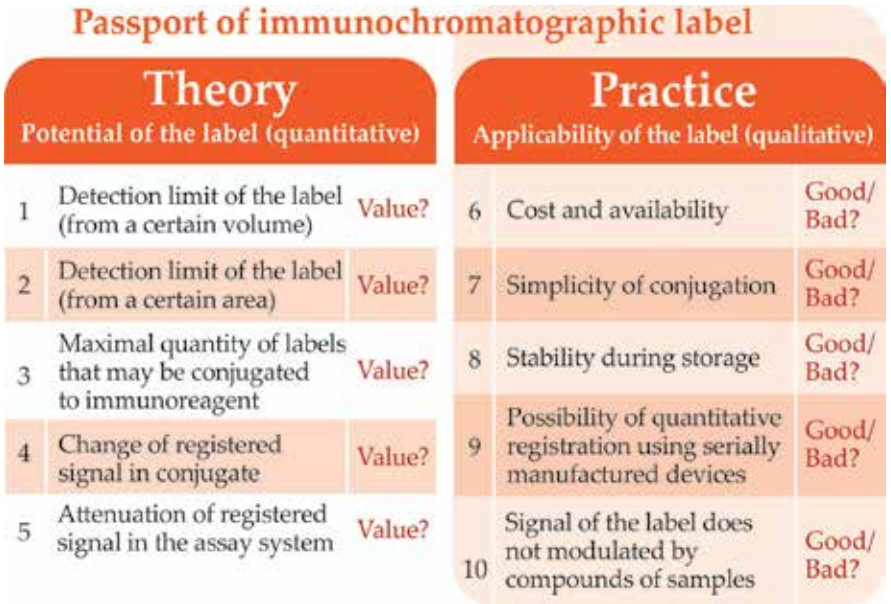

Figure 9. Characteristics of labels that determine their applicability and competitive potential in LFIA.

an assay was described in which labeled antibodies in the absence of the antigen in the sample completely bind in the first zone to the immobilized analyte. The appearance of the analyte in the sample blocks some of the antigen-binding sites of the antibodies and allows them to reach the second binding zone on the test strip, ensuring the appearance of staining (see **Figure 9**). For the case of deoxynivalenol detection, the proposed approach is 60 times more sensitive than the traditional LFIA. In Berlina et al. [90], an analysis of the food colorant Sudan was described based on the use of two conjugates of gold nanoparticles with (i) antibodies specific to Sudan and (ii) Sudan-ovalbumin conjugates. In the absence of Sudan, the conjugated Sudan-ovalbumin was coated with antibodies on the surface of the gold nanoparticle. So the interaction with the anti-mouse IgG in the test area is prevented. The added Sudan displaced the Sudan-ovalbumin causing the binding of labeled anti-Sudan antibodies in the test area and the appearance of coloration.

6. Proper response for LFIA

The response of the immunochromatographic system is the recorded signal of the label (its color or other parameters), which reflects the formation of a specific immune complex and allows for highly sensitive detection of the target analyte. Therefore, the question of proper response for LFIA is first and foremost a question of choosing a label.

The variety of molecular or colloidal labels that can be used in LFIA is extremely large [91, 92]. According to Goryacheva et al. [92], compounds such as gold nanoparticles of various shapes

and sizes, carbon nanoparticles, selenium nanoparticles, iron oxide nanoparticles, fluorescent dyes, fluorescent dye-doped nanoparticles, quantum dots, infrared emitters, up-converting emitters, nanoparticles with long-lived emission, liposomes, and enzymes may be used for this purpose. There are many articles that demonstrate the advantages of a new marker on the example of the detection of one randomly chosen analyte. However, the question of correct comparison of different labels remains open. Indeed, the differences between test systems depend not only on the label but also on the affinity of the antibodies, the regimen of intermolecular interaction, and the correctness of the choice of reactant content. Therefore, the gain achieved for one analyte does not necessarily persist after the transition to another analyte.

In this situation, it is justified to have "passports" of analytical labels, which are determined by their own properties and can be taken into account when implementing various analytical systems. The proposed list of such parameters is summarized in **Figure 9**.

Note that along with single-valued quantitative parameters reflecting the physical properties of a label, a number of qualitative parameters must be taken into account. Unfortunately, to date, researchers do not have universally recognized quantitative characteristics of existing labels and rules for a priori evaluation of proposed labels. Therefore, when deciding on responses (**Figure 10**), we are forced to follow the data of disparate comparisons of labels in different experimental developments.

Even within the framework of the use of gold nanoparticles, the developer has the opportunity to choose preparations of different sizes and shapes. The well-known recommendation [93] on the preferable use of spherical gold nanoparticles with an average diameter of 30–40 nm is confirmed by published experimental comparisons [49]. Serebrennikova et al. [94] showed the advantages of high-branched gold nanoparticles ("nanoflowers") as optical markers—a fivefold decrease in the detection limit of procalcitonin. These patterns were confirmed by Xu et al. [95], and the preferable use of long-tip (13–15 nm) nanoflowers was stated. Ji et al. [96], using gold nanoflowers, reached the detection limit of aflatoxin B1, equal to 0.32 pg./ml.

Optical markers for immunochromatography of different chemical natures are compared in a number of works. The possibilities of using carbon nanoparticles described in Van Amerongen et al. [97, 98] and Liu et al. [99], using the example of salbutamol detection, also showed the advantages of colloidal carbon compared to colloidal gold and nanogold-polyaniline-nanogold microspheres. For ractopamine detection, Hu et al. [100] showed the advantages of time-resolved fluorescent nanobeads compared with fluorescent submicrospheres, quantum dots, and colloidal gold. Effective integration of palladium nanoparticles and horseradish peroxidase with a 10-fold gain in sensitivity as compared to colloidal gold in the detection of *Listeria monocytogenes* was described by Tominaga [101]. The possibilities of high-sensitivity LFIA using graphene oxide and carboxylated graphene oxide as optical markers were shown by Yu et al. [102].

Of great interest are fluorescent markers. In many respects, this is due to the fact that with the correct choice of the wavelengths of excitation and emission, it is possible, by increasing the intensity of the exciting light, to proportionally increase the response in the practical absence (in contrast to the colorimetry) of the nonspecific signal. The gain in sensitivity achieved in

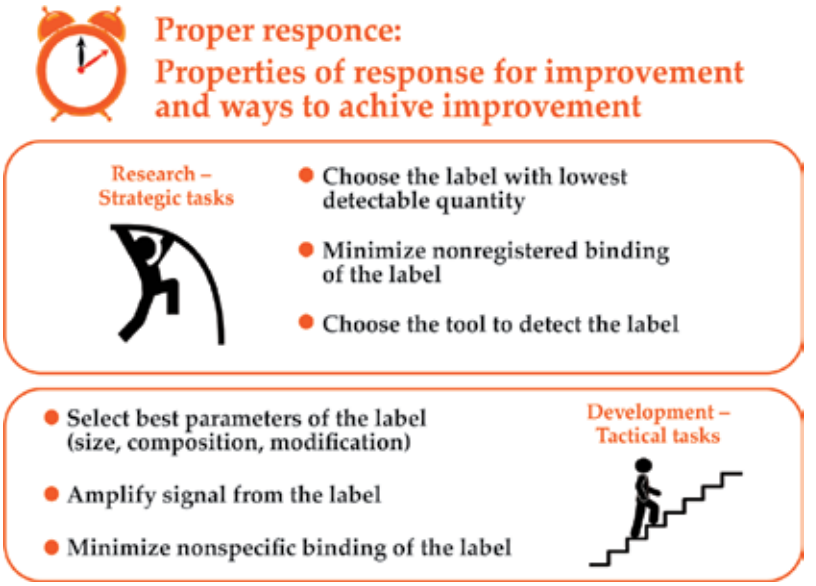

Figure 10. Main research and development tasks to obtain proper responses for LFIA.

this case is one or two orders of magnitude [103, 104]. The use of fluorescent markers in LFIA is summarized in the reviews of Pyo and Yoo [105] and Gong et al. [106]. A comparison of the analytical capabilities of quantum dot nanobeads, large-sized (50–600 nm) particles with impregnated quantum dots was given in Duan et al. [107].

Additional capabilities of high-sensitivity analysis are achieved by the registration of energy transfer with the spatial convergence of two labels—fluorescence resonance energy transfer (FRET). Systems using fluorescein isothiocyanate and gold nanoparticles were developed by Wang et al. for the detection of cancer embryonic antigens [108]. Other variants of fluorescent LFIA were also described, for example, registration of background fluorescence quenching in Chen et al. [109], silver nanoparticle-based fluorescence quenching in Jiang et al. [85], and quenching of the fluorescence of quantum dots by gold and silver nanoparticles in Anfossi et al. [84]. (See also Section 5 with their consideration as examples of competitive immunoassays with a direct dependence of the detected signal on the analyte content.)

Extremely promising is the use of surface-enhanced Raman spectroscopy (SERS) for detection of optical labels. SERS signals are based on the increase of optical absorption for reporter molecules by orders of magnitude after their immobilization on the surface of nanoparticles. The possibility of such highly sensitive analyses is demonstrated in the works of Sanchez-Purra et al. [110], Fu et al. [111], and Marks et al. [112]. Clarke et al. [113] described the combination of SERS registration with rapid vertical flow technology as an additional means of increasing sensitivity. In Maneeprakorn et al. [114], SERS detection with 4-aminothiophenol as a signal reporter lowered the detection limit by 300 times compared to traditional LFIA. In Cho et al. [115], the transition to SERS based on silver-intensifying gold nanoparticles led to a 1000-fold decrease in the detection limit. Blanco-Covian et al. [116] proposed the use of a combination of Au @ Ag core-shell nanoparticles and rhodamine B isothiocyanate in LFIA, which allowed them to perform highly sensitive

detection of pneumolysin with a detection limit of 1 pg/ml, recording the surface-enhanced resonance Raman scattering (SERRS).

Note that optical recording methods allow us to evaluate only labels that are in the upper layers of the test strip and are not shielded by membrane fibers. The loss of the optical signal depends on the properties of the material but is usually estimated [93] as about one order of magnitude. In this regard, the work of Jacinto et al. [117] is extremely interesting. They offer an electromagnetic relocation of reporter particles for amplifying an optical signal and describing the fourfold reduction in the detection limit of human chorionic gonadotropin.

This restriction is excluded for analytical methods in which registration of a label is based on other physical principles. Thus, Wang et al. [118] developed the Thermal Contrast Amplification Reader for the registration of gold nanoparticles, which, for systems of influenza and malaria diagnostics and detection of *Clostridium difficile*, showed eight times lower detection limits as compared to an optical reader. Zao et al. [119] improved the detection limit by two orders of magnitude for photoacoustic analysis compared to colorimetric measurements. The magnetic properties of the nanodispersed label in LFIA were recorded by Barnett et al. [120], Chen et al. [121], Lago-Cachon et al. [122], and other authors. Several variants of LFIA with electrochemical detection are presented in the literature, the most recent of which (the work of Zhao et al. [123]) is based on the use of a serial glucometer as a registrar. Just recently, Lin et al. proposed LFIA of myoglobin based on pressure measurement for oxygen generated by platinum nanolabels from hydrogen peroxide [124].

The capabilities of high-sensitivity detection in LFIA are not limited to the choice of a label. Additional reserves provide **amplification of the recorded signal**, which can be provided by

- treatment of the test strip with additional reagents that enhance the coloration or other detectable parameters;
- aggregation of label particles, thereby increasing their number, attached to a single immune complex;
- or initiation by the label of additional reactions, leading to the generation of the detected signal.

The existing variety of developments in this area is summarized in a review of Shan et al. [125]. The systems that implement the aggregation of several types of functionalized nanoparticles cause particular interest. Such approaches are described, for example, by Choi et al. [126] with a 100-fold gain in sensitivity for the detection of troponin I using two kinds of gold nanoparticles; by Razo et al. [20] with the generation of an optical signal by complexes of iron oxide nanoparticles (also used as a concentrating agent) and gold nanoparticles with a 32-fold decrease in the detection limit of potato virus X; by Taranova et al. [127] with a 30-fold gain in the analysis of procalcitonin due to biotin-streptavidin aggregation of gold nanoparticles; by Shi et al. [128] with complexation of gold nanoparticles of two sizes in the analysis of imidaclothiz; by Zhong et al. [129] with the formation of two layers of antibody conjugates with gold nanoparticles in the detection of melamine; and by Shen et al. [130] with aggregation of

gold nanoparticles using polyamidoamine dendrimer, which lowered the detection limit of rabbit immunoglobulin G 20 times.

The growth of the size of gold nanoparticles with the help of the catalyzed reaction of their surface between $HAuCl_4$ and NH_2OH was examined by Bu et al. [131] as a means of amplification for LFIA. The layered build-up of gold nanoparticles was described by Li et al. [132]. Anfossi et al. [133] and Panferov et al. [134, 135] considered the possibilities of silver enhancement (restoration of the silver salt on the surface of a gold nanoparticle with an increase in its size) in LFIA. In a study by Rodriguez et al. [136], the optimal regimes of silver and gold enhancements were determined to enhance the signal from the gold nanoparticles. Enzymatic amplification using alkaline phosphatase was studied by Panferov et al. [137] for LFIA of potato virus X and by Kim et al. [138] for LFIA of C-reactive protein. A feature of the latest development was the use of a water-swellable polymer for the accumulation of a colored product. An original polymerization-based amplification approach for enhancing staining was described by Lathwal and Sikes [139].

The basic requirement for amplification approaches is the maintenance of low laboriousness of analysis. Variants using additional reagents, although considered in development, should be finally transformed into devices of dry chemistry, in which all components of the test strip are applied to its membranes.

7. Proper output for LFIA

The generation of a signal reflecting the formation of immune complexes during LFIA is not the final stage of the analysis. The analysis is only completed when a diagnostically meaningful conclusion is made on the basis of this signal.

Effective use of LFIA is possible only when it is combined with modern means for documenting, storing, and processing information. In the absence of these tools, the advantages of rapid and high-performance nonlaboratory diagnostics are lost because of time-consuming processing and description of test results. Of fundamental importance is the transition from a subjective yes-no evaluation of results to automatic quantitative registration and the formation of databases that integrate the results of mass screenings or information on the dynamics of the state of patients (objects). Such systems will allow rapid collection of various indicators "at the time of request," contributing to an accurate diagnosis. Taking into account the foregoing, **Figure 11** summarizes the requirements for proper output in LFIA.

In some cases, the developer does not need to achieve maximum sensitivity but to fix the threshold that separates the positive and negative results in accordance with the regulatory requirements for MRLs. This allows the composition of conjugates used in the analysis discussed above to be varied [63]. A qualitative "yes-no" analysis can be transformed into a semiquantitative one with a change in the number of colored bands corresponding to several threshold levels. To do this, depletion of the conjugate can be used when interacting

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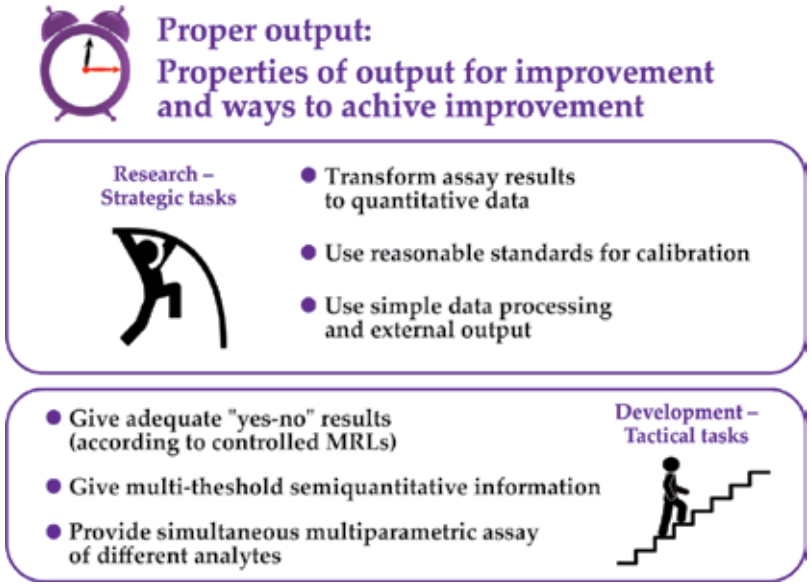

Figure 11. Main research and development tasks to obtain proper output for LFIA.

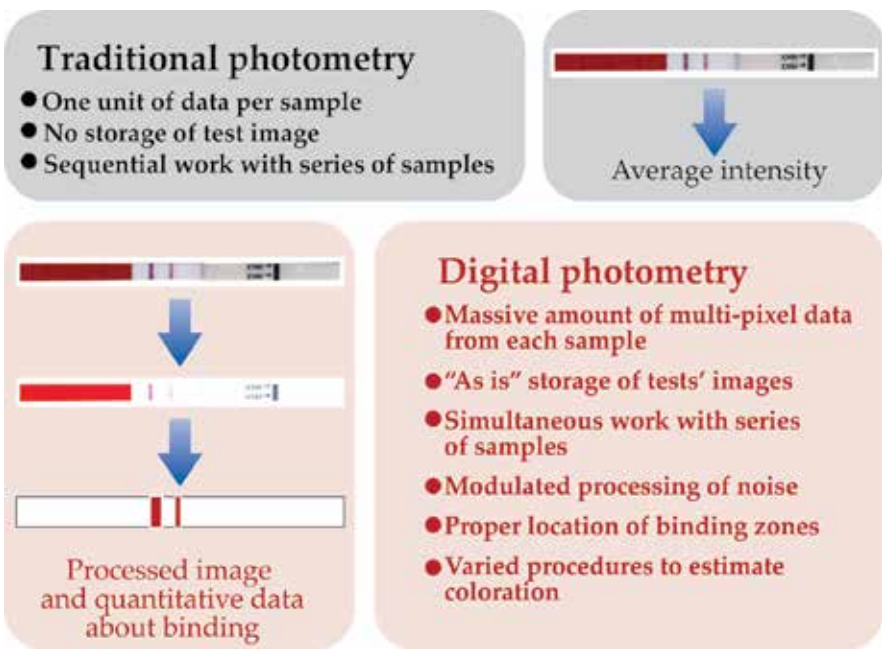

Figure 12. Comparison of traditional and digital photometry as a means for registration and processing of immunochromatographic data.

with several consecutive identical binding zones. Additional opportunities arise when using antibodies with different affinities, varying the surface density of the reagents applied in the binding zones and the distance between these zones and the beginning of the test strip. An example of an appropriate development with three thresholds of potato X virus concentrations corresponding to the degree of plant infection was described by Panferov et al. [140].

Initially, attempts were made to create detectors for membrane tests that recorded the total intensity of the staining (brightness of the reflected light) in certain sections of the test strip using a row of light-emitting diodes and individual systems of signal transformation for each diode. However, such detectors were extremely cumbersome. Blatt et al. [141] proposed a device made from 28 photosensitive sensors located along the test strip. Nowadays, the dominant means of detecting the results of LFIA, allowing a full-color image of the test strip to be received, are digital cameras. This technology is based on the use of inexpensive portable detectors or household recording devices—such as a mobile phone camera [142]. Serially produced cameras record images with a resolution of up to 2400 dpi, which corresponds to the size of an individually characterized section of less than 1 μm^2. **Figure 12** summarizes the advantages of digital photometry in LFIA.

Trends in the transformation of LFIA from the visual to the instrumental method are summarized by Cheung et al. [5]. Reviews by Quesada-Gonzalez and Merkoci [143] and Zarei [144] present the current state of analytical technologies based on the use of mobile phones/smartphones. At the same time, a significant number of manufacturers of test systems offer portable detectors that are adapted to work with their own products [8]. Of the original solutions, mention should be made of Feng et al. [145], in which the registration tool for LFIA was Google Glasses. In recent years, a number of companies have introduced cloud technologies into practice, where external servers receive data about testing results via standard communication devices and store and process this information. Thus, since 2017, Abbott has proposed a set of tools named i-STAT Alinity for distant diagnostics. Special cartridges allow 14 parameters of blood composition by bio- and immuno-chemical techniques to be controlled.

An extremely important means of increasing informativeness, although not related to an increase in sensitivity, is to conduct a multiplex analysis—that is, detection of the presence and level of several analytes using a single test strip. Data on the control of several analytes can be discriminated in space (by the position of binding zones) or by signals (by using different labels). Quantum dots are an effective tool for multi-analysis with different signals. The use of conjugated quantum dots with different spectral characteristics allows one to perform highly sensitive diagnostics with simultaneous detection of, for example, three antibiotics ("traffic light" in Taranova et al. [146]) or four mycotoxins ("rainbow" in Foubert et al. [147]).

Because the number of binding zones that can be sequentially located on one test strip while preserving the rapidity of the analysis and the reliability of the information obtained for each analyte is limited (usually no more than five zones), the transition to "two-dimensional immunochromatography" is promising—see **Figure 13**. This approach, combining the advantages of immunochromatographic tests and immunochips, is based on the formation of an ordered two-dimensional array of points with immunoreagents of different specificity on

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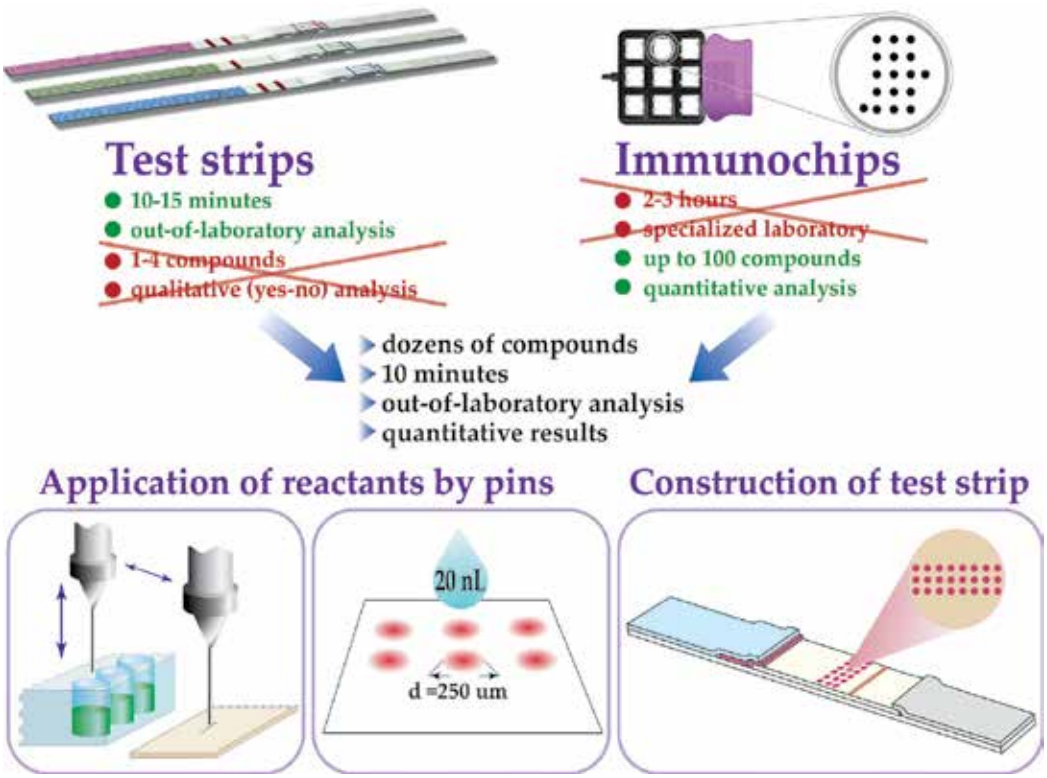

Figure 13. Concept of 2-D immunochromatography.

the membrane of a test strip. In such systems, interaction occurs in several dozens of binding zones. Due to this, the 2D immunochromatography increases the information content of LFIA results and reduces the consumption of reagents and materials for one analysis.

Examples of test systems based on the principle of "two-dimensional immunochromatography" are presented in the works of Taranova et al. [104] on the detection of drugs and Safenkova et al. [148] on the detection of phytopathogens. General approaches to multizonal LFIA were discussed in Hu et al. [149], and the current state of the development of multiplex immunoassays was discussed in Li et al. [150].

8. Further perspectives of LFIA

It would be reasonable to summarize the presented review of LFIA developments using two outcomes—strategic (research) and tactical (development) outcomes.

We may identify the following main tasks, the solutions of which are extremely important regardless of the specific analyte and type of tested samples that are of interest to the developer: To create an effective test, the developer must

- Obtain the most concentrated sample
- Select the membranes that ensure rapid movement of reagents and high intensity of staining when working with this matrix
- Select the optimal label
- Use conjugates of immunoreagents having optimal composition
- Choose the optimal location of the reagents applied to the test strip
- Find the optimal ratio of immunoreagents, combining a sufficient level of label binding and a low detection limit for the analyte.

Considering the strategic situation of the development of LFIA, we should expect test systems of the future to implement high-performance and informative analyses integrated with the tools for collection, storage, and processing of information. With the development of molecular biological methods for the production of modified and new receptors, bioanalytical systems will be able to effectively discriminate various structurally close compounds and, on the basis of their levels in the sample, make more informative diagnostic conclusions.

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Chapter 3

Microarrays as Platform for Multiplex Assays in Biomarker and Drug Discovery

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Abstract

Despite the tremendous advances in the understanding of the molecular mechanisms and the complexity of the diseases is one of the present challenges for the scientific community; then, novel strategies are required to be designed and developed for effective strategies for early diagnosis and treatment. As many cellular alterations are observed at protein level, high-throughput assays are dramatically needed for biomarker discovery. Herein, we describe advantages and limitations of protein microarrays, as proteomics strategy useful for multiplex and high-throughput protein characterization in clinical samples. Finally, a few examples are discussed; mostly of them related to currently disease biomarkers already identified in proximal fluids by protein arrays are discussed.

Keywords: biomarker discovery, multiplex detection, protein microarrays

1. Introduction

Despite the tremendous advances in the understanding of the molecular basis of diseases (such as cancer, Alzheimer, genetic diseases), there are some crucial gaps that difficult us to understand disease pathogenesis and develop effective strategies for early diagnosis and treatment [1].

The current interest in protein microarrays due, mostly in part, to the prospects that proteomics assays, based on them, allow: deciphering the altered protein expression in different

levels (tissue, cells, subcellular structures, body fluids, protein complexes,…); the development of novel biomarkers for diagnosis or early detection of diseases; the identification of new targets for therapeutics; and accelerating drug development through more effective strategies to evaluate therapeutic effect and toxicity [1].

Mainly based on the proteome alterations in disease may occur in many different ways that are not predictable from genomics analysis, and it is clear that a better understanding from genomic analysis together with an substantial impact in biomedicine.

Recently, there is a large amount of protein microarray approaches available for applications related to disease because the employment of these technologies is very useful for better diagnostics and to shorten the path for developing effective therapy. In general, protein microarrays allow to increase sensitivity, reduce sample requirement, increase high-throughput and identify protein alterations (quantitative and qualitative), such as post-translational modifications.

If we want to quantify thousands of proteins in a small number of samples, a typical proteomics discovery experiment employs a non-targeted approach (shot-gun proteomics). The hundreds of proteins that result from the comparative analysis, are differentially expressed between healthy and diseased samples. After discovery phase, the potential biomarkers proteins are reduced by performing studies on additional patients or at more time points, and/or by using another technique. Then, these potential biomarkers are verified on a set of 10–50 clinical samples. In the final step, a small number of biomarkers is "validated" on 100–500 clinical samples. Bearing in mind this conventional biomarker workflow, high-throughput assays are required in order to overcome the gap between translational research and clinical needs (from bench to the bedside) [1] (**Figure 1**).

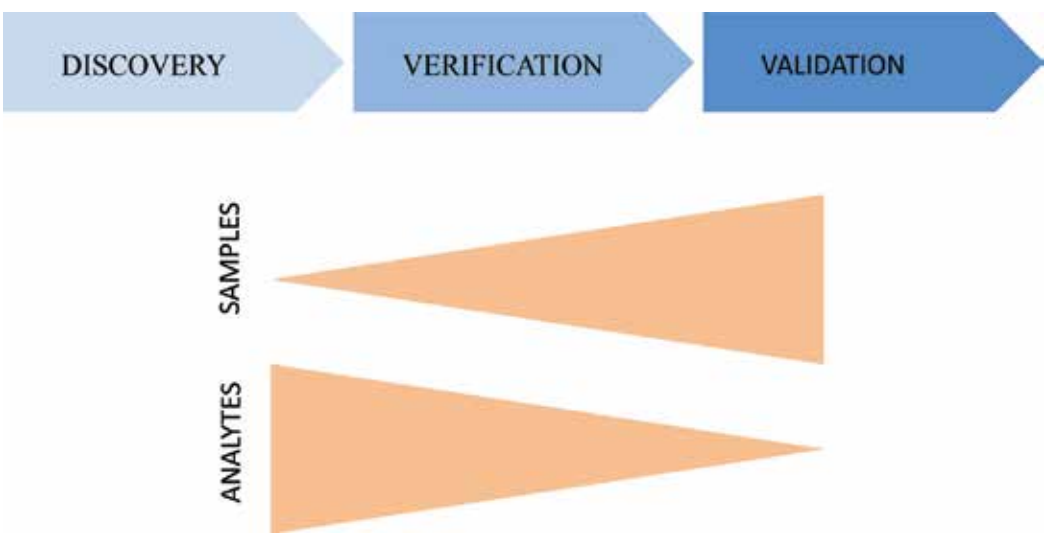

Figure 1. Stages in the development of a biomarker.

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Biomarkers or biological markers are defined as measurable characteristics that indicate the state of a biological process, differentiating if it is normal or pathological. They are all those molecules that are found in body fluids in low abundance and that are associated with specific health or disease processes [2]. The methodology to establish that a biomarker is good for clinical application is divided into three steps: discovery, verification and validation as it has been said before [3, 4].

Proteomics, the scientific discipline that studies proteins, has identified several proteins that can act as biomarkers. There are several techniques that use biomarkers and that allow an early diagnosis of the disease. These include new generation sequencing, mass spectrometry and protein arrays [5, 6]. In this chapter we will focus on the study of arrays.

Microarray technology is a term that refers to the miniaturization of thousands of assays in a single device, allowing the simultaneous and massive analysis of a large number of biomolecules in a single biological sample. They allow the study of a large number of parameters in a single test with a minimum requirement of sample and reagents, which is why they constitute a simple, fast and very sensitive sampling technique [7, 8].

The microarrays present several innovative strategies for their applications such as the identification of biomarkers and the interactions between proteins that may help in the future to routine clinical analysis.

There is a great variety of arrays which can be classified according to their content in protein microarrays, functional protein arrays and reverse phase arrays; according to their shape in planes and spheres; and also according to their detection method.

In this chapter we will explain all these types of arrays, some of their applications in biomarker discovery as well as validation/verification.

1.1. Biomarkers discovery

A biomarker is defined by the Food and Drug Administration (FDA) as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention."

It is possible to distinguished three categories depending on the application of the biomarker: diagnostic biomarkers used for disease detection, prognostic biomarkers used for predict the course of a particular disease such as, recurrence, progression, and survival and predictive biomarkers used for predict the response to treatment that could be subsequently applied in patient assessment [1].

During last years, biomarker research and its translation into the clinics have been accelerated by protein microarrays due to the extraordinary capacity for identifying of valuable biomarkers in a short period of time without the requirement of any prior in-depth knowledge into the mechanism of disease progression.

Moreover, protein microarrays are reliable for analyzing targeted/non-targeted biomarkers presented in mostly of human proximal body fluids (such as plasma, serum, synovial liquid,…)

with a wide dynamic range. In contrast with other proteomic strategies, protein microarrays avoid the sample pre-fractionation. Thus, for example, serum, plasma, urine and tissue extracts which are complex and non-fractionated proteome mixtures, could be used for experimentation. For this reason, among others, protein microarrays offer a powerful technology for functional proteomics analysis in HT format.

Microarray technologies, like DNA arrays, printed dense spots of capture ligands immobilized onto a solid support that are exposed to samples containing corresponding binding molecules (often called queries), allowing the simultaneous analysis of thousands of capture targets within the same assay. Ekins and collaborators described these binding events based on miniaturization as the key parameter. They predict that a system that uses small amounts of capture molecules and a small amount of sample could be more sensitive than a system using a hundred times more material. In fact, this is the case when $K < 0.1$ (where K is the affinity constant between ligand and target) [9].

The capture ligand is presented in a confined area of array, reducing its diffusion. The specific binding event with its target takes place with the highest signal intensities and optimal signal-to-noise ratio could be achieve in these small spots [9] (**Figure 2**). An immunoassay

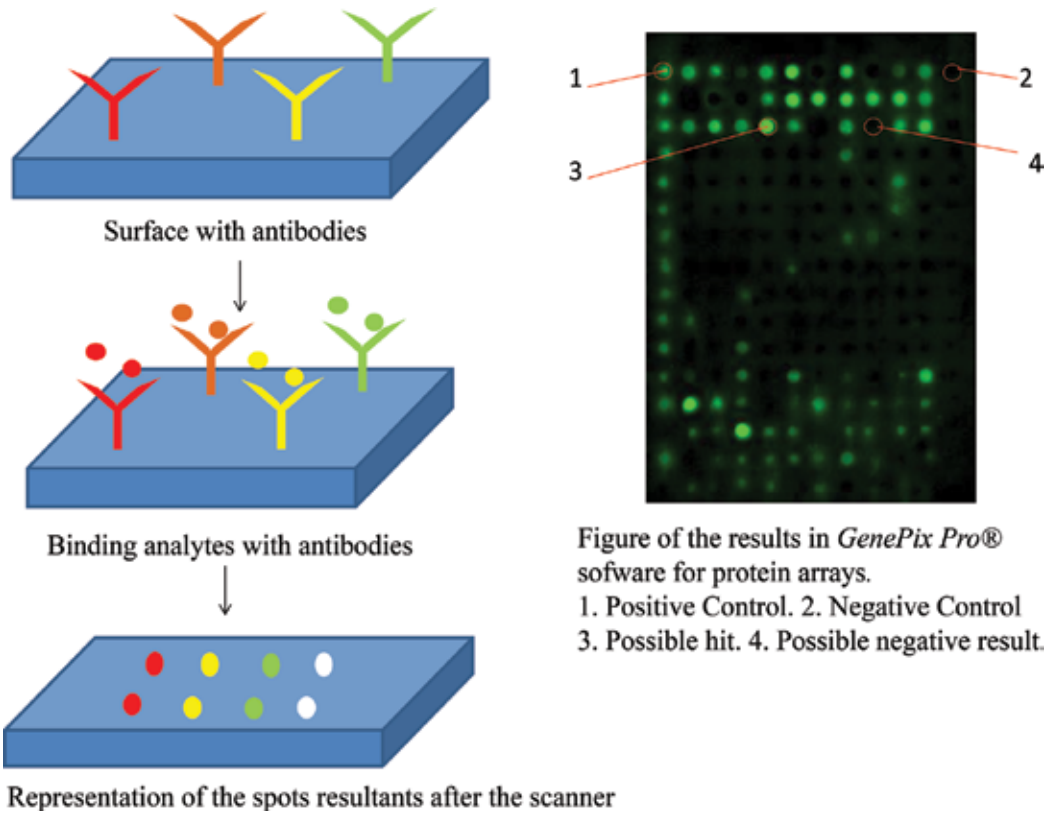

Figure 2. Process of microarray technology.

in an array format displays sensitivities in the pM to fM range, enabling test low-abundant (pg/mL) analytes in crude proteomes with a small volume of sample. In many cases, protein microarrays show a relevant advantage in clinical applications because the samples to test are minimal [10].

For that reason, protein array technology needs to use a multiplex and highly sensitive protein assay capable of handling and resolving complex proteomes with limited available sample [10].

Recently, several types of protein microarrays have been developed and applied as multiplex throughput assay in several biological characterization. Here, it is described the principal features of protein microarrays.

2. Description of protein microarrays

In general, protein microarrays are classified according to features such as content, format, detection method and according to final application such as analytical and/or functional. Here, it is described the main aspects of the different types of protein microarrays.

2.1. Arrays according to their nature of the capturing agent

In general, there are three types of arrays according to their content:

2.1.1. Analytical protein microarrays or antibody microarrays

In these arrays, antibodies are printed onto array surface and used multiplexed affinity reagents to detect and quantify proteins in complex biological samples [8, 11].

It is based on the antibody antigen interaction. Antibodies have the ability to bind to a very specific protein, so particular or rare analytes can be detected in highly heterogeneous mixtures. They are usually used to identify biomarkers which predict a biological condition such as healthy or pathologic.

So many studies have shown that only a fraction of antibodies may properly work and this may be due to the loss of antibody activity by degradation or denaturation on storage or during the printing process, or due to inappropriate antibody orientation onto the array surface [7].

2.1.2. Functional protein arrays or recombinant protein arrays

It is based in the identification of protein interactions with different molecules (proteins, DNA, lipids, drug, etc.), so recombinant proteins are printed onto array surface.

Some examples are protein in situ array (PISA), printing arrays from DNA (DAPA) nucleic acid programmable protein array (NAPPA) and multiplexed nucleic acid programmable protein array (M-NAPPA). They are essential for pharmaceutical industry because they allow to know protein interactions [11].

PISA is based on DNA amplified by PCR as a template. The DNA that encode the protein of interest contains a T7 promoter or another strong transcriptional promoter and an in-frame N-12 or C-terminal tag sequence for protein capture onto the surface. PISA offers the possibility to cell-free production of protein arrays. It means protein are produced using cell extracts directly on the surface os arrays. PISA demonstrated that multiple proteins could be produced without the need of using cells for expression followed by lysis and purification to make the proteins [11].

DAPA is a technique derived from PISA, but DAPA allows use the same DNA template slide repeatedly for printing up to 20 copies of the same protein and also DNA could be reused after prolonged periods of time. DAPA takes a long time to express proteins due to the diffusion of proteins through the membrane. This technique starts by spotting the PCR amplified DNA fragments encoding the tagged protein on one slide. This slide is sandwiched with another Ni-NTA slide where a tag-capturing agent immobilizes the expressed protein. A permeable membrane with the cell-free lysate which allows coupled transcription and translation is places between the two slides. Then, the expressed proteins are captured to the surface on the other slide through the capture ligand. Overall, DAPPA requires long time to express proteins and this technique presents the protein diffusion as strong limitation, in particular with large proteins [7, 9, 11, 12].

NAPPA uses cDNA templates cloned into expression plasmids which adds a transcriptional promoter and also an in-frame polypeptide capture tag. It has several advantages: (1) once the clone is produced as a glycerol stock it becomes an indefinitely renewable resource that could be shared with other labs; (2) if the clone is carefully sequence verified, then the resource will have long-term sequence fidelity; (3) the use of plasmids removes some of the length constraints on the epitope tags, so that functional protein tags can be used. There are many applications of NAPPA where the proteins are fused with glutathione-S-transferase (GST); nevertheless, other tags such as flag, HA, c-myc, and Halo tag have been used in specific applications. High quality supercoiled plasmid DNA is purified from bacteria cultures and printed onto an activated ester surface along with a homo-bifunctional crosslinker, bovine serum albumin (BSA) and anti-GST antibody. BSA efficiently increased the DNA binding and narrows down the unspecific interactions and anti-GST attaches the protein expressed. When cell-free expression system is added to the array, a coupled transcription/translation reaction is produced and the nascent protein is linked to the capture agent tag the C-terminal end assuring the complete translation of the protein [10, 11, 13].

Puromycin capture protein arrays (PuCA) are cell-free expression protein arrays based on the affinity of puromycin by expressed peptide/protein. First, PCR DNA is transcribed to mRNA, and a single-stranded DNA oligonucleotide modified with biotin and puromycin on each end is then hybridized to the 3′-end of the mRNA. The mRNAs are placed on a slide and immobilized by the binding of biotin to streptavidin which is previously coated on the slide. Cell extract is then dispensed on the slide for in situ translation to take place. When the ribosome reaches the hybridized oligonucleotide, it stops and incorporates the puromycin molecule to

the nascent polypeptide chain, thereby attaching the newly synthesized protein to the microarray through the DNA oligonucleotide [14].

M-NAPPA is based on combining up to five different DNA plasmids at one point. This increases the number of proteins displayed by microarrays by five. It also reduces the cost and the time spent in work. M-NAPPA would be useful in unbiased HT screening studies, such as protein-protein interactions, protein-DNA interactions, discovery of drug binding target as well as (auto)antibody biomarkers for a variety of human diseases [15].

2.1.3. Reverse phase protein arrays

In these arrays, biological samples, tissues and cell lysates are printed onto array surface. The detection is accomplished by antibodies link with specific proteins, and then link with another antibody with fluorescence. They offers the potential for rapid comparison of the levels of such proteins present among a significant number of samples on a single array [7, 8].

The problem is the interaction of the lysate matrix, therefore validated antibodies should demonstrate no cross-reactivity toward other biomolecules of the lysate (**Figure 3**).

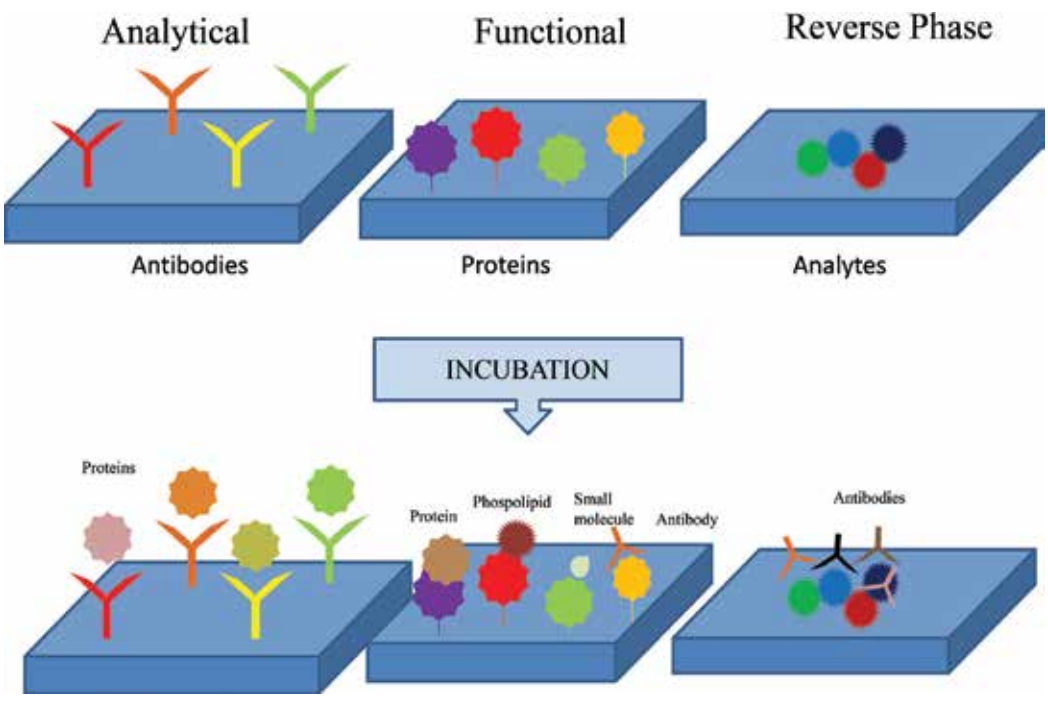

Figure 3. Arrays according to their nature of the capturing agent.

3. Arrays according to the format

3.1. Planar arrays

Planar arrays are based on high-density microspots of ligand deposited onto a solid support and separated by a minimal distance. It is necessary to know some parameters that influence in the assay performance like spot size and morphology, ligand capacity, background signal, limit of detection and spot reproducibility [13].

3.2. Microsphere arrays

Microsphere arrays are based on the simultaneous use of different populations of microspheres labeled with several fluorochromes. Each one is covered by different antibodies in order to incubate a biological sample of interest [7].

4. Arrays according to their detection method

Depending on the detection method it is classified in methods based on labels and free-label methods [7].

4.1. Methods based on labeling

4.1.1. Conventional fluorescent labels

Among them are radioisotopes and conventional fluorochromes like fluorescein, BODIPY or cyanins. Cyanines are the most used in protein arrays due to its minimal interaction with other biomolecules and its high intensity. More than two fluorochromes can be combined to identify several biomarkers at the same time. It is used for cancer biomarkers.

4.1.2. Flow cytometry sphere arrays

Flow cytometry can be used to detect soluble proteins present in body fluids or in cell lysates using microspheres. The CF has two lasers for the analysis quantitative and qualitative that allows to know the binding of target proteins with the microspheres. Comparing conventional fluorescent techniques, flow cytometry allows rapid and accurate quantitative-qualitative evaluation of a high number of proteins, with low cost and high sensitivity.

4.1.3. Magnetic spheres

These systems are an union of magnetic spheres with antibodies that allow the detection of proteins. They capture soluble proteins with high reproducibility and sensitivity, associated with low background noise, a wide dynamic range and low cost.

4.1.4. Quantum dots as fluorescence labels

The "Quantum dots" (QDs) are nano-crystals, formed by a core of semiconductor and fluorescent material coated with another semiconductor, which have very stable optical properties and a large gap between the wavelengths of emission and excitation of the complement. These nanometric particles could work like labels joining with peptides or antibodies for the recognition of cellular components. They have a high fluorescence, greater photo-stability, multicolored excitation, an adjustable and narrow emission spectrum, and their higher quantum yield, compared with organic fluorochromes. They also have several disadvantages like its high susceptibility to oxidation and photolysis as well as some risks for human health and the environment. They are used to localize tumor biomarkers.

4.1.5. Metal NPs as a label

Gold nanoparticles are used in protein arrays due to their optical properties, quantum efficiency and compatibility with a wide range of wavelengths.

4.2. Free-label methods

4.2.1. Surface plasmon resonance (SPR)

This technology is based on the generation of plasmons on a surface, which are oscillations of free electrons propagated parallel to the metal/medium interface, which allows to measure the changes in the refractive index of the sensor surface. The intensity variation of the reflected light and/or the angle of incidence tell us if there are molecular associations or dissociations, allowing to determine the relationship between the association and dissociation of biomolecules with each other. In addition, the SPR technology also allows evaluate the affinity and specificity of these interactions, facilitating the measurement of biomolecular interactions in real time with high sensitivity.

4.2.2. Microcantilevers

Microcantilevers are thin sheets of silicon coated with a gold surface associated with nanomechanical systems of biomolecular recognition. For this reason, the antibodies or proteins are immobilized on said sheets, so that when there is an interaction between these molecules and their possible ligands we can measure the variation in the position of the sheet using different optical or electronic systems.

4.2.3. Atomic force microscopy (AFM)

It is a microscopic analysis observing only the topological variations of the surface of an object. It has been used for the evaluation of the immobilization of biomolecules with a micrometric resolution, and for the detection of protein interactions on the arrays surface.

5. Arrays according to the applications

Bearing in mind the final application, protein microarrays are classified into two categories: Analytical arrays and functional arrays.

5.1. Analytical arrays

These arrays are normally used to quantitatively identify the presence of multiples protein in one single assay. Commonly, the main application is the detection of differently expressed proteins and their abundance in different samples. Biomarkers are determined by this type of arrays. They are biometric measurements, including molecular signatures, that predict a biological or clinical condition for example, normal or pathologic, often with potential diagnostic or prognostic value [9, 10, 16].

5.2. Functional protein arrays

These arrays are mainly used for the characterization and identification the specific function of proteins, as well as their interactions with other molecules (including proteins, peptides, small molecules/drug, enzyme/substrates or nucleic acids,…) [12, 17]. Moreover, functional protein arrays also allow the detection and identification of post-translational modifications (PTMs), such as glycosylation, phosphorylation, acetylation,… which typically modulate the proteins' function, regulation and/or turnover.

Advantages	Limitations
Protein arrays allow monitoring several proteins in the same assay (HT technology).	Protein arrays require validation experiments because of false positives can be detected.
Wide range of applications: serum screening, biomarker discovery and functional proteomic studies.	The highest array reported until date included only 9000 different proteins.
Easy control of experimental conditions.	Whole eukaryotic protein arrays still have not been reported.
Low sample consumption.	Difficulty to control post-transcriptional modifications.
Fast.	Arrayed proteins may not be functional on the surface.
Very sensible comparing with other HT technologies.	Lack of standar protocols.

Table 1. Advantages and limitations of protein microarrays in biomedicine.

Refers to research, functional protein arrays present the detection of multiple protein interactions with low reagent consumption in a fast and low cost fashion. On the translational side, the discovery of these interactions will promote the progress of new pharmaceutical targets, diagnostics and therapeutics. As a consequence, this technology is very interesting in the pharmaceutical industry [10] (**Table 1**).

6. Multiplex biomarker detection by protein microarray assays

During last decade, protein microarrays have been successfully employed in multiplex detection for biomarker discovery; here, it is remarked a few of these studies in order to illustrate the utility of these approaches in the field.

6.1. Oncology diseases-specific biomarkers

Haab et al. defined a panel of five serum proteins significantly expressed in serum between prostate cancer patients (33) and 20 controls [18]. In addition, this team has also identified 84 proteins with differentially relative abundance between diagnosed lung cancer patients and healthy controls [19].

In a similar approach, Wittekind and colleagues reported a set of proteins as biomarker candidates associated with hepatocellular carcinoma [20]. Nowadays, several studies have been performed and focused on biomarker identification in several oncological pathologies; for example: In ovarian cancer, 11 proteins have been identified by Amonkar et al. [21]; Sreekumar et al. identified a panel of proteins as biomarker candidates in colon carcinoma cells [22]; Díez et al. has identified differentially expressed proteins in B-cell chronic lymphocytic leukemia which are related with target therapeutics [23]. Previously, Below and coworkers have developed an antibody microarray to immunophenotype 1100 leukemia and lymphomas according to the abundance of a panel of 82 antigens or cluster of differentiation (CD) characterized at the surfaces of lymphocytes [24].

6.2. Vaccine candidates

In 2014, the firstly reported study about pathogen-host protein interactions by CID and workers. In this work, they studied the presence of post-transcriptional modifications in effector proteins, T3SS proteins, from different mutants of *Salmonella typhimurium* when they infected *in vitro* Hela. Lysate collection representing all infection conditions are printed and using several validated antibodies, they show a comparative results among the different assays according to abundance proteins or post-transcriptional modifications [25].

Another similar study, performed by Li and coworkers, has been published about identification of 149 antigens from *Yersinia pestis* presented in the serum from EV76 rabbit and to test the immunogenicity of several viral proteins [26].

In 2009 Thanawastein et al. developed an approached called Expressed protein screen for immune activators (EPSIA) which were successfully applied in the identification of novel bacterial immunostimulatory proteins from *Vibrio cholerae* [27].

Recently, Manzano et al. reported a set of novel vaccine candidates for *O. moubata* based on a systematic characterization of >2000 host-pathogen interactions, which were evaluated with self-assembled protein microarrays based on a cDNA library encoding >400 recombinant proteins of *O. moubata* [28].

In a most recent study, Montor et al. describe a work using NAPPA arrays to evaluate candidate membrane antigens in *P. aeruginosa* which could help to track the immune responses of patients infected with *P. aeruginosa* and healthy ones. In this work, 12 proteins have been identified being mostly of them related with adaptive immune response in infected patients [29].

6.3. Auto-immune diseases

In response to many pathological processes, the humoral immune system generates antibodies to self-proteins ("auto-antibodies"). These auto-antibodies are generated due to antigen over-expression, mutation, altered post-transcriptional modifications of altered degradation released from damaged tissue which lead to their recognition by the immune system. Auto-antibodies have several benefits which make them as suitable source of biomarkers: (1) They have been discovered before the appearance of clinical symptoms; (2) They are simple to identify even at low levels once their target antigen is known; (3) they are easy to reunite from blood; and (4) they could be show in higher levels and with a longer half-life than their target antigens, which may only be present in transiently in blood [10].

For example, NAPPA arrays were used for serological screening for the first time in 2007 by Anderson et al. They investigated the presence of antibodies against tumor antigens in breast cancer. The tumor-suppressor p53 is well-characterized in several solid tumors and the presence of antibodies against p53 is mainly due to mutations in its gene which lead to alterations in its half-life. By this approach, the authors presented that p53-specific antibody levels were significantly lower in healthy donors than in breast cancer patients and the response to p53 antigen was detected in Stage II disease. Also they studied that the antigen sites of p53 with several antibodies which recognized distinct epitopes of the protein to confirm that many regions of the protein expressed in NAPPA were accessible to antibodies in serum detected to them [10, 30].

In a follow-up work, also this group performed a wide screen for new auto-antibodies in breast cancer. They designed and developed 4988 candidate antigens to detect their auto-antibodies in serum samples from breast cancer patients with stage I–III disease. This screening was performed in three stage design that entailed comparing cases and controls and eliminating uninformative antigens at each stage. At the final phase, slightly more than 100 antigens were tested and 28 auto-antibodies were identified that distinguished benign breast disease from invasive cancer under blinded conditions [10, 30].

With a similar workflow, Labaer et al. developed a pilot NAPPA to assess auto-antibodies present in juvenile idiopathic arthritis (JIA) which is a disease characterized by chronic joint inflammation in children [31].

Recently, Fuentes's lab has performed a screening of auto-antibodies in osteoarthritis and arthritis rheumatoid by using NAPPA arrays and validated with other protein arrays technologies [32].

6.4. Functional biomarkers as drug targets

A decade ago, Labaer and colleagues evaluated functional properties of the proteins IVTT expressed onto the array by performing protein-protein interactions in high-throughput format. In this report, they printed an array expressing 647 unique genes in duplicate and tested for several well characterized interacting pairs including Jun-Fos and p53-MDM2 [33].

A more recent study, Manzano et al. published a work where they applied NAPPA to study protein interactions. In this study, a novel interactor partners were identified for P-selectin and phospholipase A2 and further validated [34].

Recently, a novel functional array, designed by Pascal Braun, identified novel cell signaling pathways in *Arabidopsis thaliana* by evaluating protein interactions onto this NAPPA technology [35].

In addition, a further study has just been published where evaluated multi-protein complex in NAPPA format. In this study, four novel tuberculosis-related antigens where identified in guinea pigs vaccinated with Bacillus Calmette-Guerin (BCG) and also validated with ELISA [36].

7. Conclusions and further directions

Here, we have briefly reviewed protein microarray field as suitable platform for multiplex assays in high-throughput format. Thus, the focus was on two main perspectives: (i) Key technological aspects, (ii) Biological Applications.

However, as we described previously, despite the fundamental advances in protein microarrays, allowing characterization of whole human proteome is still remaining as a challenge. Then, the information provides light on the functions of proteins and genes whose functions are currently unknown.

Overall, protein arrays may provide relevant information about the biological function of gene products. Although, it is still necessary to develop and optimize some key aspects of protein microarray in the future, other proteomics approaches could provide complimentary results.

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Conflict of interest

The authors do not declare any conflict of interest.

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Chapter 4

SLE, An Overlooked Disease: Possibilities for Early Rescue by Early Diagnosis

Reem K. Arafa and Mariam M. Ahmed

Additional information is available at the end of the chapter

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Abstract

Systemic lupus erythematosus (SLE) is a progressive autoimmune disease associated with widespread organ damage that can eventually cause death. Worldwide prevalence of SLE is difficult to report mainly due to difficulty in diagnosis as a result of its heterogeneous nature and nonspecific protean manifestations. Currently, circulating anti-DNA antibodies are the most specific diagnostic biomarkers for SLE where many detection assays are being employed in clinical practice. However, the diagnostic value of these techniques is challenged by the detection of only subpopulations of these antibodies with varying sensitivity and specificity. This is mainly attributed to differences in the antigen source and presentation and in the employed reaction conditions. This chapter will thoroughly discuss the technology, advantages, and limitations of each assay in addition to a special focus on the recently developed diagnostic technologies and novel biomarkers. Moreover, SLE will be presented as a disease model highlighting the importance of personalized medicine.

Keywords: systemic lupus erythematosus, autoimmune disease, complex pathogenesis, challenging diagnosis, anti-DNA antibodies, novel biomarkers, state-of-the-art diagnostic technologies, personalized therapy

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic multiorgan autoimmune disease with many unresolved questions regarding its pathogenesis, causes, best approaches for proper diagnosis and therapy [1–3]. It is perhaps the most heterogeneous human disease where SLE patients exhibit clinical manifestations that hugely vary on the levels of organ involvement and severity that are accompanied by differential release of autoantibodies and other serological biomarkers

[1, 4]. This is because the disease pathogenesis is highly chaotic and strikes any of the multiple stages of the immune cascade resulting in extremely wide-ranging and difficult-to-predict clinical and serological manifestations among SLE patients [1, 4]. As demonstrated through this chapter, SLE challenges the clinical community in its diagnosis, prediction of the course of the disease, extracting and monitoring reliable biomarkers, designing studies in clinical trials, and developing new therapeutics [1, 3–5]. Owing to this highly heterogeneous nature of the disease, SLE presents an ideal model for a disease that desperately calls for new developments in the state-of-the-art diagnostic technologies that can detect highly specific and reliable new biomarkers for disease diagnosis and prognosis, and it exemplifies the urge for personalized medicine that can target specific subsets of patients or specific organ involvement.

1.1. Epidemiology

Reporting on epidemiological data for SLE is not coherent among all countries, and the best informative data are obtained from North America and Europe, while less documentation has been received from Africa, Asia, Australia, and South America [1, 6]. Nevertheless, SLE is a global disease in which its incidence, prevalence, time of onset, and mortality are highly influenced by race and ethnicity [6]. For instance, in USA SLE has incidence and prevalence rates that show great variability ranging from 2 to 7.6 per 100,000 per year and 19 to 159 per 100,000 per year, respectively [7, 8]. These variations can originate from differences in ethnicity, race, and age within the studied SLE population and can also stem from differences in the employed diagnostic criteria for SLE. Similar variation trends were also observed in some European countries [9, 10].

One of the important characteristics of SLE is that it predominantly affects women more than men [11] with a disease onset that is influenced by ethnic background. For instance, in a different ethnic background-based study, it was found that the incidence of SLE in African-Caribbean females is higher at younger ages than in Asian or Caucasian females [12]. This age-specific incidence in females of different ethnicities was also seen in other studies performed in different countries [13–15]. However, the reason behind this characteristic SLE predominance in women more than in men is not entirely understood, but it was inferred to be related to hormonal factors. For example, serum prolactin was found at higher levels in SLE patients than a control group, but it is unknown how can prolactin be involved in SLE immune deregulation. Independent of gender, it was reported that generally people of African origin had a higher incidence of SLE than those of European origin [7, 8, 16].

Mortality risk is increased in SLE patients of Chinese, Hispanic, and African backgrounds with strong associations of renal damage [17, 18]. This, however, might relate to the levels of disease awareness and therapeutic adherence that might be different among different populations [6].

1.2. Pathophysiology

The chaos in biology associated with SLE, the involvement of multiple body organs along with the release of a wide array of autoantibodies has definitely challenged the advancement in understanding the disease pathophysiology. Such a comprehension is highly essential for the

identification of novel biomarkers, for efficient classification of SLE patients, and in exposing specific pathways prone for targeting to help guiding personalized therapies [1, 4, 5]. Nevertheless, great insights have been achieved through the use of mouse models of lupus and multiple genetic and epigenetic investigations [19–24] through which a mechanism for disease development has been proposed to proceed as illustrated in **Figure 1** [4].

Two important events set the basis for SLE pathophysiology: first is the loss of immune tolerance in which the immune system acquires autoantigen recognition and second the persistent release of autoantibodies that mainly target endogenous nucleic acids and associated proteins [1, 4]. A cascade of events has been postulated to underlie such two events and comprise impaired disposal of apoptotic cells, accumulation and immune recognition of nucleic acid material, deregulated lymphocyte signaling, and sustained production of interferons and other cytokines [1, 4].

Removal of apoptotic cell debris is normally a silent process. However, with impaired elimination of dead cells as in SLE, nucleic acid material becomes vastly accumulated and can acquire immunogenic properties through sustained exposure to the extracellular

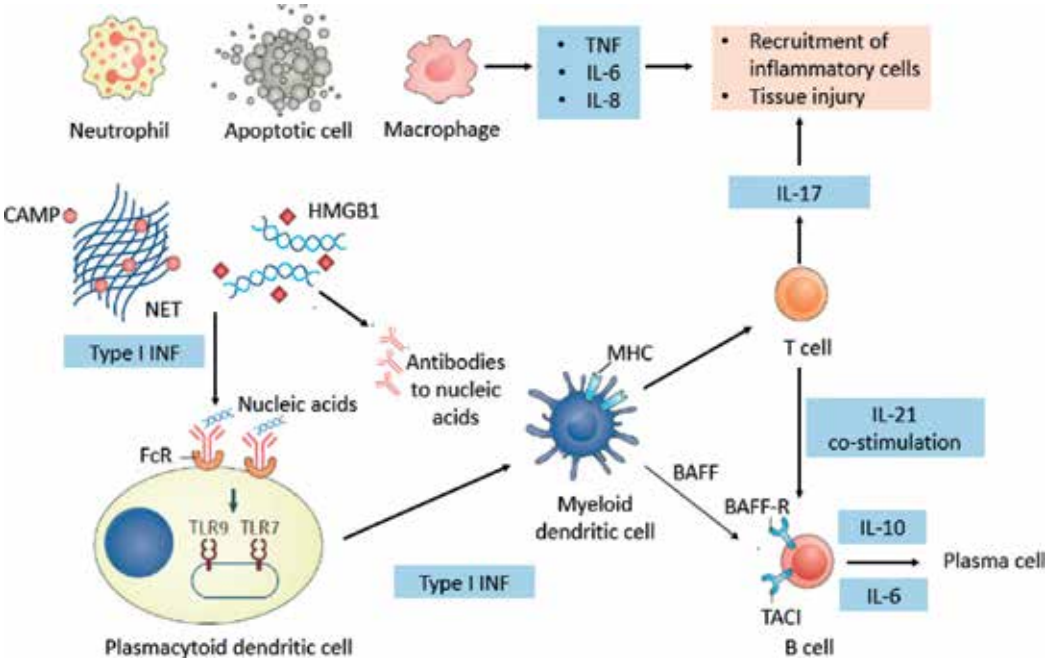

Figure 1. Underlying immune deregulation in SLE pathogenesis. Accumulated nucleic acid material containing DNA, RNA, and associated proteins that is released from apoptotic cells and neutrophils via NETosis can acquire immunogenic properties through their sustained availability in the extracellular surrounding in addition to the co-association with stimulatory molecules such as HGB1 protein. The accumulated nucleic acid material is engulfed by PCDs which in turn activate intracellular TLRs that stimulate potent release of type I INFs from PDCs which stimulate expression of BAFF that stimulates B-cell production of autoantibodies targeted against endogenous nucleic acids and associated proteins. T cells are important key players in SLE pathogenesis as they induce the release of inflammatory cytokines such as IL-17 and mediate tissue injury and destruction. Furthermore, T-cell and B-cell interactions stimulate B-cell differentiation and consequent release of pathogenic autoantibodies [4].

environment and the association with immune-stimulatory molecules such as IL-1 and the DNA-binding protein HMG-1 [25–27]. The innate immune cells plasmacytoid dendritic cells (PDCs) act by engulfing apoptotic remains containing nucleic acid material which stimulates the intracellular nucleic acid receptors Toll-like receptors (TLRs) particularly TLR7 and TLR9 [28–30]. When TLRs engage DNA or RNA, they elicit a strong release of type I interferons (IFNs) such as IFN-α which in addition to other cytokines induce autoantibody production through stimulating B-cell maturation and differentiation and can promote loss of immune tolerance and impaired immune responses [31, 32]. The activation of B cells via type I INFs has also been demonstrated by the increased expression of the B-cell-activating factor (BAFF) in response to IFN-α which stimulates B-cell activation and generation of autoantibodies [33–36]. Released autoantibodies bind their target antigens which are mainly nuclear components and form immune complexes that are recognizable by PDCs and further stimulate the release of type I IFNs amplifying INF signaling in a process known as "IFN signature" [1, 4, 27]. Many of the gene products that become actively expressed in response to type I INFs are engaged in immunoregulatory functions and were found to be highly elevated in many SLE patients with strong association of autoantibody release specifically those targeting RNA-binding proteins such as RNP, Sm, Ro, and La [37]. TLR7 specifically binds single-stranded RNA and was found to be strongly associated with the release of anti-Sm autoantibodies [38, 39]. On the other hand, TRL9 binds CpG-rich DNA sequences, was found to be highly expressed in B cells isolated from patients with severe SLE activity with an association of potent release of anti-double-stranded DNA (dsDNA) antibodies [38]. In addition to apoptotic cell death, neutrophil extracellular traps that are rich in DNA released from neutrophil cell death (NETosis) and other immunostimulatory molecules induce type I interferons through stimulating TLRs [40]. Impaired T-cell signaling is also a significant contributor to SLE pathogenesis where overly activated T cells of lupus were able to stimulate autoantibody production from B cells, activate dendritic cells, and stimulate the release of inflammatory cytokines and can thus mediate widespread tissue injury and inflammation [4, 41].

2. Biomarkers

A biomarker can be defined as an alteration in a cellular, molecular, genetic, epigenetic, biochemical, biological, or other body events that specifically accompanies a disease or condition and is amenable for quantitative and qualitative analysis. Therefore, a biomarker can be used for diagnostic, prognostic, and theranostic purposes, and the more specific it is for a disease, the more reliable it becomes [5, 42]. In SLE, the search for novel and specific biomarkers is highly crucial because as a disease of a huge range of clinical and serological manifestations, it is challenging in so many levels including accurate diagnosis, predicting disease progression, identification of disease flares, directing proper therapy, and for the discovery of new treatments [5]. Owing to this tremendous heterogeneity, it is therefore expected that no single biomarker for SLE can satisfy all the above purposes but the continuous efforts in understanding SLE pathogenesis should accumulate informative data for the discovery of novel and reliable biomarkers [43].

The most commonly employed SLE biomarkers are antinuclear antibodies (ANAs) and anti-DNA antibodies [43]. However, as will be described below, ANAs possess low specificity to SLE due to their coexistence in other autoimmune diseases as well as in some healthy individuals [44]. On the other hand, anti-DNA antibodies, despite being the most specific biomarkers for SLE [27], have poor predictive values as their levels do not always parallel disease activity [45]. Therefore, the search for novel biomarkers for SLE never ceased, and with the emergence of newer detection technologies and the advances achieved in understanding SLE pathogenesis, new biomarkers have emerged from collective efforts of genetic, epigenetic, transcriptomic, and proteomic studies as shall be described [46].

2.1. Antinuclear antibodies (ANAs)

ANAs are a large group of autoantibodies that target various nuclear antigens including DNA, RNA, proteins, or complexes of nucleic acid and proteins [44]. They can generally be categorized into two groups based on the targeted antigens, one group that recognizes DNA and DNA-associated proteins such as histones or DNA-protein complexes such as nucleosomes and another group that recognizes distinct proteins that exist in association with RNA and are thus called RNA-binding proteins (RBPs) including the small nuclear ribonucleoproteins (snRBPs) Sm, RNP, Ro, and La [47].

These nuclear antigens are normally enclosed within the nucleus. However, as described above, upon cell death, these antigens are released into the extracellular space where they elicit immune responses that lead to the generation of ANAs that target these antigens and form immune complexes that further stimulate the immune system [4]. In the context of SLE, ANA detection in patients' sera is an important diagnostic criterion where they are specified as a stand-alone criterion in SLE classification criteria that are used for SLE diagnosis [48–50] as will be described in more details in the next section.

Although, ANA positivity is detected in not less than 95% of SLE patients [44, 51], they exhibit low specificity to SLE as they are also detected in other autoimmune diseases including rheumatoid arthritis, Sjogren's syndrome, and mixed connective tissue disease [51, 52]. Furthermore, depending on the employed detection method, ANA positivity can be seen in 20–30% of healthy individuals in the general population for yet unknown reasons [44]. Therefore, the value of ANA testing for SLE diagnosis is a debatable issue because of this diminished specificity despite of its inclusion in SLE classification criteria [53].

2.2. Anti-DNA antibodies

Anti-DNA antibodies are a subgroup of ANAs that can recognize and bind cellular DNA, and their detection is almost exclusive to SLE making them the serological hallmark for the disease [27, 51, 54]. Whether anti-DNA antibodies are also released in the context of other human diseases is a question that remains unanswered [27]. Detection of anti-DNA antibodies in the sera of SLE patients has been included as a separate criterion in an optimized version of SLE classification criteria that were set to enhance the sensitivity of the old criteria, as will be described below, which highlights their importance for SLE diagnosis [50] as they exhibit 95%

specificity for SLE and are detected in at least 70% of SLE patients [45]. In addition, their value is not confined to their diagnostic role, but they are also quite valuable in probing the molecular basis of lupus autoreactivity [27] and in theranostic investigations where they can be used as deterministic factors for eligibility in clinical trials and in directing the clinical use of certain therapeutics [55, 56]. In normal immune responses, antibodies that target DNA can be seen in some cases such as that in response to viral or bacterial infections [54, 57]. However, anti-DNA antibodies in normal immunity differ from SLE anti-DNA antibodies in many aspects. In normal immunity, anti-DNA antibodies are of the IgM isotype that can recognize and bind single-stranded DNA (ssDNA) with low affinity and are nonpathogenic in nature. But in SLE pathogenesis, there is an isotype shift of the expressed anti-DNA antibodies from IgM to IgG antibodies which are detected in the majority of SLE patients. These class-switched IgG antibodies exhibit a high affinity toward double-stranded DNA that resulted from specific somatic mutations in the variable regions of these antibodies mostly in the complementarity-determining regions (CDRs) that generated positively charged amino acid residues such as asparagine, arginine, and lysine that promote enhanced binding affinity to the negatively charged DNA [27, 54].

The origin of such IgG anti-DNA antibodies was suggested to derive from antigen-specific B-cell clonal expansion where DNA was used as the selecting antigen [54]. However, the various studies that investigated the release of high-affinity anti-DNA antibodies in SLE settled on the prerequisite association of DNA with proteins to be able to elicit anti-DNA immune responses [54, 58]. This implied a role for T cells that are reactive against histones, the proteins constituting octamer complexes around which stretches of DNA are wrapped inside the nucleus, and nucleosomes which are the basic structural unit of chromatin [27, 54]. A mechanism was proposed to start with presenting DNA in complexation with a foreign protein antigen (e.g., viral or bacterial) to T cells specific to this antigen. However, in a way that is not yet fully characterized, a shift in recognition takes place toward the complexed DNA that triggers the activation of T cells specific to histones and nucleosomes [27, 54].

Various studies have demonstrated differences in selectivity patterns exhibited between anti-DNA antibodies of normal individuals and that of SLE patients. Normal anti-DNA antibodies were found to exhibit high specificity toward species-specific DNA which suggests that they bind at DNA regions that are not shared with human DNA. In contrast, SLE anti-DNA antibodies were found to nonselectively bind to a variety of investigated structurally different DNA antigens. This suggests that SLE anti-DNA antibodies might specifically bind the highly conserved phosphate backbone rather than specific nucleotide sequence [27, 54].

Therefore, as will be described later in the section of diagnostic technologies, assays that are able to detect high-affinity anti-DNA antibodies are described as assays with high specificity to SLE as it is inferred that such high-affinity anti-DNA antibodies are more reflective of SLE immune-deregulated responses [27].

Despite this high specificity of anti-DNA antibodies to SLE, their value in disease prognosis is compromised as they are poor predictors of disease activity [45]. This was demonstrated by the detection of tenacious levels of anti-DNA antibodies in SLE patients in remission [59–61] or

the presence of normal levels in patients with active disease [62]. Therefore, new biomarkers are continuously emerging, and some of the promising biomarkers are discussed below. For more detailed discussions, interested readers can be directed to these reviews [43, 46].

2.3. New spectrum of SLE biomarkers

2.3.1. Epigenetic biomarkers

Epigenetic biomarkers refer to the epigenetic changes that govern gene expression without changing the nucleotide sequence of the DNA and are specifically associated with disease development such as DNA methylation pattern, microRNA expression, and various histone modifications [5]. In the case of SLE, certain epigenetic changes are detected in SLE patients and can act as biomarkers such as the widespread DNA hypomethylation pattern in CD4 T cells [63, 64] and DNA hypomethylation of the promoters of certain genes that encode immune mediators and are associated with SLE pathogenesis such as CD40L [65], CD70 [66], perforin [67], IL-10, and IL-13 [68, 69]. With reduced DNA methylation which normally acts as a repressive signal, the affected genes become highly activated and consequently activate T and B immune cells [5].

Another potential epigenetic biomarker for SLE is the aberrant changes in histone proteins modification patterns that are normally quintessential for controlling gene expression [70, 71]. Specific changes in histone modifications have been observed in immune cells of SLE patients [5] such as the widespread hypomethylation of lysine 9 residues in H3 histone protein in CD4 T cells [72].

In addition to histone modifications, changes in miRNA expression profile are promising SLE biomarkers [5]. MiRNAs are short noncoding RNA sequences that regulate gene expression via targeting and inhibiting mRNA transcripts [73]. It was recently discovered that miRNAs play important roles in both innate and adaptive immune systems and are involved in the pathogenesis of several autoimmune diseases including SLE [74–77]. Several studies have aimed at profiling miRNA expression patterns in SLE and have identified several miRNAs that were underexpressed in CD4 T cells isolated from SLE patients such as miRNA-146a [78, 79] and miRNA-125a [80]. Reduced levels of these miRNAs were inversely correlated with SLE activity as they were associated with increased activation of type I interferon and inflammatory chemokines, respectively.

2.3.2. Cytokines as potential biomarkers

As mentioned earlier with SLE pathophysiology, type I interferons and other cytokines exhibit exacerbated activities in SLE and are key players in disease activity [1, 4]. Therefore, such mediators represent potential biomarkers and well-validated therapeutic targets in SLE.

Cytokines with the most characterized roles in SLE pathology are those of type I interferon pathway such as IFN-α and downstream-induced gene products. Aberrantly elevated levels of INF-α have been detected in cerebrospinal fluid of patients with neuropsychiatric disease in systemic lupus erythematosus in comparison to cerebrospinal fluid samples obtained from

patients with other autoimmune diseases [81] and were suggested to contribute to disease pathogenicity [82].

Using DNA microarray technology, the expression pattern of IFN-induced genes was found to be highly elevated in peripheral blood mononuclear cells obtained from SLE patients with severe disease state [83]. Positive correlation between IFN-induced genes and severity of SLE clinical manifestations and multiple organ damage was also observed in other studies [84, 85].

Among the most noticeable gene products that are regulated by type I interferons are IP-10 and sialic acid-binding Ig-like lectin 1 which were found to be associated with SLE pathogenicity and were detected in highly elevated levels in many SLE patients [86, 87].

Many other cytokines have been detected in elevated levels in the sera of many SLE patients and can thus represent potential biomarkers including IL-17 [88], IL-6, IL-10, IL-12, IL-15, and IL-21 and others [5, 43].

3. Diagnosis

Diagnosis of SLE, according to the majority of clinicians, is best established upon combinatorial approach. Thus, a combination of patients' clinical manifestations and laboratory investigations that can comprise autoantibody assays, blood tests, cell cultures, certain functional tests such as echocardiogram, or imaging such as neuroimaging must be adopted [1, 51, 89]. The decision upon any of the aforementioned investigations is guided by the patient's clinical presentations [51]. In addition, other important factors must be examined including patient's history, risk factors, SLE prevalence within the patient's demographic population, and other epidemiological data. Clinical expertise is also crucial as a highly heterogeneous disease such as SLE can be easily missed or misdiagnosed [1, 51, 89]. Some clinical manifestations are strongly associated with SLE and are therefore of high diagnostic significance such as alopecia, leukopenia, neurological involvement, oral ulcers, and serositis [51].

Nevertheless, until now no diagnostic criteria have been established for SLE due to extreme disease heterogeneity where no consistent clinical presentation or degree of disease severity appears within the cohort of SLE patients [51, 89]. Therefore, clinicians had to refer to a sort of guidelines to aid proper diagnosis and relied on SLE classification criteria such as the revised American College of Rheumatology (ACR) classification criteria for SLE for clinical diagnosis [48, 49].

The ACR classification encompasses a total of 11 criteria for defining SLE including an individual criterion for abnormally elevated titers of antinuclear antibodies that are detected by immunofluorescence immunoassay, which will be described later, or any other equivalent assay and another criterion specifying immunologic abnormalities that include the release of aberrant titers of anti-DNA antibodies or anti-Sm antibodies [48]. Based on ACR, at least 4 of the 11 criteria must be met for a patient to be classified with SLE disease [48]. However, ACR

criteria are more suited for severe cases of SLE and not patients with mild or moderate conditions as it includes the most pronounced manifestations and excludes some of the clinical presentations shown by patients at early or mid-stages which are considered important. Clinical presentations need to be taken in consideration as SLE is known to be a progressive disease that tends to exacerbate over time and establish many of the clinical and serological manifestations in an accumulating manner [1, 90]. Therefore, the ACR classification criteria have a specificity reaching 96% but with a suboptimal sensitivity of 83% [89]. Specificity here is defined as the percentage of individuals who are known to be devoid of the disease and test negative for it, while sensitivity refers to the percentage of patients who are known to have the disease and test positive for it [91]. With the aim of overcoming the limitations of the ACR classification, the Systemic Lupus International Collaborating Clinics (SLICC) classification criteria have been set in 2012 to encompass many of the clinical manifestations overlooked by ACR to reach a total of 17 criteria instead of 11 where at least 4 out of the 17 criteria must be met for a patient to be classified with SLE [50]. In SLICC, the antinuclear antibody criterion was not changed. However, the immunologic abnormalities criterion in ACR has been separated into individual criteria including a separate criterion for abnormal titers of anti-DNA antibodies but with a more strict cutoff value and a separate criterion for anti-Sm antibodies highlighting the importance of such autoantibodies in the diagnosis of SLE [50]. Nevertheless, despite an increase in sensitivity to reach 97% in SLICC compared to 83% in ACR, the specificity has been reduced to 84% compared to 96% in ACR [50]. Moreover, SLICC criteria did not improve the inclusion of SLE patients at early stages except for patients with renal nephritis damage [92].

However, it should be noted that relying on classification criteria for diagnosis is actually problematic as both disease classification and diagnosis do not generally share the ultimate aim [89, 91]. Diagnosis aims at identifying a patient's illness in terms of its causes and nature and is based on a set of diagnostic criteria that include a number of clinical symptoms and investigations that are used routinely for guiding the clinical care of patients [91]. Therefore, diagnostic criteria should have nearly 100% specificity and sensitivity [89, 91]. On the other hand, classification criteria are basically established to define a total population of patients having a specific disease that can be recruited for clinical research. They, therefore, encompass the most prominent and prevalent manifestations dropping out the rarer or less common symptoms and thus typically enjoy high specificity but at the expense of sensitivity. Consequently, using classification criteria for diagnosis can easily miss or overlook patients with the disease [1, 89, 91].

With the emergence of new biomarkers that are strongly associated with SLE pathogenesis as described above, it is highly suggested that these biomarkers will reserve their places in future developments and optimization in diagnostic criteria next to autoantibodies which will not only aid accurate diagnosis but will significantly guide patients' clinical care and management. The increasingly accumulating data in SLE biomarkers will need to be paralleled with the development of new sensitive and reliable detection technologies that are able to simultaneously detect disease biomarkers in a rapid, cost-effective, and sensitive manner [93]. In the next section, current and new trends in diagnostic technologies for SLE will be discussed.

4. Diagnostic technologies

Abnormally elevated titers of antinuclear antibodies are one of the important factors involved in SLE pathogenesis [4] and are set as stand-alone criterion in both ACR and SLICC classification criteria used for SLE diagnosis [48, 50]. Out of these antinuclear antibodies, antibodies that target double-stranded DNA are highly specific to SLE and are considered the serological hallmark for SLE [27]. Numerous technologies have been described for the detection of such autoantibodies in the sera, plasma, or other body fluids of SLE patients where some of them date back to the 1950s, and until now various state-of-the-art technologies are being described.

In principle, all assays assess the formation of immune complexes between the autoantibodies and the test antigens which can comprise isolated DNA, complex nuclear mixtures, or an array of purified, recombinant, or synthetic proteins or peptides [27, 44]. These assays, however, differ in many factors including the source of the antigenic substrate, the presentation of such substrates to the autoantibodies where they could be immobilized on solid surfaces or move freely in solution, the methodology encountered for detecting binding and the employed reaction conditions [27, 54]. The variations among the detection assays happened to give conflicting results for the same patient sample where one assay gave positive detection and another had it negative [94–96]. The most important causation of such result variability amongst the different assays is the affinity of autoantibodies towards the test antigen which is highly influenced by the reaction conditions. For example, some assays, as will be described shortly, favor the recovery of low-affinity antibodies such as ELISA, while others assays favor the recovery of high-affinity antibodies such as the Farr assay [27, 97].

Another important aspect with regards to the conflicting results obtained from the distinct assays is the diffidence in the cutoff values set by different assay for considering autoantibody levels detected significant making data from different assays difficult to compare [27]. This confusion actually roots from the vague definition of antinuclear or anti-DNA antibody positivity described in SLICC [50]. The criteria state that an anti-DNA antibody test result is considered positive if it is higher than the laboratory's reference range except for ELISA in which a test result is only positive if it is two times or more the value of the laboratory reference range [27]. That said, the criteria did not define specific tests and did not refer to accepted levels for assay sensitivity or specificity [50, 98].

These discrepancies add to the confusion of diagnosticians who already face many challenges with SLE diagnosis and urge the need for standardization among the different assays. Simplicity, time, and costs are important factors for an assay to be chosen for routine clinical use in laboratories [27].

Amid all the available assays and newly developed technologies that are described for detecting anti-DNA antibodies as the most prominent biomarkers for SLE, an important question is issued of which assay to choose and is there a gold standard to refer to? It has been reported that assays that can specifically detect high-affinity antibodies as being reflective of mature immune responses are preferred by many investigators such as the Farr assay or CLIFT [27, 54]. However, the contribution of high-affinity and low-affinity anti-DNA antibodies to

SLE pathogenesis is not yet known, and therefore looking for other biomarkers that can arise from the multiple pathways contributing to SLE pathogenicity is highly needed to complement the value of anti-DNA antibodies in diagnosis and prognosis of SLE [27, 54].

In this section, the most prominent techniques employed for the detection of antinuclear antibodies and anti-DNA antibodies that have been described decades ago till today will be presented, in addition to some investment on the futuristic highly promising state-of-the-art technologies. However, before proceeding with the discussion of the different assays, a description of the DNA substrate antigenic properties and the factors that influence such antigenicity will be discussed as they are important dynamics in assaying anti-DNA antibodies.

4.1. DNA substrate

There are general characteristics of DNA that made it possible to employ DNA isolated from distinct sources including viral, bacterial, mammalian, and even flagella as the antigen substrates in multiple anti-DNA antibody detection assays [27, 54]. For instance, DNA is generally a highly charged polymeric molecule with repetitive charges that constitute an important factor of DNA epitopes that are targeted by anti-DNA antibodies. In addition, all double-stranded DNA isolated from natural origins predominantly exist in the B-form conformation rendering them recognizable by anti-DNA antibodies [27].

That said, there are some factors that can influence DNA antigenicity rendering it more or less antigenic and can thus consequently influence the performance of detection assays [94]. For instance, single-stranded DNA has a much more flexible structure than DNA double helix which tends to be more rigid, and thus targeted epitopes are more exposed in the single-stranded forms making it more antigenic [94]. Another factor that can influence DNA antigenicity is size. Anti-DNA antibodies bind DNA through a mechanism called monogamous bivalency in which both Fab fragments of the same antibody bind the same polynucleotide chain to ensure stable binding [27]. The distance between two Fab sites is 136 angstroms which corresponds to a stretch of 40 bp (**Figure 2**) [27]. Therefore, short oligonucleotides can be inefficient for binding anti-DNA antibodies, and longer DNA substrates can be essential for binding [27, 94]. Furthermore, certain isolated DNA such as that from *Crithidia luciliae* protozoal cells display a bent conformation resembling that of nucleosomes allowing binding only a subset of autoantibodies [27].

4.2. Immunofluorescence assay (IFA)

IFA (also known as indirect IFA) is an assay that dates back to the 1960s that was developed with the aim of providing sensitive and reliable means for the detection of ANAs [99]. As mentioned earlier, ANA positivity is one of the classification criteria described for SLE diagnosis, and IFA was mentioned by name in the ACR classification for detecting ANA [48]. Moreover, it is considered the gold standard for ANA testing [44, 48, 49, 90]. IFA involves fixation of culture cells on a slide to serve as the source of antigens targeted by ANAs, and

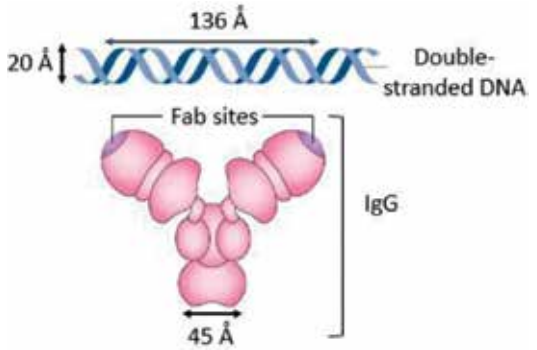

Figure 2. Monogamous bivalency binding of anti-DNA antibodies to DNA. Anti-DNA antibodies bind to the DNA via a mechanism known as monogamous bivalency in which both Fab sites bind the same stretch of polynucleotides. The distance between two Fab sites is 136 angstroms which corresponds to 40 base pairs. Accordingly, DNA fragments of shorter sizes are not efficient as substrates in anti-DNA antibody assays [27].

HEp-2 cells are currently employed for this purpose as they express a wide spectrum of antigens (**Figure 3**) [44]. Fixed cells are then incubated with serial dilutions of the patient's serum or plasma to promote the formation of immune complexes which is then followed by washing off unbound antibodies. The formed immune complexes are then detected by adding an anti-IgG antibody that is conjugated with a fluorescent agent and are visualized by fluorescence microscopy [44]. Positivity is assigned through determining the endpoint titer which is defined as the reciprocal of the last dilution that gave a fluorescence signal above the cutoff value [44, 100]. The specificity of binding can be inferred from examining the staining pattern which reflects the relative location of the antigen [44].

However, IFA is challenged by certain limitations that can compromise its specificity. First, whole cells are used as the source of test antigens which leads to the detection of other

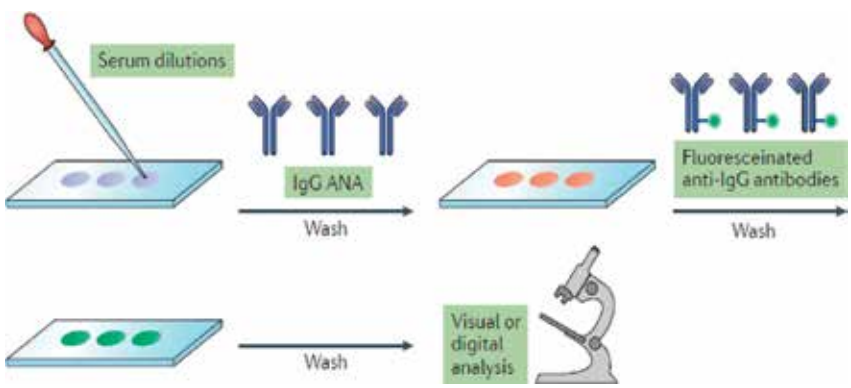

Figure 3. Immunofluorescence assay (IFA). IFA starts by fixation of culture cells on a glass slide which serves in providing the nuclear antigens that are targeted by ANAs in the patient's serum. Next, different dilutions of the patient's serum are incubated with the cells and are followed by a washing step to remove unbound antibodies. Immune complexes are detected by adding fluorescently labeled anti-IgG antibodies which are followed by a second washing step to remove unbound anti-IgG antibodies. Immune complexes are then visualized and analyzed through fluorescence microscopy [44].

antibodies targeting cytoplasmic and mitotic antigens that complicate the analysis [44]. Furthermore, certain antigens can be underexpressed in these cells which limit the detection of certain autoantibodies [101]. Moreover, IFA execution is subject to variability in experimental conditions including cell fixation protocols, concentrations of assayed cells, different assays of the commercially available kits, and the specificity of the anti-IgG antibodies [44]. Therefore, many efforts have been devoted at formulating other assays that can detect ANAs in a more robust and less technically demanding fashion and capable of high-throughput screening such as enzyme-linked immunosorbent assay (ELISA).

4.3. Enzyme-linked immunosorbent assay (ELISA)

ELISA is one of the most renowned techniques that enjoy a high versatility that allowed its use in a variety of biomedical applications including the detection of ANAs and anti-DNA antibodies in patients with SLE and other autoimmune diseases [102–107]. ELISA is based on coating a solid surface with the antigens of interest such as DNA substrates or an array of nuclear antigens so that they are tightly bound and can withstand subsequent washing steps. After immobilization, the test sample containing autoantibodies such as a patient's serum is added to promote binding of autoantibodies with their respective antigens, and the formed immune complexes can be detected through the addition of an anti-IgG antibody that is either conjugated with a peroxidase or alkaline phosphatase enzyme or a fluorophore [44, 94].

ELISA has high sensitivity and can detect a wide spectrum of antibodies owing to the efficient exposure of surface-immobilized DNA substrates making them readily available in high concentrations for binding and can thus capture both high- and low-affinity antibodies [27, 44, 94]. Therefore, ELISA can be a good choice for initial screening [27]. Moreover, it is easy to perform and allows quick, quantitative, and high-throughput analysis of autoantibodies [44]. However, ELISA mediates the recovery of low-affinity anti-DNA antibodies which compromises its specificity to SLE [54]. This can be due to a variety of reasons that can relate to the reaction conditions and the structure and source of the DNA substrate [54]. In addition, the increased sensitivity of ELISA leads to the generation of false-positive results due to cross-reactivity [27, 44].

4.4. Farr radioimmunoassay assay

Farr assay has been first introduced in 1968 for the detection of anti-DNA antibodies [108], and until now it is preferred by many clinicians for assaying anti-DNA antibodies as it mediates the selective recovery of high-affinity antibodies which have been described to be reflective of mature immune responses [27]. The principle of the Farr assay is based on the incubation of a solution of radiolabeled DNA such as ^{14}C-DNA with patient's serum sample to promote the formation of immune complexes between anti-DNA antibodies and the DNA substrate. After incubation, immune complexes are precipitated with a saturated solution of ammonium sulfate, and the fraction of the initial radiolabeled DNA that has precipitated with autoantibodies is used to indicate the amount of anti-DNA antibodies in the serum sample [109]. With the ability to only recover high-affinity antibodies, Farr assay exhibits high specificity for SLE, but consequently its sensitivity is not the best when compared with other assays such as ELISA

that is able to detect both high- and low-affinity antibodies [27]. In addition, because of the hazardous radioactive material used in DNA labeling and the troublesome associated with its disposal, researches aimed at the development and optimization of other assays including ELISA which at the time suffered inconsistencies with the results owing the lack of standardization protocols with antigen immobilization [93]. However, as will be discussed below, ELISA has been eventually optimized and became one of the popular biomedical techniques in assaying autoantibodies [44].

4.5. PEG precipitation assay

In this assay, the same principle of the Farr assay is applied where a solution of radiolabeled DNA is used as the test antigen for anti-DNA antibodies in the serum sample. However, instead of using ammonium sulfate for precipitation, polyethylene glycol is used as the precipitating agent, which mainly leads to the recovery of low-affinity anti-DNA antibodies in contrast to the Farr assay [110].

4.6. Crithidia luciliae immunofluorescence test (CLIFT)

CLIFT is an assay that was first introduced in 1975 [111], and it is similar to the IFA assay described above except for the source of the used antigenic substrate. The assay employs *Crithidia luciliae* protozoal cells as the source of antigenic DNA substrate as they possess a giant mitochondrion called kinetoplast that contains a giant mass of mitochondrial DNA. The kinetoplast was considered as a good substrate for the detection of anti-DNA autoantibodies because it is unlikely to be associated with nuclear antigens and can thus serve as a source of naked double-stranded DNA [111]. The assay proceeds exactly as IFA where the *Crithidia luciliae* cells are fixed on a glass slide and a series of dilutions of the patient's serum is incubated with the cells and detection is mediated through the addition of fluorescently labeled anti-IgG antibodies. CLIFT has been described to be highly specific to SLE similar to the Farr assay [97]. However, DNA of *Crithidia luciliae* was described to have a bent conformation similar to nucleosomal DNA which can result in the recovery of only a subset of anti-DNA antibodies, and thus the assay was described to have a low sensitivity [27, 97].

4.7. Multiplex assays

Multiplex assays refer to the technologies that permit the simultaneous profiling of a repertoire of antigens in just a single test [112]. In the context of SLE where patients can express as many as 200 distinct antibodies targeting multiple antigens [44], such multiplex technologies provide concomitant determination of antibody specificities in a high-throughput, rapid, and cost-effective manner and can be highly advantageous in the discovery of novel biomarkers and monitoring disease activity [52, 93, 112]. Multiplex assays involve different settings in which some of them are already implicated in clinical use such as LINE immunoassays and microbeads assays, while other newer multiplex technologies are also rapidly evolving such as microfluidics and nanobarcodes [93, 112].

4.7.1. LINE immunoassays (LIA)

In LIA, selected distinct antigens that can be synthetic, recombinant, or purified proteins or peptides are immobilized onto a nylon membrane or other protein-binding surfaces. Individual antigens are attached in parallel "lines" where each line represents a specific antigen. These lines are then cut from the membrane forming thin strips. The same serum sample is then added to each individual strip to identify all autoantibodies reactive against the panel of strips. The formed immune complexes are detected through the addition of a secondary antibody that is conjugated to an enzyme or a fluorescent label. Therefore, LIA allows the simultaneous detection of multiple autoantibodies in the same patient sample [44, 112].

4.7.2. Addressable laser bead immunoassays

Addressable laser bead immunoassays (**Figure 4**) [112] are based on coupling distinct antigens (up to 100) onto microbeads that come in multiple laser reactive colors where each antigen is coupled to a specific color of beads creating an "addressable" color code that is used for the identification of each antigen [93]. After antigen coupling, all beads are stabilized, collected into a microtiter well, and incubated with the sample, and the formed immune complexes are detected by a fluorescently tagged secondary antibody. Samples are then analyzed using a dual laser system that utilizes flow cytometry and digital signal processing. Each bead is hit with two laser beams where one detects the specific color code or address of the microbead that is used to identify the specific antigen, and the other laser beam detects and quantifies the fluorescence signal coming from the secondary antibody. The generated data provides quantitative and qualitative analysis of each autoantibody [93].

Both LIA and addressable laser bead immunoassays use defined sets of identified antigens which allows the detection of specific autoantibodies associated with a certain disease such as anti-DNA antibodies in SLE which is of high diagnostic value and can thus overcome one of the main limitations of IFA and ELISA in which assigning absolute specificities to an

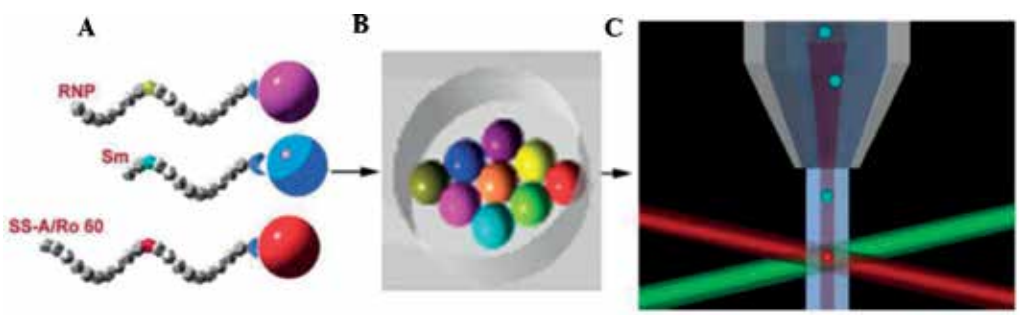

Figure 4. Addressable laser bead immunoassay (ALBIA). (A) ALBIA involves coupling of each individual antigen onto microbeads of laser active color so that antigens can be "addressable" by the color of the beads. (B) After coupling, all beads are combined into the microtiter well where they are incubated with the test sample such as patient's serum sample to promote formation of immune complexes which are detected by a secondary antibody that is fluorescently labeled. (C) Autoantibody-antigen complexes that are coupled to the beads are analyzed using a dual laser system where one detects the specific color code of the microbead and can thus be used to identify the specific antigen and the other laser beam detects and quantifies the fluorescence signal coming from the bound secondary antibody [112].

ANA-positive result is not possible. Furthermore, these assays are less time-consuming, are amenable for automation, and allow high-throughput analysis. However, this specificity stemming from the use of defined set of antigens can come at the expense of sensitivity where many autoantibodies can be missed. For example, SLE patients can release up to 200 different autoantibodies where only few of them are detected [113].

4.7.3. Autoantigen microarrays

Autoantigen microarrays are high-throughput assays that allow simultaneous detection of different autoantibodies in autoimmune diseases including SLE (**Figure 5**) [52]. The technique involves printing a distinct array of antigens into a surface that is coated with substrate that becomes covalently attached to the antigens. Printed antigens on the surface of microarrays are performed in a way that retains their reactivity with other molecules. In SLE, the array of antigens can include nuclear antigens including RNA and DNA and associated proteins, other cellular proteins, and specific targeted epitopes where the immobilized antigens can be purified or recombinant proteins or synthetic peptides. Different samples can be screened including serum, plasma, or other biological fluids such as cerebrospinal fluids or saliva for simultaneous detection of autoantibodies. Identification of immune complexes is mediated through the addition of secondary antihuman antibodies that are fluorescently labeled. Distinct isotypes of

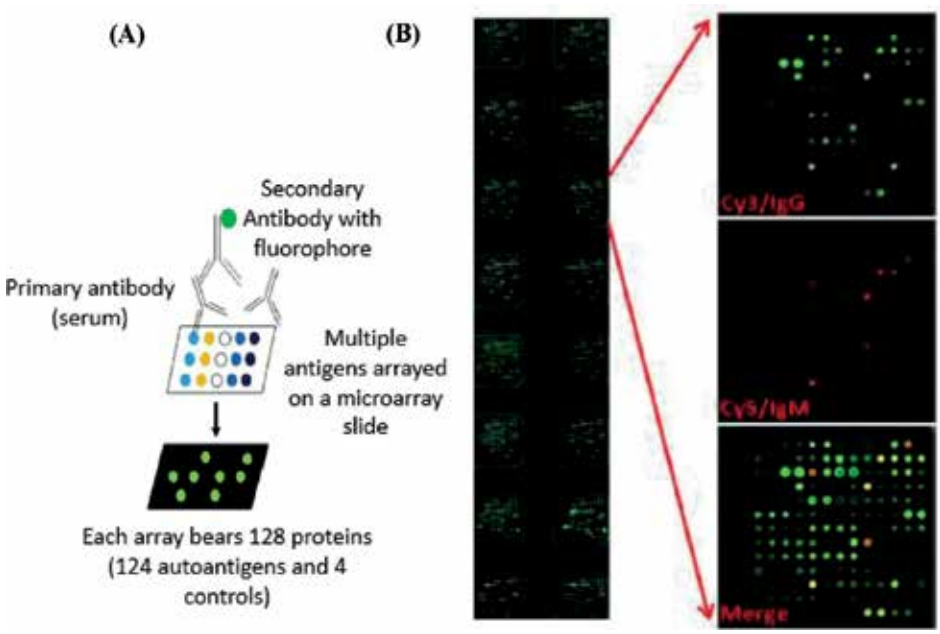

Figure 5. Simultaneous detection of autoantibodies using autoantigen microarrays. (A) Multiple autoantigens are printed onto a microarray surface and are incubated with patients' serum sample that contains autoantibodies. The formed immune complexes are identified through the addition of secondary antibodies that are labeled with a fluorophore so that each spot with a positive reaction produces a fluorescent signal. (B) Different isotypes of autoantibodies such as IgG and IgM can be visualized using secondary anti-IgG and anti-IgM that are labeled with differently colored fluorophores such as Cy5 and Cy3. Merging the images will show the different isotypes in the same sample [52].

autoantibodies such as IgG, IgM, or others can be specified by using secondary antibodies that are conjugated to different colored fluorophores. Autoantigen microarrays are not only useful in showing the differential detection of autoantibodies, but specific associations of certain autoantibodies with certain SLE manifestations can be made in addition to the identification of novel biomarkers. However, microarray chips can be challenging to be produced in consistent and reproducible manner for commercialization.

4.7.4. Microfluidics and nanobarcodes

With the aim of developing assays that are highly amenable for automation and point-of-care usage with the ability to concomitantly detect many analytes that are prepared in minute volumes with very high sensitivity, technologies such as microfluidics and nanobarcodes are implemented as bioassays for many molecules including the detection of autoantibodies in autoimmune diseases [112, 114, 115]. These assays are not yet implemented in clinical use, but they are highly promising as near-future diagnostics.

Microfluidics (also known as lab-on-a-chip microtechnologies) from its name is a technology that involves the analysis of ultra-low amounts of sample that are applied into specific devices that are fabricated to be in tens to hundreds of micrometers in dimensions [112]. These devices can be made in different configurations such as channels, pumps, pipes, or valves. The technology of microfluidics makes use of the flow characteristics of fluids in such micro-sized channels such as laminar flow and increased surface tension and capillary forces to move the sample through the microdevice [116]. In the detection of autoantibodies, the walls of such microdevices are coated with an array of antigens, and sample containing autoantibodies is applied to flow through the microdevice where immobilized antigens catch their respective antigens. A washing step is then applied to remove unbound antibodies followed by the application of a labeled secondary antibody, and signal can be detected by specific detectors [112, 114].

Nanobarcodes fall under the bigger science of nanotechnology in which systems, devices, or materials are fabricated in the nano-range to render them new and enhanced properties [117]. Unlike microfluidics that are fabricated at the micron scale, nanobarcodes are made even smaller to the nanoscale [117]. In the detection of autoantibodies, nanobarcodes consist of different stripes where each stripe is composed of a different metal such as gold, silver, platinum, or nickel that are electroplated into templates [115]. Different antigens are coupled to each metal stripe which are then incubated with the serum sample allowing the binding of each autoantibody to its respective coupled antigen. Detection of immune complexes is achieved via the addition of secondary antibody that is fluorescently labeled, and different antigens can be identified by fluorescence microscopy through the differential reflectivity of each stripe creating a pattern that resembles that of a barcode [112, 115, 117].

Microfluidics and nanobarcodes offer many advantages including the application of minute amounts of the sample and reagents which reduce costs and minimize chemical waste. Moreover, these devices are amenable to complete automation and maybe well applied as point-of-care diagnostics without the need of specialized labs and technical skills. In addition, they produce data that allow simultaneous comparison of the different analytes and at the same time reliable due to the inclusion of multiple internal controls [112, 115, 116].

5. Personalized therapy in SLE

SLE is actually a highly representative model for diseases that are in crucial need for personalized therapies as it is one of the highly heterogeneous and complex human diseases with chaotic pathogenesis [1, 4]. Although under the same disease umbrella, SLE patients are not homogenous cohorts that can be classified, treated, or managed equally as they show marked discrepancies in their responses to the same treatment, manifestations of disease severity, type and levels of circulating biomarkers, organ involvement, and the underlying pathogenic mechanisms that are highly influenced by genetic, environmental, and other risk factors [1, 118]. Currently, SLE patients are routinely treated with potent immunosuppressive agents that can cause adverse side effects which tend to be even more aggressive than the disease itself [41]. With the aim of achieving optimum management of SLE patients, it is therefore very wise to stratify these patients into subsets that share common pathogenic pathways which can be best accommodated with targeted or *personalized* therapeutic approaches that do not only increase treatment efficacy but also present safer alternatives to the nonselective immune-toxic steroids that are currently employed for the management of SLE patients [3, 118].

Recent gains in understanding SLE immunopathology have exposed certain deregulated immune trends that are now known to be common in subsets of SLE patients where some of these trends are now well characterized and paved the way for the exposure of various targets that are highly promising in personalized therapeutic approaches (**Figure 6**) [3]. Over the past 10 years, various medications made their way through preclinical and early clinical testing for the treatment of SLE patients, but unfortunately almost none of them was met with success during the later stages of clinical trials. This can be attributed to the highly heterogeneous nature of SLE patients which make study designs for clinical trials a very difficult mission [119, 120].

In this section of the chapter, highly promising therapeutic targets for specific subsets of SLE patients and recent therapeutic developments that hold a great potential in personalized medicine targeting specific cohorts of SLE patients will be discussed.

5.1. New potential therapeutic targets in SLE

As previously learnt, type I IFNs including IFN-α are greatly implicated in SLE pathogenesis and mediate a variety of downstream-deregulated immune responses including the release of autoantibodies [4]. However, highly elevated levels of type I INFs are only found in 40–50% of SLE patients constituting a subset of patients that can be particularly responsive to therapies targeting type I INFs and other mediators implicated in their pathway such as TLRs and IFN receptors [118, 121, 122]. One such agent is the anti-IFN-α monoclonal antibody sifalimumab which showed promising clinical activity in phase I and phase II clinical trials with high tolerance and safety profile [3, 123]. Other agents include the monoclonal anti-IFN-α antibodies rontalizumab [124] and AGS-009 which have finished phase II and phase I studies, respectively, with promising clinical results [3].

Another promising target is BAFF cytokine (also called BLyS) which is essential for B-cell maturation and function [4]. Differential circulating levels of this cytokine have been observed among SLE patients of different ethnic backgrounds where it was particularly elevated in

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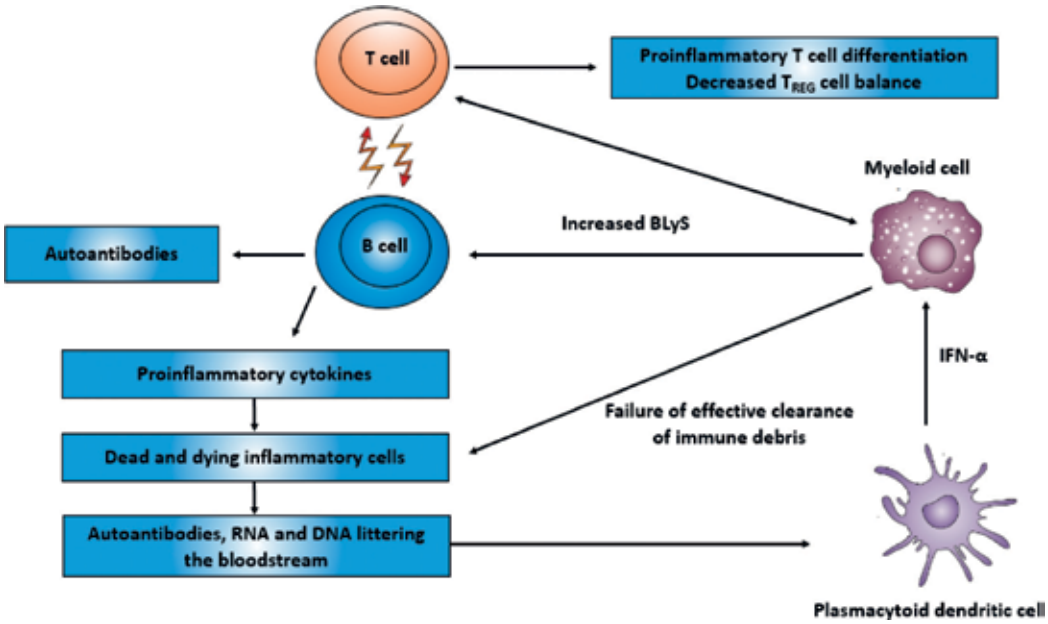

Figure 6. Several therapeutic approaches target distinct immune modulators in the pathogenesis of systemic lupus erythematosus. Deregulated immune cascade in SLE involves activation of TLRs in plasmacytoid dendritic cells mediated by internalized nucleic acids that are released from dead cells. Activated TLR induces potent release of type I INF such as INF-α which activates myeloid cells that act to release elevated amounts of the B-cell activator factor BAFF (also called BLyS). BLyS stimulates B-cell maturation and release of autoantibodies. Further activation of B cells is mediated via B-cell and T-cell interactions that can ultimately lead to loss of immune tolerance. Several agents have been described for targeting key players in the above immune cascade where some have paved their way in clinical use; others are still under clinical investigations, while some were not met with clinical successes [3].

patients of African background in comparison to patients of European background [125]. One of the agents that has gained approval in many countries for the treatment of SLE patients is the anti-BAFF monoclonal antibody belimumab [3] that has shown considerable efficacy and tolerability in randomized placebo-controlled phase III clinical trials in SLE patients against a control group [55, 126]. Many other agents that target BAFF are also still under clinical investigations and have shown promising results including blisibimod which has currently passed phase II clinical trials for SLE [3, 127]. Another agent that target B cells and showed highly encouraging early results for the treatment of SLE but has unfortunately failed in advanced stages of clinical trials is the chimeric antibody Rituximab [3]. Rituximab showed highly promising results in reducing SLE activity particularly in patients with lupus nephritis [128, 129] and has been largely prescribed to SLE patients all over the world with a decision that was mainly based on clinical experience and open-label studies [3]. However, in randomized placebo-controlled clinical trials, rituximab failed to provide efficacy in moderate to severe SLE patients with and without renal nephritis [130, 131]. Nevertheless, these results could be potentially misleading owing to some issues in the study design as it was shown that both test and control groups were receiving strong immunosuppressive agents including high doses of glucocorticoids [3, 118]. Nevertheless, rituximab is still prescribed by some clinicians, which also suggests the probability that this treatment works best at certain subsets of lupus patients, and further investigations should be implemented [118].

Finally, T cells and associated stimulatory pathways play a key role in the deregulated immune cascade in SLE pathogenesis [1, 4] and are thus highly promising therapeutic targets in SLE. Many attempts have been made in generating therapeutics targeting T cells including anti-CD40 ligand antibodies such as CDP7657 which is currently under clinical investigations in phase I study for SLE [3]. Other anti-T-cell approaches are being attempted including small molecule inhibitor drugs such as quinoline-3-carboxamide derivatives [132] and analogues of sphingosine-1-phosphate [133] which are still under clinical developments.

6. Conclusion

SLE is a chronic and highly progressive autoimmune disease that carries a high risk of early death [1–3]. The incidence and prevalence of SLE are highly influenced by many factors such as race, ethnicity, age, gender, and patients' demographics [6]. Although informative reporting on SLE epidemiology is inconsistent among the different countries, it is recognized as a global disease that faces so many clinical challenges in its diagnosis, prognosis, monitoring, and management [1]. The challenging nature of SLE originates from its chaotic immunopathology in which the affected stage in the immune cascade and the extent of deregulation are highly variable among SLE patients who as a consequence express a wide array of nonhomogeneous highly protean clinical and serological manifestations making them impossible to be recognized as a single cohort of patients that can be managed equally [1, 4]. Consequently, setting specific diagnostic criteria is very challenging, and until now no defined diagnostic guidelines have been established for SLE [51, 89]. However, as a reference, clinicians have used SLE classification criteria for diagnostic purposes such as the ACR or the more recent SLICC [48, 49, 92]. Nevertheless, using classification criteria for diagnosis is problematic because they are set to include the most prevalent manifestations of the disease that occur during late or severe stages and neglect many of the early or mid-stage symptoms making them highly specific but not sensitive [89, 91]. This cannot be well suited for SLE owing to its progressive nature where many of its pathologies accrue overtime and therefore referring to classification criteria for diagnosis can dismiss many SLE patients specifically those at the early stages of the disease [1, 89, 91]. Another challenge facing SLE is the insufficient availability of reliable and specific biomarkers which are highly needed for the highly heterogeneous nature of SLE where it is highly improbable that only a single biomarker can be indicative of the wide array of manifestations [5]. Therefore, the search for distinct and specific biomarkers that can accurately mediate early diagnosis, predict disease development and emergence of disease flares, monitor disease activity, indicate specific organ damage, and guide therapies, guide reliable inclusion in eligibility criteria for conducting clinical trials, and evaluate patients' responses to novel therapeutics is highly needed and never ceased [5, 43]. Conventionally, ANAs and anti-DNA antibodies are routinely used as biomarkers for SLE as they occur in at least 70 and 95% of SLE patients, respectively, and their detection is included in the classification criteria for SLE [48, 50, 51]. However, ANAs are highly unspecific for SLE as they are detected in other autoimmune diseases in addition to a not so small proportion of the general population [44]. Anti-DNA antibodies are currently the most specific biomarkers for SLE, and their detection outside SLE is not yet found [27]. Nevertheless, anti-DNA antibodies are poor predictors for SLE

activity as it happens that elevated levels of anti-DNA antibodies accompany patients in remission while normal levels accompany flared disease activity [45, 59, 61, 62]. Many promising novel biomarkers are emerging such as type I INFs which are highly elevated in a subset of SLE patients or some epigenetic biomarkers that are associated with SLE progression such as DNA methylation pattern, microRNA expression, and various histone modifications [5]. However, efforts devoted to overcome the abovementioned challenges of SLE along those employed at the discovery of novel biomarkers will not be possible without being rivaled with developments in state-of-the-art technologies that can accurately detect and monitor biomarkers with high sensitivity and specificity and in a manner that is cost-effective, rapid, easy to perform, and amenable to high-throughput screening [27, 44]. Currently, many of the technologies available for SLE diagnosis and monitoring are set to detect ANAs and anti-DNA antibodies [27, 44]. According to many investigators, the Farr assay is considered the gold standard for being able to detect high-affinity autoantibodies which are described to be more reflective of mature immune responses [27]. Nevertheless, a variety of techniques are available that all set to measure the formation of autoantibody-antigen immune complexes but differ in the source of the antigenic substrates, the way that such substrates are being exposed to the autoantibodies in the sample, the employed reaction conditions, and in the principle of detection [27, 54]. These discrepancies in the setup of the distinct assays render the differential sensitivities and specificities toward the recovered or detected autoantibodies in which a particular autoantibody can be tested positive in one assay and negative in another for the same sample [94–96]. The most important contributor for such conflicting results is the affinity of autoantibodies where some assays employ reaction settings that favor the recovery of low-affinity antibodies such as ELISA, while others favor the recovery of high-affinity antibodies such as the Farr assay [27, 97]. Therefore, the results obtained from different assays are difficult to compare and add to the confusion already facing SLE diagnosis. With the emergence of novel biomarkers, the need for multiplex technologies that permit the simultaneous detection of many antigens in just a single test in a rapid, cost-effective, and high-throughput fashion intensifies to accommodate the multiple parameters introduced with the increased variability of detected biomarkers [112, 113, 134]. Many multiplex technologies such as autoantigen arrays, nanobarcodes, microfluidics, and addressable laser immunoassays are strongly emerging with a highly promising potential for clinical diagnosis and monitoring of SLE [93, 112]. Finally, SLE most accurately exemplifies a disease model that in crucial need for the development of personalized therapies owing to the highly versatile clinical manifestations of SLE patients who are not possible to be treated equally [3, 118]. Potent immunosuppressants are currently employed for the nonspecific management of SLE patients, but they are associated with many adverse side effects that can even be more aggressive than the disease itself [41]. The better understanding of SLE pathophysiology has helped in stratifying SLE patients into subsets that share common immune-pathologies and thus can guide many of the emerging highly promising personalized therapeutic approaches [3, 118].

Conflict of interest

The authors declare no conflict of interest.

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