Biomarkers for Serum Diagnosis of Infectious Diseases and Their Potential Application in Novel Sensor Platforms

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ABSTRACT: Nanotechnological tools and biomarkers for diagnosis and prognosis, as well as strategies for disease control and monitoring populations at higher risk, are continuous worldwide challenges for infectious diseases. Phage display and monoclonal antibody combinatorial libraries are important sources for biomarker discovery and for improved diagnostic strategies. Mimetic peptides were selected against polyclonal antibodies from patients with dengue fever, leprosy, and leishmaniasis as model diseases, and from immunized chickens with total antigens from all three pathogens. Selected single or combined multi-epitope peptide biomarkers were further associated with four different sensor platforms, classified as affinity biosensors, that may be suitable as general protocols for field diagnosis. We have also developed two methods for nanoparticle agglutination assays (a particle gel agglutination test and a magnetic microparticle [MMP]-enzyme-linked immunosorbent assay [ELISA]) and two electrochemical biosensors (impedimetric and amperometric) for DNA and antibody detection. For the agglutination tests, micro- and nanoparticles were coupled with filamentous bacteriophages displaying the selected mimotopes on their surfaces, which has favored the formation of the antigen-antibody or peptide-protein complexes, amplifying the optical detection in ELISA assays or after the chromatographic separation of the microagglutinates. We have also demonstrated a proof-of-concept for the electrochemical biosensors by using electrodes modified with novel functionalized polymers. These electrochemical biosensors have proven to be fast, very sensitive, and specific for the detection of pathogen DNA and circulating antibodies of patients, which may become important in a wide range of diagnostic devices for many infectious agents.

KEY WORDS: recombinant peptides, particle gel agglutination assay, magnetic nanoparticle-ELISA, electrochemical sensors, infectious diseases.

I. INTRODUCTION

Infectious diseases are becoming major threat worldwide due to the fast dissemination and adaptation of pathogens favored by the unrestricted cultural globalization, increasing migration and trading, moving across the borders, and exceeding limits of survival. Therefore, infectious diseases

ABBREVIATIONS

AFM, atomic force microscopy; CL, cutaneous leishmaniasis; EIS, electrochemical impedance spectroscopy; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; MMP, magnetic microparticle; PaGIA, particle gel immunoassay; PCR, polymerase chain reaction; PPaGIA, phage-particle gel immunoassay; SELEX, systematic evolution of ligands by exponential enrichment; ssDNA, single-stranded DNA; VL, visceral leishmaniasis
are a matter of national security and may have a great impact on public health and the world economy.

The search for universal and robust diagnostic platforms and for highly specific and sensitive markers has been the biggest challenges in the medical field, due to variable disease spectra, different pathogenetic backgrounds, and complex interactions with vectors and environments, which may result in very diverse phenotypes, with a possibility of turning specific world regions into hyper-endemic ones, especially where poverty is a contributing factor to the disease.

At the moment, most approaches have looked for a common phenotype, which is probably the only chance to interpret or to find a unique profile or marker for the disease spectrum. However, the notion of diagnostics based on one marker has changed, and multiple targets with increased affinity and specificity may result in improved detection. This review aims to demonstrate the development of highly specific markers and their use in combination with novel strategies of affinity biosensors that may be divided into DNA biosensors and immunoassays. We use as models for infectious disease diagnostics dengue fever, leprosy, and leishmaniasis, which are caused by the dengue virus, a bacterium, and a protozoan parasite, respectively.

Dengue fever is caused by infection with the mosquito- (Aedes aegypti) borne dengue viruses, which are members of the genus Flavivirus and the family Flaviviridae. There are four distinct serotypes that can be distinguished by serological molecular methods. In recent years, dengue fever or its severe forms, dengue hemorrhagic fever and dengue shock syndrome, have emerged as a major public health problem. People living in epidemic areas may be susceptible to up to four infections in their lifetimes, and it is not unusual to find more than one serotype co-circulating in an area. Thus, rapid diagnosis and identification of the circulating serotype is crucial for the management of the disease.

Leprosy, a chronic infectious disease of humans caused by the bacterium Mycobacterium leprae, is still a major health problem in Asia, Latin America, and Africa. It is insidious, initially affecting the peripheral nervous system, with patients exhibiting contrasting clinical, immunological, and pathological manifestations. Based on immunopathological criteria, patients are divided into six clinical categories: indeterminate, tuberculoid, borderline-tuberculoid, mid-borderline, borderline-lepromatous, and lepromatous. The lack of effective primary prevention measures has led scientists to search for tests that may detect M. leprae at early stages in order to interrupt its transmission and to prevent nerve damage and deformity of patients.

Leishmaniasis remains a major public health problem, particularly in developing countries, despite progress in diagnosis and treatment. The impact of leishmaniasis on human health has been grossly underestimated for many years, and it has now been classified by the World Health Organization (WHO) as one of the most neglected tropical diseases. Leishmania spp. are obligate intercellular parasites of the macrophages and cause a number of important human diseases ranging from self-healing cutaneous leishmaniasis (CL) to diffuse cutaneous and mucosal manifestations or disseminated and often fatal visceral leishmaniasis (VL). The latter can range from anthroponozoonosis in India and Central Africa to zoonosis transmitted by domestic dogs in the Mediterranean and America. VL affects 88 countries, from which 72 are classified as developing countries. Ninety percent of VL cases occur in just five countries: Bangladesh, India, Nepal, Sudan, and Brazil; similarly, 90% of CL cases occur in just seven countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia, and Syria. The diagnosis of VL cannot always be made on the basis of clinical symptoms alone, because VL shares clinical features with other diseases such as malaria, typhoid fever, and tuberculosis, which commonly occur in the same areas. Rapid and unfailing indirect diagnoses are necessary tools for zoonotic VL because of the large variability of clinical symptoms and the presence of asymptomatic but infective dogs.

In addition to being neglected diseases, diagnostics and epidemiological behavior of the three diseases are further complicated due to their mode of transmission (dengue virus and leishmaniasis are vector-borne diseases and leprosy is spread through direct contact) and their complex clinical spectra, with variable symptoms, differential immune response, and additional complications after treatment or cure.

Although the diagnosis of all four diseases is accomplished based on clinical symptoms, serology, and/or histopathology (M. leprae), nucleic
acid detection has been slowly incorporated into the clinical setting as a confirmatory test for early diagnosis, depending on the target organ. For example, detection of *M. leprae* DNA in the peripheral blood of household contacts serves as a predictor of subclinical infection, and contacts may be submitted to chemophylaxis.1

The current tendency to carry out field monitoring has driven the development of biosensors as new analytical tools able to provide fast, reliable, and sensitive measurements at a lower cost.14 These biosensors may be defined as analytical devices incorporating a biological material, a biologically derived material, or a biomimic intimately associated with or integrated within a physicochemical transducer or transducing microsystem.15

We explore in this review the potential use of biosensors and discuss their recent improvements as a fast, reliable, sensitive, and specific diagnostic strategy by associating them with the most advanced technologies to produce and incorporate biological probes and targets.

II. NOVEL MOLECULES FOR DIAGNOSTICS

The full genome sequencing of several organisms, the discrimination of single nucleotide polymorphisms, the determination of many transcriptomic and proteomic profiles of affected individuals for various diseases, and an understanding of developmental stages of tissues under different environments have allowed important improvements in detection, which may become powerful markers when associated with nanotechnology tools.

At the moment, there are two main strategies for obtaining short and highly specific ligands or markers: the first is to select nucleic acids in a method called systematic evolution of ligands by exponential enrichment (SELEX), and the other uses proteins (peptides and antibody fragments) from combinatorial libraries by phage display.

A. Aptamers: Nucleic Acids and Peptides

1. **Nucleic Acid Aptamers**

Nucleic acid aptamers are artificial ligands showing specific binding affinity for amino acids, drugs, proteins, and other small molecules, which can be screened through the SELEX process from random RNA or DNA libraries by repetitive binding of the oligonucleotides to target molecules.16,17

Oligonucleotide aptamers are 40- to 60-bp, single-strand DNA with high affinity to specific molecular targets and equilibrium constants (*Kd*) in peptide binding ranging from 1 pM to 1 nM.18 In addition to their similarity to monoclonal antibodies, DNA aptamers are chemically more stable, withstand harsh environmental conditions, can be boiled and frozen without loss of activity, have less demanding syntheses, and can be improved in structure using conjugated molecules or nucleic acid modifications as aptasensors.

Recent progress and perspectives in the aptamer selection, strategies for improving their stability and modification, and incorporation into biosensors as affinity biocomponents have been systematically discussed, with a focus on analytical applications such as chromatography, enzyme-linked immunosorbent assay (ELISA)-type assays, optical biosensors, electrochemical biosensors, and affinity polymerase chain reaction (PCR).19 This review also points out the advantages of the electrochemical transduction system, which offers high sensitivity, fast response, low cost of production, the possibility of miniaturization, and a large range of different techniques for detection that can be selected according to different requirements.

2. **Peptide Aptamers**

Peptide aptamers are artificial recognition molecules that consist of a variable peptide sequence inserted into a constant scaffold protein. The features that distinguish peptide aptamers from other classes of constrained combinatorial proteins (such as antibodies, antibody fragments and other nonantibody scaffold-based molecules) include their small size, their simple design, and their disulfide-independent folding. The latter enables them to function inside living cells, unlike antibodies.20

Aptamers have potential application as a recognition element in analytical and diagnostic assays because they can be easily screened, designed, and evolved in vitro.21-24 DNA/RNA aptamers are selected from more than 10<sup>15</sup> individual nucleic acid molecules for different functionalities, and
peptide aptamers are usually selected from yeast—
two hybrid methods to bind to a wide range of cellular, viral, and bacterial target proteins involved in a variety of regulatory pathways. The excellent recognition specificity and high binding affinity typical of peptide aptamers suggest that they could be used in many protein detection methods for which antibodies are currently used.

Novel strategies have been developed by selecting specific and functional peptides through a series of technologies; phage display has become the most prominent and will be the focus of this review.

B. Phage Display: Mimotopes, True Epitopes, and Combinatorial Antibody Fragments

Antigenicity is defined as the ability of the peptide or whole antigen to bind to the selector monoclonal antibody or target. Phage display technology has been widely and extensively used to identify ligands that bind specifically biological molecules through cycles of selections.

Large repertoires of random sequences displayed on the phage surface (phage display libraries) offer the advantage that large numbers of sequences can be screened in a relatively short time. Binders of target molecules are identified by a selection procedure (biopanning) that is based on affinity selection. The principle of the method consists of isolating phages that bind specifically to the target molecule and identifying their insert by sequencing. Among the many selected phage clones, these assays identify those that may be of further interest as binders, agonists or antagonists, antigens or immunogens.

The most exciting potential of phage display peptide libraries is to obtain small peptide molecules that mimic an antigen (mimotopes), at least with respect to a particular epitope. In addition to their interest as research tools, such mimotopes could in principle be useful as diagnostic tools or for eliciting antibodies to a predefined epitope. However, the reduction of the phage insert sequence to a short peptide that can compete with the antigenic—and in particular with the immunogenic—properties of the natural antigen faces considerable difficulties.

Identifying true antigen sequences based on mimotopes remains a very difficult task because of the structural and chemical mimicry of the selected sequences, and because the binder is not always a protein; however, these mimotopes have demonstrated great potential as diagnostic reagents.

When the pathogen or the immunodominant antigen is unknown, sequence homology may be used to reveal the antigen or the etiological agent. This is only the case when mimotopes mimic linear epitopes in which a significant degree of homology to the natural antigen can be expected. However, reports on the elucidation of the etiological agent are limited because most of the serum antibodies are conformational and select mimotopes present little resemblance to the natural antigen.

Another strategy for developing novel biomarkers for the diagnosis of infectious disease is the use of antibody fragments such as Fab (fragment antigen binding) and scFv (single chain fragment variable), which are displayed on M13-derived filamentous phages. Immunization of chickens is the method of choice for the production of monoclonal IgY antibodies by phage display for the construction of antibody libraries. The use of polyclonal IgY, especially for the detection of molecules from specimens such as blood or serum, provides a minor background due to the lack of interaction with heterophilic antibodies, rheumatoid factor, human antimouse antibodies, and complement components, and may be the method of choice for monoclonal antibody production.

The construction of these combinatorial antibody fragment libraries from amplified mRNA of immunoglobulin G (IgG) genes by recombinant PCR is much simpler than in mammals, since IgG from chickens (IgY, which can be easily purified from egg yolk) presents only one gene for the light and heavy chains, simplifying the process by using only two pairs of primers.

Our group has with great success in constructing libraries of IgY fragments expressing scFv on the M13 phage surface, starting with mRNA from the spleens of chickens immunized with total extracts of Leishmania dengue viruses and M. leprae parasites. These antibody libraries have been used to select specific scFv/Fab ligands to different antigens in order to characterize and validate them in new platforms for rapid diagnosis.
This review will be focused on the development of peptide markers and sensor platforms aiming the improvement of diagnosis in leishmaniasis, dengue fever, and leprosy.

1. Leishmaniasis

For leishmaniasis, the choice of antigen is important because the usage of crude antigens or whole parasites often results in a low specificity in detecting disease-specific anti-leishmanial antibody. In the last few years, several *Leishmania* antigens have been characterized in order to improve the vaccine potential and diagnosis of human and canine leishmaniasis. The recombinant protein, rK39, contains a 39-amino acid repeat that is part of a predominant protein in *Leishmania chagasi* amastigotes. Patients with VL have high antibody levels to rk39 repeat and have been used in several immunoassay formats. Although the antigen has been reported to be satisfactory, results have varied considerably in different disease-endemic areas. For reasons that remain unclear, Sudanese patients seem to develop lower titers of antibodies against rK39 than Indian patients, although the format of the test might be a factor, as other brands of immunochromatographic tests performed better in this region. Several recombinant antigens have been developed recently in an attempt to discover new vaccine candidates and improve the diagnosis of leishmaniasis.

Recombinant proteins in vaccines have been intensively tested since the 1990s, and have been used against nearly all forms of disease and parasite species. DNA vaccines, compared with recombinant proteins, are more stable, have a lower cost of production, and have the flexibility for combining multiple genes. Antigens such as LACK, LeIF, TSA, LmSTI1, H1, CpA + CpB, and NH36 are promising candidates for DNA vaccines in the forthcoming years.

We have identified specific peptides by phage display selection that are reactive to canine and human VL antibodies, which could become potential vaccine immunogens or antigens for diagnostics. Selections to human VL mimotopes were processed into two independent reactions with the recombinant antigen rk39 and total antigens of *L. chagasi*. Individual clones were analyzed by dot-blot analysis in competition with the *L. chagasi* antigens. Peptide analysis revealed a high degree of sequence similarities between important proteins involved in this disease, such as gp46, gp63, kinesin K39, and LACK antigens (data not shown). Dot-blot analyses of all clones showed no reactivity with CL or control sera (Fig. 1). The clones with better reactivity to VL antibodies were tested in a competitive assay showing differential performances. Some of these clones presented high affinity to the same epitopes recognized by

![FIGURE 1. Immunodot-blot assays for validations of mimotope reactivity selected from phage display libraries against polyclonal sera from leishmaniasis patients. The clone 7A presented a 3-mer motif of the repetitive core of the rk39 antigen, known as LcKin (kinesin type *L. chagasi* antigen), also showing the highest reactivity, similar to that observed for the rK39. N, negative sera.](image)
L. chagasi antigens and competed with them. The selected mimotopes may present great potential for new diagnosis platforms to human VL, and may also be used as immunogens in a future polyvalent vaccine.

III. PHAGE-PARTICLE GEL IMMUNOASSAY

The principle of the particle gel immunoassay (PaGIA) was derived from passive hemagglutination assays that have proved to be the fastest and most suitable method for detecting human red blood cell antibodies. The hemagglutination technology was based on the recognition and binding of erythrocyte receptors to specific autoantibodies from patients with multiple transfusions, which are separated by size from nonreactive erythrocytes by centrifugation through a gel matrix of chromatographic microcolumns, resulting in a positive reaction when the microagglutinates are captured at the top of the column and the negative reaction is characterized by a flow through of the nonaggregated erythrocytes detected at the bottom of the column (Fig. 2).

Improvements in this gel test were accomplished by conjugating peptides with beads, generating the PaGIA assay, which presented a higher sensitivity and specificity than regular ELISA tests for detecting antibodies against specific targets. This test was based on functionalized three-micron polystyrene beads with peptides that capture specific reactive immunoglobulins, first demonstrated with Chagas disease. However, this immunoassay has been restricted to a few disease diagnostics, including those that use antibodies conjugated to microbeads, but it has not been used as a universal serology platform, probably due to problems with probe conformation, size, and avidity.

This problem was supplanted by a novel approach using a phage-particle gel immunoassay (PPaGIA), which uses a specific peptide fused to the pIII capsid protein of the filamentous phage M13. This new approach uses the phage particle as a spacer carrying the antigen on its surface on one side only, probably increasing the immunoglobulation process. In fact, the cross-linked phage particles may produce a self-assembly complex of phages and their conjugated microparticles, and when incubated in the presence of reactive immunglobulins, particles were retained in the column, increasing the sensitivity of the reaction. Several applications were demonstrated elsewhere, such as exemplified for leishmaniasis diagnosis, with a pIII-fused peptide that was specifically selected to detect the VL form of the disease (Fig. 1). Circulating antibodies from VL patient sera were detected by PPaGIA with a specific mimotope clone that presented a similar motif of the rK39 antigen (Fig. 3).

IV. MAGNETIC MICROPARTICLE-ELISA

Improved techniques in micromachining and microtechnology have opened new avenues in the field of biochemical analysis in the past 10 years. These techniques have been exploited in creating microreaction cells, microarrays, and microchips, all of which share common advantages: smaller volumes for analyses and reagents to be consumed, reduced waste, cost-effectiveness, increased number of analyses per volume unit, and speed.

Nanoparticles, as alternative solid and solution phases, are equally suitable for miniature and immune/multiplex assay formats and as drug carriers. Among these, important scientific advancements have been performed by reproducing an ELISA with a magnetic processing system for diagnosis of toxoplasmosis, multiplexed and microparticle-based analyses, and magnetic techniques for the isolation and purification of proteins and peptides.

A variety of particle sizes and composition, large surface areas, speed, and the ease and flexibility of production aspects and coating procedures on different surfaces favor the use of monosized polymer microparticles.

Magnetic microspheres exhibit a superparamagnetic property in which the individual dipoles align under a magnetic field, exhibiting magnetizations similar to those of the bulk magnetic material; however, in contrast to bulk materials, these suspensions exhibit no residual magnetization once the field is removed. The magnetic particles’ sizes may range from 50 nm to 20 μm, and they have been widely exploited for many biomedical applications such as DNA isolation, cell separation, immunoassay, hyperthermia, magnetic resonance imaging agents, and drug delivery.
FIGURE 2. Hemagglutination assay with the ID-Micro Typing System using ID-Cards (DiaMed, Cressier sur Morat, Switzerland). For detecting antibodies in the serum or plasma of a patient, reagents containing blood cells having known antigens are mixed with a serum sample. The reactants are incubated for a period of time sufficient to permit agglutination of the red blood cells, which occurs when antibodies against those antigens are present. The mixture is then centrifuged, and if agglutinated blood cells are present, such agglutinates are clearly visible at the top of the gel matrix, indicating the presence of antibodies in the sample directed against the known antigens on the red blood cells. If no antibodies are present, agglutination does not occur, and this is indicated by the absence of agglutinated red cells after centrifugation precipitating to the bottom of the gel matrix. The digital images are from ID-Cards (sephacryl matrix) after centrifugation of the microagglutination patients’ sera reaction against red blood cells with known surface specific antigens. A, Strong microagglutination is observed on top of the column; B, weak microagglutination reaction results in distribution of agglutinates at various distances from the top of the gel matrix; C, no agglutination is observed accumulating at the bottom of the column; D, digital image of the gel test assay with four columns of the ID-Card.
Magnetic separation techniques have several advantages in comparison with the standard separation protocols. The process is very simple, with only a few handling steps. Magnetic bead carriers with immobilized ligands exhibiting affinity for the target compound are usually used to perform the isolation procedure. A short incubation period is required for the recognition of the target by the ligand bound to the magnetic particles, forming a complex that is easily and rapidly separated from the sample using an appropriate magnetic field (magnet). After washing out the contaminants, the isolated target compound(s) can be eluted and used for further work.

As a general rule, for the immobilization of small molecules, we use an M13 filamentous bacteriophage (800 micron) because the amino groups with epoxy-activated supports via nucleophilic addition will react with a greater efficiency by using increasing concentrations of salts (e.g., potassium or sodium phosphate). An alternative to that is the use of increasing amounts of ligands in solution and altering solution conditions such as pH, temperature, ionic strength, and coexisting substrates in order to increase the rate of the immobilization process.

We use a magnetic microparticle (MMP)-ELISA approach to specifically enrich and validate peptides that were selected by phage display against positive IgGs from dengue fever patients. We chose magnetic beads conjugated with the protein G to bind antibodies at their Fc region (Fig. 4A), so the Fab region of antibodies can be correctly exposed to the specific peptides fused to the pIII protein of the phage capsid, and true binding to the positive sera can be demonstrated. The correct

**FIGURE 3.** Digital image of the PPaGIA. Circulating antibodies from leishmaniasis patients were detected with specific antigen phage particles (mimotope clone 7A, see Fig. 1) for visceral leishmaniasis (VL), which presents a similar motif of the rK39 antigen. A, positive serum from a patient with VL (Calazar) detected by specific phage particles (agglutination at the top); B, positive serum from a patient with cutaneous leishmaniasis (CL) probed with specific phage particles (no reaction); C, positive serum from a patient with VL probed with nonspecific phage particles (no reaction); D, negative serum probed with specific phage particles (no reaction).
orientation of the phage surface allows the direct capture of the reactive antibody sera, which may also be of potential use with antibody fragments that can be tailored for solid-phase coupling by the introduction of a site-specific group, thus lowering the background binding and causing no chemical contamination, high coupling efficiency, and proper orientation of cross-linked antibodies.  

Magnetic bead-based immunoassays offer an attractive alternative to antibody-based array systems. For example, this assay can be used for quantitation of specific antibodies in patient sera, or antibody-coated beads can also be used as platforms for immunoassays for infectious disease such as dengue fever, leprosy, and leishmaniasis.  

In addition to the MMP immunoassay used for screening clones with specific epitopes that bound to positive IgG from dengue fever patients, a second method was developed to detect positive sera from leprosy patients.  

The first immunoassay used protein-G-coupled magnetic microsphere (2.8 μm) to bind positive and negative HPLC-purified IgG from dengue fever patient sera and controls, respectively. Detection of mimetic peptides of virus antigens displayed on the M13 surface was done by using secondary antibody anti-phage. The clones were previously selected against monoclonal antibodies that specifically recognize the four dengue virus subtypes. Screening by MMP-ELISA presented better results than those from conventional ELISA tests. The microsphere-based approach has several advantages over the conventional ELISA test, including: smaller volumes, enabling the use of fewer reagents; a homogeneous assay format, requiring no washing steps; and absolute quantification of binding using fluorescent standard particles. Furthermore, the use of different surfaces for selection and detection could contribute to the lowering of nonspecific binding of the detection system. The small size and large surface area of the suspended beads increase the efficiency of antigen-antibody interaction by approximating the conditions that are observed in solution.  

For the second platform of the MMP-ELISA, we coated the magnetic epoxy microsphere with a bacteriophage carrying a mimetic peptide of the PGL-1 (phenolic glycolipid-1, a specific \textit{M. leprae} antigen) fused to the pIII protein, which has been used for the detection of circulating antibodies in positive sera from leprosy multibacillary patients.  

M13 bacteriophages are large filamentous structures about 1-μm long and 9-nm wide, and thus their binding to the immunocomplex immobilized on the surface of microtiter plates is strongly limited by diffusion or steric hindrance by co-immobilized, nonspecific subpopulations of antibody. The 2.8-μm diameter size and the dispersed nature of the magnetic beads allow for a nearly “in solution” reaction optimizing the access of the phage-bearing peptide to the immobilized immunocomplex. In addition, the phage particle presents a large surface formed by more than 2700 copies of the major coat protein pVIII. This serves as a highly multivalent scaffold for binding of the anti-phage antibody-HRP (horseradish peroxidase), resulting in significant signal amplification.  

V. ELECTROCHEMICAL BIOSENSORS  

A biosensor is defined by the International Union of Pure and Applied Chemistry (IUPAC) as a “device that uses specific biochemical reactions
mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals.” The device is formed by a transduction element integrated to a biological recognition element (biochemical receptor), providing specific analytical information.

Biosensors are usually classified into various basic groups according to the signal transduction and to the biorecognition principles. On the basis of the transducing element, biosensors can be categorized as optical, electrochemical, piezoelectric, or thermal.

Electrochemical biosensors are emerging as the most interesting sensors because they are more amenable to miniaturization, have compatible instrument sensitivity, allow measurements in turbid media, and have no environment interferences such as temperature and humidity. Drawbacks are that poor coupling of biochemical recognition materials may affect sensitivity and selectivity, and they have limited dynamic range and stability, both of which problems justify the continuous investigation of new polymers, conjugation schemes, and the application of novel recognition elements such as DNA aptamers and short peptides in order to improve detection in electrochemical biosensors.

Considering the biorecognition principle, biosensors are classified as catalytic biosensors if the recognition element is an enzyme, a vegetal or animal tissue, or cells derived from microorganisms (bacteria, fungi, or yeast); affinity biosensors have a recognition element based on biospecific complex formation—for example, DNA fragments and oligonucleotides (genosensors) or antibody, antigen, or hapten (immunosensors).

After this brief introduction to biosensors, this review presents new approaches based on the development of novel polymeric materials and strategies applied to detection of the dengue virus RNA, *M. leprae* DNA, and *Leishmania* antibodies using amperometric and impedimetric biosensors.

**A. Development of Polymeric Films for Sensor Application**

Polymers are an important class of materials with electrical properties that allow a wide range of electronic, optoelectronic, and biotechnological applications, such as in rechargeable batteries, electronic displays, solar cells, ion-exchange membranes in fuel cells, capacitors, field-effect-transistors, printed circuit boards, chemical sensors, drug-release systems, and biosensors.

Conducting polymers present unusual properties such as electrical conductivity, low ionization potential, low-energy optical transitions, and high electron affinity due to the charge mobility along the backbone of the polymer chain promoted by the delocalization of the electrons by the overlap of the orbital of successive carbon atoms with π bonding.

Nonconducting polymers present high resistivity, permeselectivity, and self-limited growth. These polymers have properties that make them useful for interesting applications such as increasing electrocatalytic properties for the development of analytical devices and support matrices for the immobilization of biomolecules.

The methods for polymer production can be chemical or electrochemical. The electrochemical synthesis of polymers has become a very attractive route because it is a fast and easy method. It also presents high reproducibility and stability and thickness control, and the reactions can be carried out at room temperature.

Electrodes chemically modified by functionalized polymers are excellent materials for development of biosensors because they are relatively cheap materials, the techniques for their production are simple, they can be deposited on various types of substrates, the thickness and homogeneity of the films are easily controlled, and the choice of different molecular structures allows for the construction of films with different characteristics.

Electropolymerization studies using different pH values of polymer formation are important to produce different polymeric films on the electrode surface. For example, the electrooxidation of phenols and derivatives in basic medium occurs at more cathodic potentials than in acid medium, due to the increase in the electronic cloud by the presence of an oxygen atom in the phenoxide form, increasing the resonance effect on the aromatic ring.

The electropolymerization of aromatic compounds has been investigated, and previous
studies have indicated that monomers containing aromatic groups directly bonded to oxygen are easier to polymerize and present high reproducibility and mechanical resistance of the obtained film, allowing for higher stability of the modified electrode.97,103,104

Recently, we have reported in situ preparation of electrodes coated with conducting films of poly-2-, poly-3-, and poly-4-aminophenol,96-99,102 poly-4-methoxyphenethylamine,93 poly-4-hydroxyphenylacetic acid,92,105 and polytyramine.100 Those results showed that polymers prepared in acid medium have interesting properties, such as high thickness and excellent conductivity. We have also observed that graphite electrodes coated with these polymers were much more efficient for biomolecule immobilization when compared with noncoated graphite surfaces. Therefore, the immobilization of biomolecules onto an appropriate substrate is an important process due to its significant influence on sensitivity, detection resolution, and reproducibility.

The combination of electrodes with functionalized polymers is a promising strategy for immobilization of markers in the development of genosensors and also for immunosensors and other biological recognition elements, which will be discussed further below.

B. DNA Biosensors

DNA diagnostics has become an important area of molecular biology and biotechnology studies. The detection of specific sequences in human, viral, and bacterial nucleic acids is becoming increasingly important in several areas, with applications ranging from the detection of disease-causing and food-contaminating organisms to forensic and environmental research.106 Nucleic acids have received increasing interest as bioreceptors for biosensors and biochips for specific sequence detection.107-109 In general, the surface of the substrate and/or DNA ends has been modified for stable immobilization of DNA probes.110

Conventional methods for the analysis of specific gene sequences are based on either direct sequencing or on DNA hybridization and amplification methods. However, because of its simplicity, the latter option is more commonly used in diagnostics.106

Nucleic acid amplification techniques are powerful tools for pathogen-detection assays. The combination of excellent sensitivity and specificity, low contamination risk, and medium requirements for manipulation and speed has made real-time PCR technology an appealing alternative to conventional testing methods. However, when PCR is used to amplify a target sample, the main focus should refer to the choice of the target sequence, the assay format, the nucleic acid extraction method, sample type, the ideal sampling strategy, and conditions of manipulation. However, several factors, such as nucleic acid contamination of reagents or laboratory materials, minimum laboratory requirements to keep its performance, great influence of the environment, and high costs, have limited the use of this technology until now. The analysis can be even more complicated when the Southern blot methodology is considered, which is highly influenced by the concentration of both probes and samples, type of membrane, labeling procedure (fluorescence vs. radioactivity), and hybridization conditions.

Beyond this limitation, conventional PCR analysis still uses carcinogenic agents such as ethidium bromide in gels with UV detection, and Southern blot analysis also uses radioactive probes. Although sensitive methodologies, they are hazardous to human health and to the environment.111,112 In order to circumvent these problems, the development of genosensors (or DNA biosensors) has been given attention due to their advantages over other techniques, including rapid application in genetic analysis, low cost, and the possibility of mass production. These devices combine immobilized single-stranded DNA (ssDNA) sequences as a probe on the sensor surface used as a biological recognition agent, conferring selectivity, and a transducer that provides sensitivity and converts the recognition of the sequence-specific hybridization into a measurable electronic signal.

DNA biosensors hold enormous potential for clinical, pharmaceutical, environmental, and forensic applications. This technique can be used as an alternative to PCR, gel electrophoresis, and radioactive tracers, because it is highly sensitive and selective for the detection of mutant genes or genes associated with hereditary or infectious diseases.111,113
Electrochemical DNA biosensors are based on electrochemical studies of nucleic acids and their adsorption on different types of electrode materials. The electrochemical detection of DNA hybridization can be performed by direct oxidation of the DNA bases or by indicators of hybridization: mediators, intercalators, enzymes, or nanoparticles.

These indicators can bind DNA through reversible physical intercalation between base pairs or through electrostatic interaction in well-defined binding sites. The effect is a differential accumulation of the indicator in the DNA layer near the surface of the electrode when ssDNA or double-stranded DNA is attached, which correlates with different voltammetric peak currents, indicating the hybridization occurrence.

In our group, studies using cyclic voltammetry, electrochemical impedance spectroscopy, electrochemical quartz crystal microbalance, UV-Vis spectroscopy, infrared spectroscopy, scanning electron microscopy, and atomic force microscopy have been combined to study electropolymerization and its applications in the incorporation and electrooxidation of DNA nucleotides, oligonucleotides and conserved genomic sequences of the dengue virus, leprosy, and leishmaniasis.

Studies have shown that graphite electrodes modified with polymer matrices are efficient platforms to immobilize nucleotides that are oxidizable on these surfaces. All four bases (adenine, guanine, cytosine, and thymine) can be determined simultaneously by differential pulse voltammetry onto graphite electrodes modified with poly-aminophenols (Fig. 5). These studies showed that the graphite electrodes modified with poly-aminophenols are more efficient in immobilizing biomolecules compared with noncoated graphite surfaces, and that the detection of nucleotides incorporated onto the graphite electrode modified with poly-4-hydroxyphenylacetic acid is dependent on the buffer used in the analysis (Fig. 6). Higher oxidation currents were obtained with AMP in phosphate buffer solution (pH 7.5) and GMP in acetate buffer solution (pH 4.5).

Studies of oligonucleotide hybridization showed that the oxidation peak currents of guanosine and adenosine gradually decreased after hybridization, indicating that during the oligonucleotide hybridization, hydrogen bonds were formed between complementary sequences, leading to a duplex inside of which it is more difficult to oxidize the bases, decreasing the
oxidation peak current of the guanosine and adenosine. Another reason for the higher current values obtained with ssDNA is that it presents higher proximity and a higher degree of adsorption onto the electrode surface due to its higher conformational flexibility (Fig. 7), facilitating the charge transfer between the nucleotides and the electrode.\textsuperscript{118,119}

1. Genosensors for Leprosy

Tools of molecular biology and immunology have been of great value for the diagnosis of leprosy and for epidemiological research.\textsuperscript{120,121} PCR and ELISA have been used as complementary techniques to detect and classify the clinical forms of leprosy, but none has presented high sensitivity and specificity.\textsuperscript{122,123} Additionally, these techniques still demand time, qualified people, and a relatively high cost.\textsuperscript{124} Such problems can be circumvented by using other tools of lower cost that are simple, sensitive, and easily handled as biosensors.

A new approach for specific gene detection of \textit{M. leprae} was developed by our group using immobilizing ssDNA 78 bases long (RLEP3, specific sequence of the \textit{M. leprae} genome,) on graphite electrode coated with poly-4-aminophenol. The interaction with ssDNA or dsDNA of \textit{M. leprae} and the indicator methylene blue was evaluated electrochemically in different concentrations of the complementary target (Fig. 8). The hybridization results presented a significant reduction in the

**FIGURE 6.** Surface response analysis for (a) AMP and (b) GMP as a function of the pH and current peak values.

**FIGURE 7.** Schematic representation of topographical changes in the polymer after the immobilization of oligonucleotide onto the surface of the modified electrode (a) before (ssDNA) or (b) after hybridization (duplex formation).
oxidation peak current of the indicator. The lowest concentration detected of the complementary target was approximately 100 pmol L⁻¹.

2. Genosensors for Dengue Virus

Studies have been performed by using specific PCR amplification products of DENV-1 (dengue virus-1) that target a conserved genomic sequence of DENV-1 onto graphite electrode modified with poly-4-hydroxyphenylacetic acid, using both direct electrochemical oxidation of guanine or redox electroactive indicator (ethidium bromide). The electrochemical detection of a specific sequence for DENV-1² presented a linear range from 12 to 42 nmol L⁻¹ with a detection limit of 7.12 nmol L⁻¹ (Fig. 9).

Atomic force microscopy (AFM) has also been used to investigate the interaction between the probe and the complementary target, and results showed important topographical changes in the surface of the modified graphite electrodes. It was also possible...
to visualize greater rugosity differences between the electrodes containing the probe and after hybridization of the complementary target using this technique (Fig. 10). The rugosity was clearly increased when hybridization was observed.

C. Immunosensors

Immunosensors are affinity ligand-based biosensor solid-state devices in which the immunochemical reaction is coupled to a transducer. The fundamental basis of all immunosensors is the specificity of the molecular recognition of antigens by antibodies to form a stable complex like those observed in other immunoassay methodologies.125

The following serological tests are prominent in clinical practice by using antigens or antibodies as probes: indirect hemagglutination, indirect immunofluorescence, ELISA, and western-blot analysis. However, these traditional methods are time-consuming and may have sensitivity problems.

The immobilization of antigens and antibodies on the surface of transducers led to the development of immunosensors for several substrates of interest in the biological, clinical, and industrial areas.126-129 The methods that employ immunosensors are very rapid and have both high specificity and sensitivity.130 In addition, they have the advantage of requiring small sample volumes, allowing the incorporation of additional samples in the analysis, thus lowering costs compared with conventional analytical methods.

1. Immunosensors for Leishmaniasis

The leishmania kinesin antigen, rK39, is found in L. chagasi. Antibodies that recognize the rK39 antigen have been detected by ELISA in nearly 98% of the investigated sera from patients with VL in Brazil, Sudan, China, and Pakistan, but were virtually absent in South-American patients with

FIGURE 10. AFM topographical images of modified graphite electrode with poly-4-hydroxyphenylacetic acid: (a) without biomolecules after immobilization and hybridization with biomolecules: (b) poly(GA) before hybridization; (c) poly(GA):poly(CT), after hybridization; (d) DENV-1 ssDNA, before hybridization; (e) DENV-1 dsDNA after hybridization. poly(GA) and poly(CT) represent synthetic single-stranded DNA molecules composed by GA sequences (GAGAGAGAGAGAGA), and CT sequences (CTCTCTCTCTCT), respectively.
mucocutaneous leishmaniasis and CL, as well as
in patients in Turkey with CL.131

One of the most significant advantages of impedance detection for biosensing is that antibody-antigen binding can be directly detected, allowing the development of immunosensors. Various types of immunosensors that use detection by electrochemical impedance spectroscopy (EIS) measurements, known as impedimetric immunosensors, have been proposed.132,133 These immunosensors are based in the changes of the electrical properties of antigen/antibody layers immobilized onto electrodes.

Our group studied the detection of antigen rK39 by EIS. Figure 11 shows the electrochemical impedance spectra, known as complex plane diagram (Nyquist), for the modified graphite electrodes with poly-4-hydroxyphenylacetic acid films containing the rK39 antigen. Specific, positive IgG binding was clearly demonstrated by comparing the Nyquist curve with the nonspecific IgG binding. For all plots, a semicircle at a high-frequency range followed by a straight line with a declination close to 45°, a behavior know as Warburg, can be seen. The equivalent circuit for the experimental data simulation was: \( R_t[(R_{ct,1}W)Q_{dl,1}](R_{2}Q_{2}) \).

A correlation of the chi-square values (\( \chi^2 \)) at 10^{-2} order supporting a low statistic error for the simulation was observed. The charge transference of resistance values, \( R_{ct} \), for the electrode only in the presence of rK39 was 120 \( \Omega \). An increase in the charge was observed when specific positive sera (IgG+) was added onto the electrode with immobilized rK39, which was estimated to be 300 \( \Omega \) (+150%). This was due to a barrier for the electron transfer of \([\text{Fe(CN)}_6^{3-/4-}] \) at the electrode interface resulting from insulated rK39/IgG+, which was blocked by the formation of bilayers on the electrode surface. When nonspecific serum (negative IgG) was adsorbed, the \( R_{ct} \) determined was 95.0 \( \Omega \) (–20%). These values are evidence that specific antibody was successfully assembled onto the electrode surface.

The double-layer capacitance, \( C_{dl} \), for the electrode in the presence of rK39 was 0.67 mF. A decrease in the \( C_{dl,1} \) values for specific and nonspecific sera was 0.38 and 0.98 mF, respectively, demonstrating that significant changes had occurred in the outer electrode area. The decreased \( C_{dl,1} \) value suggests a smaller number of oxidizable/reducible superficial groups. This result suggests that the interaction between rK39 and the positive IgG was favored compared with the nonspecific IgG used, increasing the \( C_{dl,1} \) value. A similar behavior for \( R_2 \) and \( Q_2 \) was observed. This second set (\( R_2Q_2 \)) described the response of the inner superficial area of the film, as can be seen in other works containing biomolecules adsorbed onto polymeric films.92,93

VI. CONCLUSIONS

The development of a successful portable biosensor would be of immense value, because such a system would form a platform for the analysis of a wide range of analytes. In principle, different sensors could be constructed by changing the electrode that must be conjugated with different biomarkers. However, many commercial markers still present low specificity or sensitivity, and may not be adequate for fast diagnostic systems.

This review has discussed recent technologies for the acquisition of novel biological diversity by
using combinatorial techniques to obtain highly specific ligands based on proteins and nucleic acids selected by phage display and SELEX, respectively. These molecules are ideal for use in many sensor platforms, because they are easy to produce on a large scale and are highly stable and specific.

Biosensors that can rapidly detect infectious agents or the patients’ immune response are essential tools with which to monitor the health conditions of a population at risk for specific diseases, and may also provide an early warning signal that prevents or anticipates the real threat of biohazard agents during epidemiological monitoring programs, which may promote flexible, agile, and rapid actions.

There is no simple and universal method at the moment that can detect many diseases in one single platform. However, we have presented novel technologies that can be implemented in all kinds of laboratories and point-of-cares, contributing to a fast and precise diagnosis.

We have shown important applications of surfaces that can be functionalized with markers with improved detection systems ranging from microparticles with immunoagglutination tests to polymeric films with electrochemical detection. These strategies have relied on the recognition pattern (affinity) between molecules such as DNA-DNA, DNA-RNA, proteins-proteins, or proteins-antibodies.

Detection based on latex beads immobilized with phage particles carrying peptides or antibodies significantly improved once immunoagglutination became more sensitive than that observed in ELISA assays, as it can be visually detected in microchromatographic columns. Similarly, magnetic microparticles have significantly improved immunoassays when phage-carrying peptides were used. The correct orientation of the phage clone or the antibody as primary markers coupled to the magnetic particle may indicate its use either for phage display validation or for diagnostic purposes.

On the other hand, considering the recent advances in electrochemistry, we have finally prototyped a biosensor platform that can be universally used for DNA and protein detection. This new sensor platform presents different electrodes with specific parameters for each disease investigated, which consider considered polymer synthesis, mediators or intercalators, and the type of molecules to be detected.

The DNA sensor (genosensor) was feasible and presented similar sensitivity as that detected by real-time PCR. However, it is important to consider that this technology also requires DNA processing for its detection because the ideal sensor should detect DNA on crude samples, and this is not yet available. The detection of specific $M. \text{leprae}$ and dengue virus DNA based on the voltammetric response was mediated by the intercalators methylene blue and ethidium bromide, and the DNA-DNA interaction (duplex formation) onto the bioelectrode presented a negative and a positive current change after the DNA hybridization, respectively. These electrochemistry biosensors have been demonstrated to be faster than PCR, with a lower cost, and do not require labeling of nucleic acids (markers or targets). At the moment, the novel bioelectrodes present a sensitivity that reaches a minimum of 100 pmol/L and a response time of 15 min.

The same electrochemical approach was used to detect circulating antibodies in the serum of patients with VL. The interaction of the serum with the rK39 antigen ($L. \text{chagasi}$) adsorbed onto the electrode was detected by electrochemical impedance spectroscopy, which has showed a significant modification on the Nyquist plot upon addition of the complementary target (serum antibody), resulting in increasing resistance transference and decreasing double-layer capacitance.

The electrochemical biosensor has proven to be fast, very sensitive, and specific for the detection of pathogen DNA and circulating antibodies in patients, and may become an important and a widely used diagnostic device for many infectious agents.

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REFERENCES

30. Carlander D, Larsson A. Avian antibodies can


