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REVIEW

Recent advances in immunodiagnostics based on biosensor technologies—from central laboratory to the point of care

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Abstract

Immunological methods are widely applied in medical diagnostics for the detection and quantification of a plethora of analytes. Associated analytical challenges usually require these assays to be performed in a central laboratory. During the last several years, however, the clinical demand for rapid immunodiagnostics to be performed in the immediate proximity of the patient has been constantly increasing. Biosensors constitute one of the key technologies enabling the necessary, yet challenging transition of immunodiagnostic tests from the central laboratory to the point of care. This review is intended to provide insights into the current state of this transition process with a focus on the role of biosensor-based systems. To begin with, an overview on standard immunodiagnostic tests presently employed in the central laboratory and at the point of care is given. The review then moves on to demonstrate how biosensor technologies are reshaping this landscape. Single analyte as well as multiplexed immunosensors applicable to point of care scenarios are presented. A section on the areas of clinical application then creates the bridge to day-to-day diagnostic practice. Finally, the depicted developments are critically weighed and future perspectives discussed in order to give the reader a firm idea on the forthcoming trends to be expected in this diagnostic field.

Keywords Biosensor techniques · Immunoassays · Immunosensors · In vitro diagnostics · Near-patient testing · Multiplexed detection · Nanomaterials · Point-of-care testing · POCT

Abbreviations

Ab	Antibody
AFP	Alpha-fetoprotein
AKI	Acute kidney injury
ALP	Alkaline phosphatase
BPE	Bipolar electrode
BNP	B-type natriuretic peptides
CA199	Cancer antigen 19-9
CBP	Calcium-binding protein
CDx	Companion diagnostics
CEA	Carcinoembryonic antigen
$CK\text{-}MB_{mass}$	Creatinine kinase MB
CLIA	Clinical Laboratory Improvement Amendments
ECL	Electrochemiluminescence
FRET	Förster resonance energy transfer
GMR	Giant magnetoresistance
HRP	Horseradish peroxidase
IGFBP-7	Insulin-like growth factor-binding protein 7
ICT	Immunochromatographic test
IFN-γ	Interferon-γ
IL-n	Interleukin n
ISF	Interstitial fluid
IVD	In vitro diagnostics
KIM-1	Kidney injury molecule-1
LFA	Lateral flow immunoassays
LFD	Lateral flow device
LLOD	Lower limits of detection
MW	Molecular weight
NGAL	Neutrophil gelatinase-associated lipocalin
NWA	Nanowell array
PMMA	Poly(methyl methacrylate)

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POCT	Point-of-care testing
PSA	Prostate-specific antigen
PTFE	Polytetrafluoroethylene
SAW	Surface acoustic wave
SPCE	Screen-printed carbon electrode
SPE	Screen-printed electrode
SPR	Surface plasmon resonance
SWNT	Single-wall carbon nanotube
TDM	Therapeutic drug monitoring
TNF	Tumor necrosis factor
TPA	Tripropylamine
TSH	Thyroid-stimulating hormone
TIMP-2	Tissue inhibitor of metalloproteinases-2

Introduction

In general, the extraordinary specificity of naturally occurring polyclonal or monoclonal antibodies (Abs) against antigenic structures (epitopes) is the foundation for immunoassay and immunosensor technologies. When employed in point-of-care testing (POCT) devices, the immunological reaction principle enables the qualitative or quantitative assessment of the concentration of an analyte/measurand. Immunodiagnostics, performed as POCT, are nowadays used in hospitals, doctor's offices, and by patients themselves [1]. Immunodiagnostic methods for POCT applications can be categorized as homogeneous and heterogeneous immunoassays, particle agglutination and immunochromatographic tests, and as immunosensors.

Immunosensors constitute a specific type of biosensors. Generally, biosensors are composed of a solid-state immobilized recognition element allowing for the biospecific interaction with an analyte in solution and a transducer enabling the monitoring of this interaction. Various recognition elements have been described for biosensors ranging from receptors and enzymes to living cells. In case antibodies or their fragments are either employed as recognition element or constitute the analyte, biosensors are referred to as immunosensors [2].

The predominantly applied recognition element in immunodiagnostics are still Abs, either polyclonal derived from an animal host, or monoclonal derived from the hybridoma method or produced from recombinant Ab technologies. There are, however, disadvantages and situations where Abs cannot be produced or lack the required specificity or affinity in the assay. Here, the recent advantages of tailored protein and DNA/RNA technologies may help for a suitable selection of alternate biorecognition elements (e.g., oligonucleotide aptamers). For a detailed description of these technologies, see the article "Highly Sensitive Diagnostics at the Point of Care Employing Alternative Recognition Elements and Smartphones: Hype, Trend, or Revolution?" by Thaler and Luppa also published in this volume.

The assay methodology for immunodiagnostics, to be applied to POCT, is nowadays mainly focused on immunosensors, either for quantification of single analytes or for multiplexing a single-digit or low double-digit number (but not hundreds) of different parameters [3] (Fig. 1). A typical example for multiplexed POCT already arrived in clinical routine constitutes the "shortness of breath panels" offered by several manufacturers. These test systems enable the simultaneous determination of the analytes relevant for this clinical situation: cardiac troponins, creatine kinase MB, myoglobin, BNP, and D-dimer. In that regard, most of the published and patented new techniques have not arrived on the in vitro diagnostic (IVD) market just yet. At the same time, immunological rapid tests, such as the particle agglutination assays and the immunochromatographic tests, are still the workhorses for various clinical applications, but should be refined in terms of improved sensitivities. The extent of the necessary improvement of analytic sensitivities, however, cannot be specified in further detail as it varies with the analyte and the question the analyte is used for.

The areas of clinical applications for immunodiagnostic POCT methods are becoming increasingly complex in healthcare, since they offer novel possibilities for prevention, diagnosis, and monitoring of diseased subjects [4, 5]. As stated by Vashist, many guidelines for acute clinical procedures already witness the upcoming trend for the integration of POCT into established clinical practice [6]. POCT can ameliorate medical therapeutic procedures in terms of more patient-centered care, and an improved outcome by accelerated clinical decisions. It is now generally accepted that in many emergency departments, individual POC analyses reduce the length of stay and significantly reduce the number of hospital admissions [7, 8]. But it must be kept in mind, that immunological POCT methods are applied in hospitals, general practice, and by the patients themselves (so-called home testing). In this segment, high annual compound growth rates of 6–8% are documented.

To summarize the underlying analytical principles of immunosensors, a general scheme is given in Fig. 2. It must be mentioned, however, that the scheme neither claims completeness nor considers the significant group of homogeneous immunoassays.

An excellent example to demonstrate the progresses made by applying nanotechnology—in terms of optimization of speed, analytical sensitivity, and accuracy of immunoassay formats—is a publication of Sakamoto et al. from 2014 [9]. The authors show that reactions with sandwich immunoassays using antibody-functionalized, fluorescent magnetic microbeads (consisting of a conglomerate of ferrite nanoparticles and fluorescent europium complexes) yielded significantly faster assay times due to accelerating the antigen-antibody reaction.

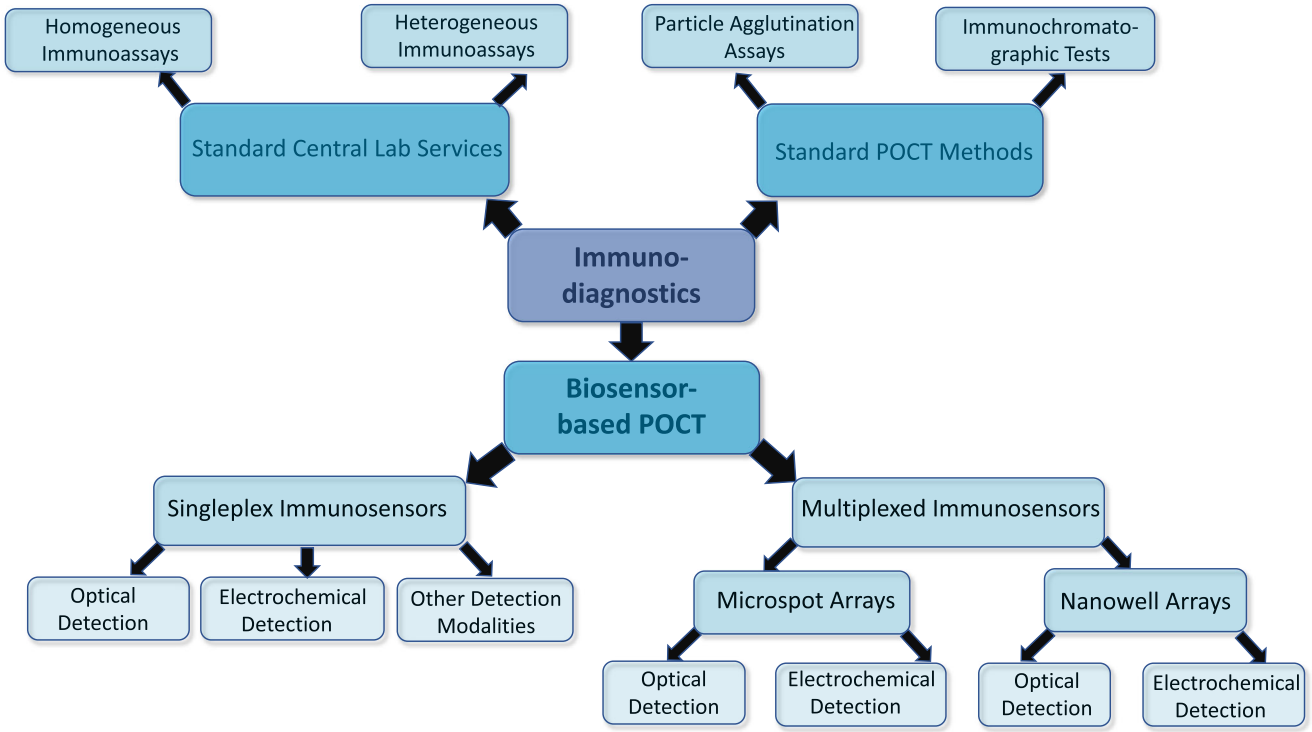

Fig. 1 Assay methodologies for immunodiagnostics

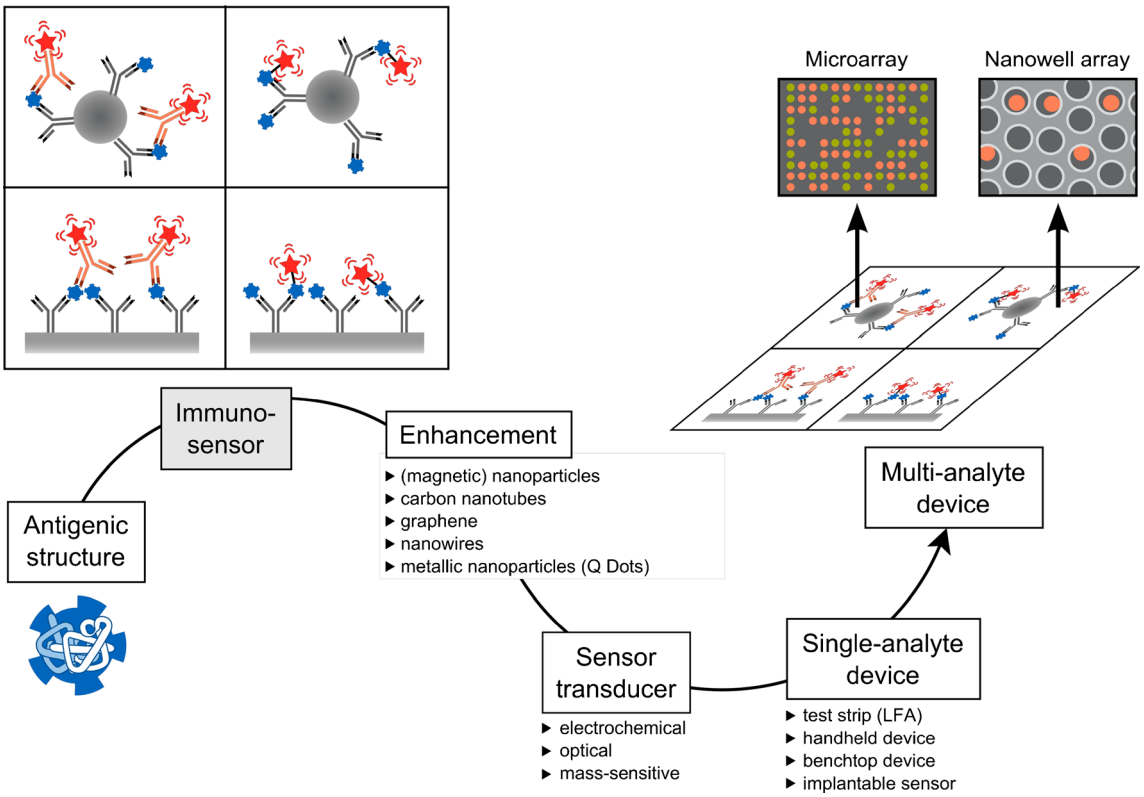

Fig. 2 Analytical principles of immunodiagnostics. Panels on the top left illustrate the principle of non-competitive bead-based assays (top left), competitive bead-based assays (top right), non-competitive solid-phase assays (bottom left), and competitive solid-phase assays (bottom right)

Antibodies

Abs, also known as immunoglobulins, are naturally occurring large, Y-shaped glycoproteins produced and secreted into plasma by lymphatic plasma cells. The most important Iγ classes (IgA, IgG, and IgM) contain 1, 2, or 5 Y-subunits. Each subunit contains four polypeptide chains: two identical heavy chains (molecular weight (MW) approx. 50 kDa each) and two identical light chains (MW approx. 25 kDa each); single disulfide bondages connect each light and heavy chain pair. Due to their function in the human immune system to neutralize pathogens, they are able to recognize antigenic epitopes > 1.5 kDa via the two Fabs highly variable antigen-binding regions (paratops) with highest specificity [10].

Affinity and avidity of an Ab to specific antigenic structures are measures of the strength of the biochemical binding forces in the resultant monovalent or multivalent immune complexes. For most biospecific interactions under conventional conditions (concerning pH, temperature, and buffer solution), the association constants are up to 10^{15} L/mol and as such quite high.

Important for diagnostic applications is the fact that there are two types of Abs: Monoclonal (mAb) and polyclonal (pAb). Both types are naturally occurring, but can also be constructed by use of artificial protein production techniques. mAb are more specific for a single epitope which results in lower cross-reactivities. pAb, as a result of a heterogeneous immunological response in host animals to larger antigenic structures, may bind multiple epitopes.

For the application of Abs as biorecognition elements in biosensor-based POCT immunodiagnostics, an important prerequisite is the ability of Abs to be (covalently) bonded to a sensor surface. Therefore, Abs must be screened for their respective binding specificity after being immobilized [11]. Li and Chen [12] described current bioconjugation methods, such as physisorption, affinity attachment, random cross-linking, and specific covalent conjugation, to ensure the specificity of target molecular recognition. An alternative strategy to prevent a significant loss of performance of the immobilized Ab is the indirect binding of Abs to solid-state surfaces by the use of a site-specific Fc biotinylation and subsequent binding of the biotinylated Ab to (strept)avidin-coated surfaces. Biotinylation reagents are available for targeting specific functional groups or residues, including primary amines, sulfhydryls, carboxyls, and carbohydrates. These procedures result in stable conjugates retaining full immunologic activity [13].

The unique capacity of Abs for a highly specific molecular recognition together with the sophisticated production modes, either in host animals or by use of the hybridoma technique, has made them the cardinal biorecognition elements in immunodiagnostics (immunoassays, immunohistochemistry, indirect immunofluorescence, etc.) already for decades [11].

Both mAbs and pAbs are extraordinarily valuable tools for immunodiagnostics, not only due to the specific molecular interaction with analytes, but also due to the beneficial chemical stability and durability when assembled in assay kits. These properties together with the vast range of available Abs against most different antigen specificities at reasonable costs guarantee high versatility for all kinds of analytical demands.

However, there are circumstances where an Ab may not be available or does not exhibit the required antigen specificity or affinity. This is the case, e.g., for small analytes such as steroid hormones. Alternative immunological strategies such as antimetatype antibodies may be beneficial in this context [14]. There is also a common observation of many immunoassay developers that commercially available Abs sometimes suffer from poor characterization and gross lot-to-lot variations [15]. The authors claim that these binding reagents must be defined by their sequences and be produced as recombinant proteins. Additionally, monospecificity as the pivotal characteristic of mAbs is sometimes questionable. Distinct genetic diversities in Ab heavy (VH) and light chain (VL) genes in individual hybridomas were recently reported [16]. Hence, hybridomas may express one or more additional functional VH and/or VL in significant amounts. This leads to compromised specificities and binding signal-to-noise ratios of the affected mAbs.

The hybridoma technique for the generation of mAbs has some limitations:

- Some antigens are toxic or poorly immunogenic for the mammalian host immune cells.
- By use of mAbs from murine origin, heterophilic Abs may interfere in the immunoassay [13]. Naturally occurring anti-mouse Abs in patient serum often cause unpredicted interferences with the assay format applied. As a result, grossly erroneous results of the immunoassay measurements may result.
- mAbs produced from mammalian cells may contain additional animal pathogens.
- The hybridoma technique requires high expertise and is cost-expensive.

Some of the limitations of classical Abs as diagnostic tools could be avoided by the construction of single-domain (sdAbs, from Camelidae) or single-chain variable fragment (scFv or antigen-binding fragment (Fab)) molecules. The primary solution for the limitations of the hybridoma technology, however, is the production of human mAbs by use of the recombinant Ab technology. This technology became popular in recent years and offers major benefits over conventional production methods. The term "recombinant Ab" implies entire Ab or Ab fragments generated by recombination of Ab-coding genes. Commonly, Fab fragments and scFv are produced for IVD applications.

Modern techniques originate from very large libraries of Ab genes ($>10^9$), such as the Human Combinatorial Antibody Library (HuCAL), a powerful synthetic Ab collection [17]. Naturally, such libraries cannot be directly screened for binding properties. Therefore, the Abs that may bind to a certain antigen are to be identified by a selection protocol. The most widely used selection method is "Phage display" [18]. This refers to a method in which the selection of host cells (predominantly *E. coli*, but also yeast is applicable) containing the preferred antibody fragment is performed. By use of genetic fusion, the expressed recombinant Ab is linked to the surface protein III of a filamentous *E. coli* bacteriophage (e.g., M13). This allows for a fast production of scFv and Fab fragment recombinant mAbs.

In recent years, the analytical techniques to ensure affinity, specificity, and lot-to-lot consistency of produced Ab batches were also optimized. Examples are surface-plasmon resonance (SPR) sensor-based interaction analysis systems such as the Octet instruments (Fortebio, Fremont, CA, USA) or the Nanotemper Dianthus (Munich, Germany) approach: molecular interaction analysis by measuring temperature-related intensity changes (TRIC) of fluorescence signals.

Standard immunodiagnostics

Homogeneous and heterogeneous immunoassays in the central lab

Immunoassay techniques employing polyclonal as well as monoclonal antibodies are widely used in a plethora of settings ranging from basic research to clinical medicine [19, 20] (see also Fig. 1). Heterogeneous non-competitive sandwich assays with bound-from-free separation are the most commonly used type for analyzing peptide or protein analytes in the clinical diagnostic laboratory. Fluorescence and chemiluminescence techniques are mainly used for signaling with labeled primary or secondary Abs or labeled tracers.

In contrast, homogeneous immunoassays without bound-from-free separation of immune complexes often utilize fluorescence polarization techniques [21] or the Förster resonance energy transfer (FRET) principle. In FRET, two different fluorescent dyes must be in close contact to each other. The energy transfer occurs if these fluorescent molecules overlap in their spectral emission (donor) and excitation (acceptor) ranges and is inversely proportional to the distance between donor and acceptor. The spatial proximity can be achieved by formation of an immune complex.

Paramagnetic nanoparticles, coated with specific Abs, have engendered immunoassay procedures with high analytical sensitivity. One example of a novel detection method is a biosensor, presented by Edan Instruments (Shenzhen, China), using magnetic beads to measure immunocomplex formation by means of giant magnetoresistance (GMR). GMR, first described in 1988/1989 by Baibich et al. [22] and Binasch et al. [23], is observed in electrically conductive, alternating magnetic and non-magnetic layers of nanometer thinness. Due to the antiparallel alignment of the magnetization of the two magnetic layers (antiferromagnetic coupling), the electrical resistance in the entire structure is significantly higher compared with a magnetized state. When an external magnetic field is applied, the antiferromagnetic coupling is canceled out and the magnetic moments are oriented in the same direction. As a result, the electrical resistance is significantly decreased.

Homogenous as well as heterogeneous immunoassays proved to be powerful tools for day-to-day routine in clinical diagnostic laboratories. This is due to their reliability, flexibility with respect to possible analytes, low limits of detection, adaptability to automatic analyzer platforms, and reasonable prices. However, even with today's sophisticated immunoassay systems, error rate due to analytical interference is estimated to be between 0.4 and 4%. These analytical interferences arise from factors confined to individual samples and as such are not detected by conventional quality control measures. Their identification is further complicated by their reproducibility within the respective assay, their sporadic nature, and the chance of yielding plausible results. In order to avoid patient harm, several approaches have been suggested, which, however, still are not able to definitely exclude irregular analytical errors [24, 25].

Immunological rapid tests

Before introduction of biosensor methods into POCT immunodiagnostic applications, also rapid test methods were—and still are—based on the immunoassay principle. Appropriate formats for use are particle agglutination and immune chromatography. Prominent examples are the pregnancy test and the variety of microbial antigen detection assays in body fluids. The detection of antigens with a high molar mass relies on a sandwich immune complex, where the first Ab to a particular analyte epitope is labeled, and a second capture Ab to a second epitope on the same antigenic structure is immobilized on the lateral flow strip. Qualitative lateral flow assays (LFA) are based on signal techniques which rely on a visible color (such as colloidal gold or various particle-based systems) to identify the presence or absence of specific analytes.

An analytical problem when using capillary blood as sample material is the fact that interstitial fluid will be admixed to various extents when blood is drawn. As a result, changes in concentrations of the analytes are often observed. Another drawback is the lack of valid reference ranges for many measurands, determined by the use of test strip [26].

Particle agglutination assays

Latex (polystyrene) carrier microbeads (approx. 0.8 μm diameter) are decorated with specific Abs by physisorption. Microbial antigens as analyte structures in the patient sample will instantly bind to the latex particles. This agglutination appears as a macroscopic precipitate in the milky suspension. For quantification, the particle agglutination tests can be performed on microscope slides or even in microtiter plates. These immunodiagnostic devices are successfully applied for diagnosing rotaviruses, but also for multiplexed antigen detections, particularly in the case of meningitis panels: The POCT methods allow the simultaneous detection of the most important infectious agents causing meningitis, such as *Streptococcus pneumoniae*, *Neisseria meningitidis*—serotypes A-C, Y, and W135—and *Haemophilus influenzae* type b.

Immunochromatographic tests

Immunochromatographic strip tests (ICT) are known under various names in literature: lateral flow assays (LFA), lateral flow devices (LFD), or dipstick assays. The analytical basis for these tests is a thin-layer chromatographic separation step: In general, the Abs are immobilized on a membrane and the body fluids ((capillary) blood, urine, cerebrospinal fluid) are moved onto the membrane by capillary force after adding the liquid sample to the strip. The principle is illustrated in Fig. 3. ICT formats are easy to use and allow rapid qualitative, semi-quantitative, or quantitative analyses.

The sensitivity of ICT can be enhanced by numerous technical modifications: use of labels with higher specific signal intensity, such as paramagnetic microparticles, carbon particles, colloidal metal particles, or blue latex particles [27].

For LFDs, also immunocomplex-induced color changes can be exploited [28]. The immune reaction with the analyte directly changes the reflective properties of the silicone surface and the different layers applied.

There are also quantitative LFAs for the assessment of cortisol and IgA in *saliva* on the market. Saliva as easy-to-get patient sample matrix attracts more and more attention for POCT analyses. However, an important drawback of the use of saliva is that concentrations of many analytes in saliva are often much lower than in serum/plasma. This is mainly due to the fact that saliva can be seen as ultrafiltrate of blood with low-protein content.

In the case of steroid hormones, the important advantage of saliva as sample material as compared with serum/plasma is its ability to reflect the free or biologically active hormone fraction, whereas in blood levels of the bound or complexed hormone may be detected. The most frequently applied parameter of this category is cortisol, a stress marker used in psychiatric or psychosomatic patients [29].

A simple, robust, and ultrafast determination of autoantibodies in saliva has been reported by Burbelo et al. [30]. The authors established an immunocapture method, which employs the capturing of protein A/G-coated paramagnetic beads bound to autoantibody/luciferase-labeled antigen complexes. In the presented tube assay to be read out with a traditional luminometer, Renilla luciferase is employed and detected with coelenterazine substrate. Additionally, a further assay variant

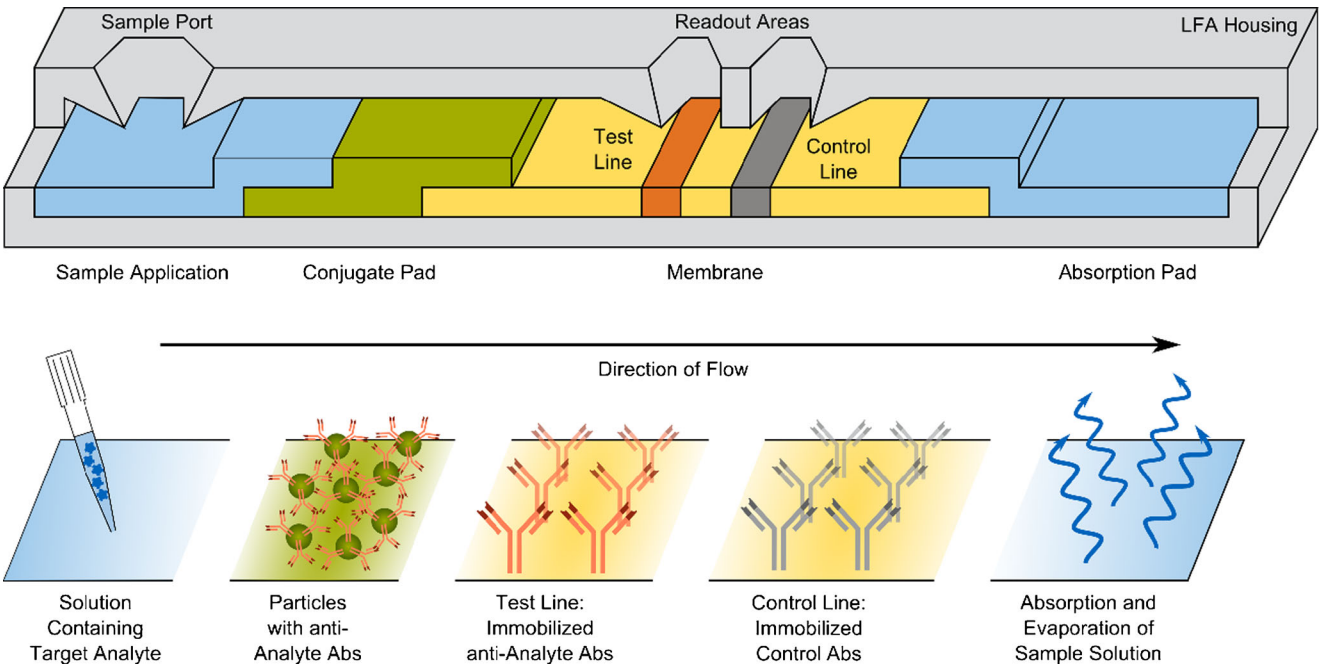

Fig. 3 Principle of immunochromatographic strip tests

based on the NanoLuc luciferase (Promega, Madison, WI, USA) and furimazine is depicted, which offers the possibility to be read out with a handheld luminometer.

POCT methods have also a high value in the field of infectious diseases. In this field, LFAs provide—on a positive assumption—useful information about the infectious agent and the initial treatment. Kozel [31] has tabulated tests granted Clinical Laboratory Improvement Amendments (CLIA) waiver status in the USA. Among them are LFAs for the detection of infections with group A *Streptococcus*, *Helicobacter pylori*, influenza virus types A and B, RSV, HIV-1 and HIV-2, *Trichomonas vaginalis*, *Borrelia burgdorferi*, *Treponema pallidum*, or HCV. For more details, see also "Medical sectors for application."

Biosensor-based immunodiagnostics for POCT

Singleplex immunosensors

The immunological antigen-antibody complex formation allows for the qualitative detection of a measurand. Depending on the assay format, also quantitative assessments of analyte concentration by immunosensors are possible [2]. From the broad spectrum of biosensor techniques, in POCT, mostly electrochemical (potentiometric, amperometric, or conductometric, capacitive) and optical sensors are employed [4, 32]. Microgravimetric (quartz crystal microbalance) and thermometric sensors, on the contrary, play no significant role as methods for POCT. Applications of immunosensors in clinical laboratory diagnostics are already numerous, as depicted in a review of Justino et al. [33] and in a survey of available POCT methods, recently published [3].

Electrochemical immunosensors have been proven as analytical tools of high sensitivity, low costs, and fast results, and, thus, are suitable for POCT [34]. Cho et al. reviewed the advantages of modern nanotechnology which allows for the development of innovative electrochemical biosensors with enhanced sensitivity [35]. Main components are various nanomaterials (such as carbon nanotubes, graphene (oxide), indium tin oxide, nanowires, or metallic nanoparticles) that facilitate the electron transfer and exhibit increased capacities for carrying various molecule labels. Additionally, the prevention of non-specific binding phenomena of proteins onto electrode surfaces by sophisticated supramolecular architectures such as self-assembled monolayers has made significant advances in the last years, as reviewed by Contreras-Naranjo and Aguilar [36].

Optical biosensors are operated as direct (label-free) or indirect (labeled) immunosensors. In the label-free type, the photonic signal is generated directly by the interaction of the analyte with the transducer surface. One would assume direct immunosensors to be in widespread use for POCT applications, because of their simple reagent concept. Their actual use in POCT immunodiagnostics, however, is surprisingly limited. This concept offers analytical advantages, as long as an adequate concentration range can be accomplished for the measurand. The label-based indirect approach involves the use of an optical label, being generated by a colorimetric, fluorescent, or (chemi) luminescent method. Here again, nanoparticles will advance the photonics characteristics of optical biosensors in the upcoming years mainly by multiplying the number of antibody binding sites due to increased carry capacity.

The underlying photonic modalities are SPR, evanescent wave fluorescence, interferometry, ellipsometry, reflectometric interference spectroscopy, and surface-enhanced Raman scattering [37, 38]. Moreover, quantum dots (photoluminescent semiconductor nanocrystals) are frequently used in immunoassays for research questions. Due to their unique optical properties and their size-adjustable absorption/emission characteristics, they can also be used for a multiplexed analytical modality of multiple analytes. These methodologies utilize the FRET detection mode since it offers the opportunity to specifically turn-on or turn-off the measurable quantum dot emissions upon measurand interaction. This enables sensitive and specific detections [39]. Quantum dot–based assays to date, however, do not have any relevance for POCT devices used in clinical routine.

Chen et al. [40] described an ultrasensitive bioluminescent immunosensor for POCT applications using a sophisticated double enzyme immunoassay system including alkaline phosphatase (ALP) and luciferase. In the presence of an analyte, the ALP attached to the surface of the immune-nanocomplex (built from antibody-coated nanoparticles and ALP-bearing nanoparticles with a second Ab) dephosphorylates added ATP. As a consequence, in the presence of luciferin, no ATP-luciferin-luciferase bioluminescent reaction occurs. In the absence of the analyte, the bioluminescent readout reaches a maximum. The double-enzymes-mediated bioluminescent immunosensor performing the measurements is highly sensitive up to the femtomolar range. The system can be minimized to the size of a handheld device and readily lends itself as a suitable example of innovative POCT.

As for classical immunoassays, there are many options of immune-labeling of Abs or tracers for immunosensors. The most reliable of these labels are enzymes such as peroxidase, glucose-oxidase/-dehydrogenase, ALP, catalase, or luciferase. Fluorescent labels include rhodamine, fluorescein, Cy5, ruthenium diimine complexes, and others. Laser-induced fluorometric resonance energy transfer between two different fluorophores can specifically be employed in fiber optic sensors [41].

Both electrochemical and optical biosensors have to be based upon sophisticated microfluidic biochips. Nowadays,

microfluidics as an enabling technology has reached the potential to integrate complex workflows into a single miniaturized cartridge that operates without additional hands-on time after adding patient samples [42]. In order to develop microchannel structures for multiplexed analysis, the choice of the solid-state substrate is crucial. Besides glass, quartz, or silicon, often polymeric materials such as polytetrafluoroethylene (PTFE), photosensitive silicone, or poly(methyl methacrylate) (PMMA) are used. Additionally, biopolymers such as calcium alginate and cross-linked gelatin can be employed [43].

Multiplexed immunosensors

Clinical decisions are rarely made due to the results of one single biomarker, but rather based on a scenario-dependent panel of biomarkers. As such, multiplexing of analytes is most desirable for POCT applications. Beyond that, multiplexed POCT may also offer some organizational advantages with respect to turnaround-time and cost [44]. Generally, microarrays consist of analytical spots on a sensor chip with each spot having a selective recognition element for a specific protein measurand. Typically, the microarray spots consist of primary Abs to capture the analyte. After washing to reduce non-specific binding events, labeled secondary Abs are added to bind to the respective analytes. The subsequent signal generation must use a robust technology. Biosensor techniques based on optical or electrical signaling are therefore preferred.

Multiplexed electrochemical protein detection is usually realized in a so-called barcode approach where detection Abs are modified with different metal nanoparticles that are subsequently dissolved and the metal ions (or quantum dots) detected by anodic stripping voltammetry [45]. Attractive alternatives are multi-electrode arrays. Here, a different Ab is immobilized on each electrode. There are many examples of publications describing such analytical systems and these have been reviewed recently [45].

The group of Knopp et al. [46] demonstrated an immunosensing platform for the multiplexed detection of the tumor markers carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) in serum using biofunctionalized magnetic graphene nanosheets together with horseradish peroxidase (HRP)-coupled thionine and HRP-ferrocene nano-gold hollow microspheres as distinguishable signal tags.

Wan et al. chose a similar strategy by applying 16-channel screen-printed carbon electrodes (SPCE) [47]. Each element contains a working electrode being modified with different capture Abs for two sandwich type assays for prostate-specific antigen (PSA) and interleukin 8 (IL-8), a carbon counter and a silver reference electrode. In Fig. 4, the SPCE array is illustrated.

Electrochemiluminescence (ECL) is often used for signaling of immunosensors due to the superior performance characteristics of assays with the $Ru(bpy)_3^{2+}$/tripropylamine (TPA) reagent system.

Screen-printed electrodes (SPE) were used by Feng et al. [48]. An ECL detection method was also employed to establish an immunosensor for the two tumor markers CEA and cancer antigen 19-9 (CA199). The sensor was modified with $Ru(bpy)_3^{2+}$-silica@poly-L-lysine-gold composite nanoparticles as labels and the respective antigen bound to it by electropolymerization of the electrode with the tumor marker.

Wu et al. employed a closed bipolar electrode (BPE) system for a multiplexed biosensor [49]. Indium tin oxide electrodes were used with Au film modification on the cathodes of the BPE array. Nanobioprobes including aptamer or Ab and a novel ECL tag, produced by doping thionine in silica nanoparticles, were introduced into the cathodes by immunoreaction or DNA hybridization. The nanobioprobes served as recognition elements and signal amplification indicators and mediated the ECL signals of $Ru(bpy)_3^{2+}$/TPA on the anodes of the BPE array. The system used an Ab sandwich assay to detect AFP and PSA, and a DNA aptamer to detect both ATP and thrombin.

Sardesai et al. described the fabrication of an ECL immunosensor array featuring capture antibody-decorated single-wall carbon nanotube (SWNT) forests, residing in the bottoms of microwells with hydrophobic polymer walls [50]. $Ru(bpy)_3^{2+}$-silica nanoparticles functionalized with secondary Abs were used for the ECL signaling, detected by a CCD camera. This system enabled the measurement of PSA and IL-6 in serum simultaneously in 16 microwells.

Significant analytical problems of the described multiplexed techniques for protein analytes are:

- Difficult preservation of antigenicity of protein or peptide antigenic structures
- Non-specific interactions caused by the sample matrix
- Different measurand concentration ranges impair a universal detection mode
- Complex detection modes (e.g., CCD camera) increase system complexity.

To overcome the obstacles stated in the first three bullets, *nanowell arrays* (e.g., in the form of array electrodes) could be a solution for future applications as high-throughput tools for diagnostics.

The so-called nanowell arrays, first introduced by Rissin et al., are suitable to reach multiplicity and ultra-sensitivity for diagnostic purposes. They already have arrived in medical laboratories with the associated equipment being available in different sizes (e.g., Simoa SR-X and HD-1 analyzers from Quanterix, Lexington, MA, USA) [51]. This laboratory analyzer is able to process single and multiplexed digital immunoassays of 16 measurands of interest for cardiovascular, cancer, infectious disease, neurology, and inflammation

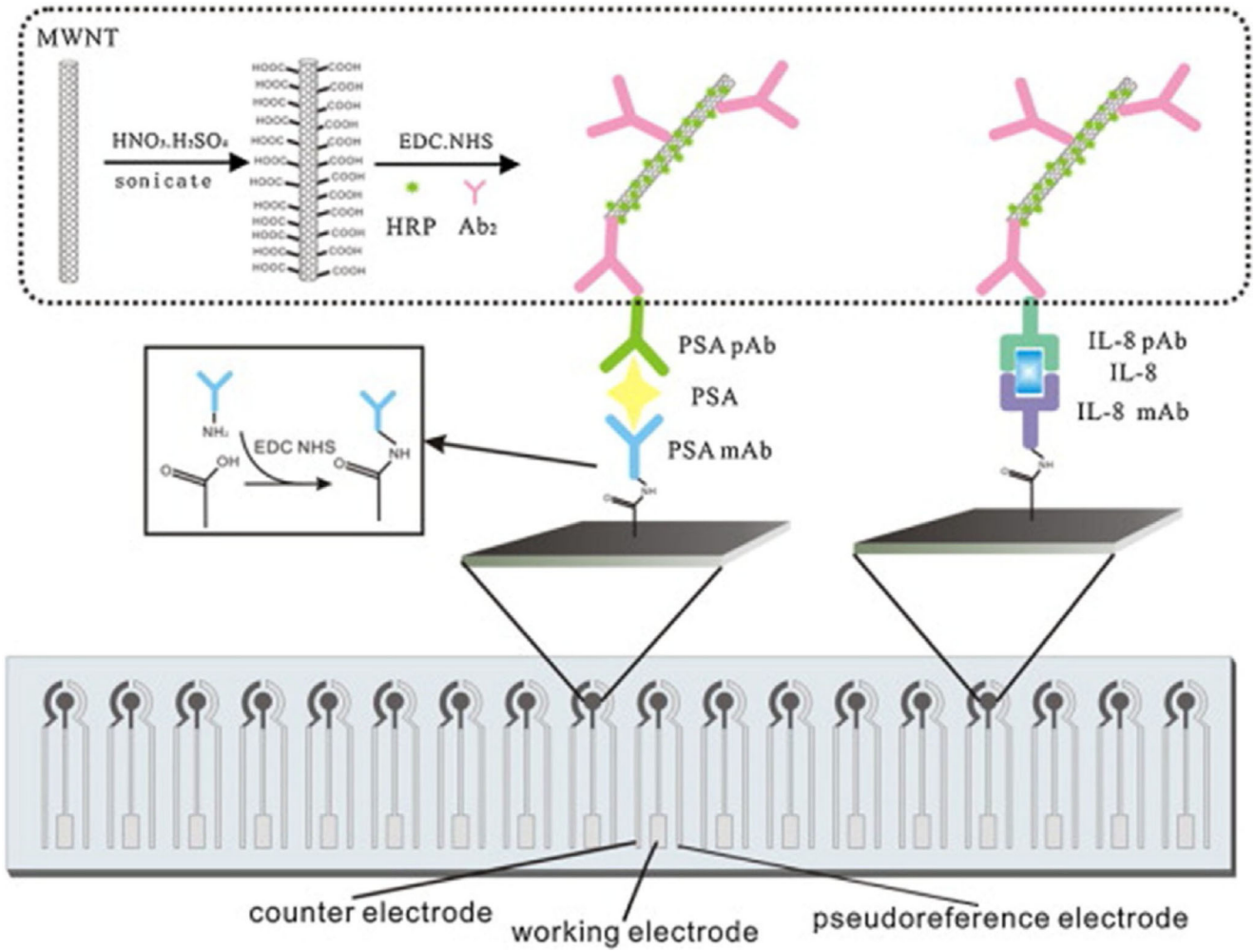

Fig. 4 Example for a multiplexing electrochemical immunosensor using a disposable screen-printed carbon electrode (SPCE) array as the detection platform according to [45]. Reproduced with permission from Elsevier

applications [52]. Being able to detect single immunocomplexes in arrays of femtoliter wells, this novel technique has enabled the measurement of clinically important analytes in serum at subfemtomolar concentrations. Although this technique has not yet reached practical suitability for POCT, it is expected to be of high relevance for future developments of POCT devices.

Seo et al. presented an electrochemical nanowell array (NWA) assay, where the small size of the nanowells within the array permits extremely sensitive and reliable sensing of a small quantity of biomolecules [53]. The array format significantly enhances the sensitivity and reliability for electrochemically detecting biomolecules. Remarkably, the NWA approach is also suitable for the detection of other biomolecules and can be extended to other types of biosensors than electrochemical sensors. Additionally, it can easily be adapted for simultaneous detection of multiple analytes.

Furthermore, there is a big variety of lateral flow, planar, or bead-based protein arrays on the market. Most popular are microsphere-based suspension formats, such as the Luminex xMAP system [54]. These arrays enable high-throughput detection of a huge number of peptide/protein and nucleic acid analytes. For time-critical POCT, however, future applications of these formats are difficult to imagine: The interpretation of results would overstrain the diagnostician. One cannot exclude, however, that artificial intelligence may facilitate this interpretation in the far distant future.

Areas of clinical applications for POCT

A comprehensive summary of clinical applications of POCT has recently been published [3]. Immunodiagnostic POCT methods are nowadays applied in hospitals, in doctor's offices, and by patients themselves. The increasing demand of POCT in various markets around the globe has been documented many times [55, 56]. In contrast, e.g., the adoption in Germany is significantly lower due to licensing procedures and restrictive reimbursement for general practitioners [57].

Medical sectors for application

The most prevalent POCT immunosensor application is the determination of *cardiac markers* in emergency rooms, intensive care units, and in left heart cath labs where therapeutic decisions are made based on the serum concentrations of these biomarkers. Currently, there are qualitative and quantitative tests available for troponins T and I as well as for creatinine kinase MB (CK-MB_{mass}) [58]. Due to the clinical evidence that the sole determination of troponin T or I is superior in the diagnosis of acute myocardial infarction, CK-MB_{mass} has become less important [59]. In recent years, the b-type natriuretic peptides (BNP and N-terminal BNP (NT-pro-BNP)) have been established as specific and prognostic valuable biomarkers for chronic heart failure [60]. To date, determination of cardiac markers via POCT applications exhibits a high degree of acceptance in emergency rooms of small- to medium-sized hospitals, i.e., in scenarios where services of a central laboratory are not readily available round the clock. This is mainly due to the significantly reduced turnaround-time helping to improve patient outcomes.

As already mentioned earlier, immunological POCT methods have a high value also in *infectious diseases* to detect pathogens or Abs against infectious agents. In this field, rapid tests provide—on a positive assumption—useful information about the infectious agent and the initial treatment. Banerjee and Jaiswal reviewed recent advances in nanoparticle-based LFA for infectious agents [61]. They conclude that LFA allow a wide range of qualitative as well as quantitative identification of pathogens and present comprehensive tables for various bacterial, fungal, viral, and protozoal infections.

In general, two types of assays are to be distinguished: LFAs against microbial, parasitic or viral antigens, or serological assays directed against acquired patient Abs to infectious agents [40]; [61]. Due to the fact that the LFA platform is extremely versatile, hundreds of different POCT assays have been developed against a plethora of various infectious agents or even infection-related Abs. To give only two examples: First, sensitivity (98 to 100%) and specificity (86 to 100%, one outlier 75%) of immunological POCT methods for HIV infection is largely comparable to standard methods. As false-positive results cannot be excluded with the rapid tests, it is however recommended to confirm positive results with another rapid or conventional test [62]. Second, the diagnosis of streptococcal pharyngitis on clinical symptoms alone is not reliable. A conventional microbiological culture takes 1–3 days. Therefore, easy-to-perform streptococcus rapid tests have become increasingly widespread especially in pediatric scenarios. The tests are characterized by a high specificity and improved sensitivity [63]. Enthusiasm about the multitude of available POCT tests for infectious diseases, however, should not obscure the need for an evidence-based approach for implementation of these tests into diagnostic strategies. Especially when testing for infectious agents, it has to be considered whether the diagnostic result impacts therapy and a negative test result definitely excludes an ongoing infection. In case of nucleic-acid-testing POCT, relevance of genotype resistance testing for the phenotype has to be evaluated. And finally, POCT testing for infectious diseases has to be cost-effective in the clinical setting. Results of the discussions and considerations outlined above will necessarily also differ depending on the level of development of the respective country, e.g., industrialized or developing country.

POCT is also frequently applied for *drug of abuse testing*. Simultaneous detection of the most relevant substance groups is not only helpful in the differential diagnosis of impaired consciousness, but also in monitoring patients in a drug-withdrawal status. However, the higher detection limit of such tests should be kept in mind when interpreting the results. Compared with standard laboratory methods, some of the commercially available POCT methods are less sensitive. Testing is only performed on substance groups and not on a single substance. Moreover, it has to be taken into account that the detection of new synthetic designer drugs is not guaranteed with such tests. Therefore, false-negative and false-positive results cannot be excluded.

The described new technological approaches may help to improve measurements at very low concentrations. Stenken and Poschenrieder reviewed recent analytical developments for the measurements of *cytokines* [64]. Cytokine determinations will probably have a high importance as acute markers for the application of POCT methods in the future. In particular, there is a high demand for the simultaneous detection of various cytokines in order to elucidate the inflammation state of a patient in more detail (the so-called secretome analysis). Therefore, besides Luminex bead-based assays, there is a great interest in developing multiplexed cytokine arrays. Captured Abs can be immobilized onto solid-state surfaces and allow the development of cytokine arrays that are able to detect interferon-γ (IFN-γ), IL-1β, IL-2, IL-4, IL-6, IL-12, IL-13, and TNF in the low picograms per milliliter (pg/ml) range when using Cy5-labeled streptavidin and biotinylated detection Abs [65]. Due to the analytical problem to detect cytokine basal serum concentrations even within the low pg/mL range, the analytical aim is to significantly extend the lower limits of detection (LLOD) for cytokine immunoassays. This would increase the clinical usefulness of such assays. Different improvements have been proposed including use of ECL or even immune-PCR methods that use the amplification of nucleic acids [64]. A plethora of methods and schemes of immuno-PCR applications have been reported in the literature [66]. Compared with classical immunoassay methods, the immuno-PCR amplification methods provide up to 10^5-fold improvements in the LLOD. The transferability of this assay format into a POCT design, however, is questionable.

In high-performance *sports medicine*, POCT is nowadays also frequently applied. The main objective is a suitable framework of multiple indicators of internal and external stress situations during training and recovery phases in order to adjust the training program most effectively.

Düking et al. recommend a series of clinical chemistry parameters for close monitoring of an athlete [67]. Among them are measurands that can be measured employing POCT immunodiagnostics, such as high-sensitive C-reactive protein, salivary IgA, ferritin, salivary cortisol, myoglobin, and salivary testosterone.

In order to establish a POCT application for the *therapeutic drug monitoring* (TDM) of immunosuppressants, the group of Moreno-Bondi developed a sensitive rapid homogeneous fluorescence polarization immunoassay for the free mycophenolic acid fraction determination, continuously microdialyzed from human blood [21]. The assay employs the competition of free mycophenolic acid (from patient blood) and mycophenolic acid labeled with an emissive near-infrared fluorescent dye for the binding of anti-mycophenolic acid antibodies. Obviously, this attempt seems to be promising due to the fact that homogeneous assays would simplify the functioning of a respective POCT device. Another attempt to revolutionize TDM was postulated by Kiang [68]. The group used multiple hydrogel-forming microneedles (to be placed onto a skin area) for extraction of analytes from the interstitial fluid (ISF). ISF has a plasma-like composition but is characterized by low protein content. Therefore, therapeutic drugs are mostly present in ISF in a free, not protein complexed form.

A further TDM application is the patient-near measurement of insulin concentrations, important for the management of insulin pumps, and insulin monitoring systems in diabetic patients. The clinical need focuses on managing diabetic, prediabetic, and pancreatic disorders. Singh and Krishnan described the development of electrochemical and optical immunosensors for minimally invasive measurements [69]. By utilizing Abs or aptamers in combination with nanomaterials, the authors could demonstrate various suitable electrochemical and SPR-based immunoassays.

Further examples of immunological POCT applications are listed in Table 1 (modified from [3]).

Settings of application

Hospital

In contrast to emergency tests, such as blood gas analyses, immunological POCT methods are usually applied to compensate for suboptimally organized structures. If a central laboratory is present, such as in most large hospitals, the need for immunologic POCT use would depend, for instance, on the possibilities of sample transportation and transmitting measurement results. In smaller hospitals, POCT can be used to compensate at least in part for the lack of an emergency laboratory. Measurements of cardiac troponins but also C-reactive protein, pregnancy tests, and several other analytes can be found. POCT for detection of Abs, proteins, and hormones can usually be dispensed within hospital settings and are only helpful in isolated cases, for instance to detect Streptococci infections or for drug-of-abuse screening.

Doctor's office

The POCT-based determination of cardiac troponins or D-dimer to rule out acute coronary syndrome and deep vein thrombosis/pulmonary embolism, respectively, offers certain advantages within primary patient care. As in hospitals, immunologic POCT in the doctor's office, however, is usually not used for emergencies but to improve organizational processes. When measuring analytes, such as HbA1c at the POC, the patient does not need to schedule a second appointment to discuss the previous test results. Simple blood cell counts (e.g., number of thrombocytes) prior to chemotherapy are a further helpful application of POCT in the doctor's office [70].

Home testing

On the one hand, the self-monitoring of blood glucose by patients with diabetes mellitus and the self-measurement of INR in warfarin/phenprocoumon therapy are well established for more than two decades. Thereby, patient care could be significantly improved [56, 71, 72]. On the other hand, the benefit of immunological rapid tests is less clear. Fertility monitors in females with a long history of infertility have proven to be successful [73].

To prevent the risk of false-negative or false-positive results by inadequate use of tests by laypersons or by the use of less sensitive or specific POCT systems, immunologic tests for infectious diseases, tumor markers, or hormones are better to be performed in a medical laboratory than at the POC.

Specific settings

Immunologic POCT may also be applied in diagnostics of infectious diseases after large-scale disasters, such as a bioterrorist attack by aiding rescuers in triaging victims. The same applies to diagnostics in the case of natural epidemics such as Ebola and other outbreaks of infectious diseases [74, 75].

In order to improve training programs, immunodiagnostic POCT methods are also applied in sports medicine. Stress indicators may help to adjust the training program most effectively (see "Medical sectors for application") [67].

Table 1 Examples of characteristic measurands, which can be assessed using immunological POCT methods (immunosensors, immunoassays, immunochromatographic tests), modified from [2]

Application	Examples
Cardiac markers	Cardiac troponins, NT-pro-BNP, CK-MB_{mass}, interleukin 1 receptor-like 1 (ST2)
Coagulation	D-dimer
Pregnancy	Human chorionic gonadotropin
Inflammation	C-reactive protein, procalcitonin, interleukin 6
Infectious diseases	Antigen detection: *Streptococci* (group A and B, pneumococcus), *Clostridium difficile* (toxin or antigen), *Chlamydia spp*., *Neisseria gonorrhoeae*, influenza, respiratory syncytial virus, *Plasmodium spp.*, *lLegionellae*, rotavirus
	Ab detection: Hepatitis, human immunodeficiency virus, Epstein-Barr virus (mostly heterophilic Abs), *Treponema pallidum* hemagglutination assay, *Helicobacter pylori*, *Mycobacterium tuberculosis*
Endocrinology	Follicle-stimulating hormone, luteinizing hormone, estriol-3-glucuronide, pregnanediol glucuronide, parathyroid hormone
Immunology	Immunoglobulin E (IgE), allergen-specific IgE, transglutaminase/gliadin Abs, lactoferrin
Rheumatology	Antistreptolysin, rheumatoid factor, Abs against mutated citrullinated vimentin
Tumor markers	Prostate-specific antigen, fecal occult blood; nuclear matrix protein 22 and bladder tumor antigen in urine
Drug-of-abuse screening	Group screening with amphetamines, barbiturates, benzodiazepines, buprenorphine, cocaine, methamphetamine, ecstasy, morphine, methadone, tricyclic antidepressants, cannabis

Critiques and perspectives

Central and POCT laboratory services complement each other. In hospitals, both are important for optimizing diagnostic processes. In many emergency rooms, explicitly, POCT offers diagnostic advantages for the clinician and may improve patient outcomes. This is made possible by the higher process speed of the clinical decision. Price summarizes this fact in the phrase that "the innovation offered by POCT is through the impact of delivering services" [76]. Due to the still existing analytical challenges for the determination of analytes, mostly in the nano- to attomolar concentration ranges, the nascent immunodiagnostic modality should be still under the control of laboratory and healthcare professionals. POCT coordinators from the central laboratory ensure adequate comparability of the analytical performance characteristics of immunodiagnostic POCT methods and central lab-based devices. In this regard, assessment criteria for comparison measurements and implementation of POCT were already described elsewhere [77].

The pending challenges for developers of novel biosensor-based immunodiagnostics for POCT applications constitute often not so much analytical features. Development of an assay for a given analyte that is suitable for POCT with respect to sensitivity, specificity, and reliability is usually feasible. The more important question, however, is about the analyte to be determined: which novel biomarker (or panel of biomarkers) creates genuine added value for the diagnostic process? As such, the search for novel ultralow concentrated biomarkers is the hot lead in POCT development. These markers pose the opportunity to meet clinical needs where the current immunodiagnostic analytes are not sufficiently informative for diagnosis or in the follow-up monitoring of therapy. To give an example: Immunodiagnostics for the measurement of serum concentrations of thyroid-stimulating hormone (TSH) or cardiac troponins have proven to be extremely useful in endocrinology and cardiology. This success evokes the desire in nephrologists to revolutionize the early diagnosis of acute kidney injury (AKI) [78]. The candidate biomarkers kidney injury molecule-1 (KIM-1), neutrophil gelatinase–associated lipocalin (NGAL), tissue inhibitor of metalloproteinases-2 (TIMP-2), and insulin-like growth factor–binding protein 7 (IGFBP-7) were already defined, but none of them or their astute combinations have yet reached significant impact on the diagnostic process of AKI.

As already pointed out in "Multiplexed immunosensors," multiplexed biosensor analytics cause problems in the efficient analysis and interpretation of the substantial dataset generated. Therefore, these techniques are best kept in the hands of experts in central laboratories rather than as a POCT application. This can certainly be generalized also in other areas of immunodiagnostics. In this regard, comprehensive "baseline profiles" produce many false-positive findings as inevitable result from random orders. Thus, experienced diagnosticians are aware of this drawback from large-scale biochemical requests. Here, limitations for clinical applications can only be overcome after subjecting new immunodiagnostic methods to impartial and evidence-based scrutiny.

Emerging trends for immunodiagnostic POCT in the next decade will surely encompass (semi) continuous monitoring of various parameters (e.g., fertility hormones) or therapeutic drug monitoring as self-testing

measurements of various medications (e.g., antiepileptics or antibiotics). Such developments are certainly directed towards the need of the patient and those involved in the care of him. Another trend is towards companion diagnostics (CDx). CDx means diagnostic measurements applied as a companion to a corresponding therapeutic drug for an individual cancer patient in order to estimate its safe and effective use. Whereas until today, most CDx are performed in the pathology lab as tissue-based diagnostics, in the future, more serum-based tests will be established.

Immunodiagnostic POCT applications will have further success in the highly regulated healthcare systems provided that these assays can be run economically in clinical routine work. This applies in particular for hospital applications that only affordable reagents, low-cost microfluidic cartridges, and less-expensive analyzers will be competitive with the comprehensive and cost-effective services of the central laboratory. When establishing novel POCT applications, developers should pay special attention to clinical validation and implementation strategies [8]. This validation mainly takes into account clinical outcomes, costs, or even staff and patient satisfaction.

The idea of wearable immunosensors for patients with chronic diseases such as diabetes mellitus, arthritis, chronic kidney disease, depression, and chronic obstructive pulmonary disease will probably remain a dream. Key enabling technology might be stretchable biosensors that endure the extreme deformations experienced by the human skin. But also, an implantable format for immunosensors is conceivable. All these POCT applications, however, demand for higher accuracy, enhanced stability, and low costs. The creation of such devices will only be realizable after adoption of novel design rules, material, and production processes.

In summary The analytical techniques of POCT immunodiagnostics are premised on diagnostic Abs. The attractive feature is thereby the outstanding sensitivity and selectivity. Novel biomedical engineering processes and innovative analytical concepts enable the analytical transition from classical immunoassays to POCT-appropriate immunosensors, lateral flow assays, and multiplexing platforms.

The (potential) improvement in the treatment and care of a patient by applying these new biosensor-based POCT methods has to be evaluated for each single case in thoroughly performed clinical studies. The first crucial question to be asked is about the diagnostic benefit of the biochemical marker itself. The second question is about the benefit of testing in the specific setting of application. And not before a satisfactory response is given in relation to this issue, the third question has to be answered: should the measurement be performed in a centralized medical laboratory or at the point of care?

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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