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Emerging multianalyte biosensors for the simultaneous detection of protein and nucleic acid biomarkers

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ABSTRACT

Traditionally, biosensors are designed to detect one specific analyte. Nevertheless, disease progression is regulated in a highly interactive way by different classes of biomolecules like proteins and nucleic acids. Therefore, a more comprehensive analysis of biomarkers from a single sample is of utmost importance to further improve both, the accuracy of diagnosis as well as the therapeutic success. This review summarizes fundamentals like biorecognition and sensing strategies for the simultaneous detection of proteins and nucleic acids and discusses challenges related to multianalyte biosensor development. We present an overview of the current state of biosensors for the combined detection of protein and nucleic acid biomarkers associated with widespread diseases, among them cancer and infectious diseases. Furthermore, we outline the multianalyte analysis in the rapidly evolving field of single-cell multiomics, to stress its significance for the future discovery and validation of biomarkers. Finally, we provide a critical perspective on the performance and translation potential of multianalyte biosensors for medical diagnostics.

1. Introduction

The diagnosis of diseases, as well as the prognosis of treatment response, depends on the accurate detection of biomarkers from various biomolecule classes, including proteins and nucleic acids. In general, an early diagnosis allows more early-stage interventions and thus, is fundamental for a successful outcome of the treatment. Hence, *in vitro* diagnostic (IVD) tests have become an indispensable tool in hospitals, laboratories, and home care. Numerous IVD tests are available on the market to analyze biomarkers from various body specimens (i.e. samples) including tissue or body fluids like blood or urine (Kosack et al., 2017; Rohr et al., 2016). In recent years, especially the demand to detect multiple analytes from a single sample (multianalyte detection) has gained in importance. The majority of IVD tests are designed, nevertheless, to detect only one single biomolecule class by using technologies such as polymerase chain reaction (PCR) or immunoassays (Coskun et al., 2019; Dincer et al., 2017).

However, biological processes such as disease development are regulated in a highly interactive way by different biomolecules. Therefore, the analysis of a comprehensive set of biomarkers (including different biomolecule classes) can provide a better picture, enabling an accurate diagnosis and treatment of diseases (Cohen et al., 2018; Falconnet et al., 2015; Langelier et al., 2018; Liu et al., 2020). For example, the treatment outcome of a cancer patient could be improved by increasing the treatment target specificity. A study suggested that this could be achieved by testing patients for highly correlating mRNA transcripts before treating them with a drug against the corresponding protein (Kosti et al., 2016). Also in terms of infectious diseases, it can be very beneficial, to combine the detection of pathogens via nucleic acid analysis with the detection of inflammation biomarkers of the host to enable an early detection of an acute infection and a target-specific therapy (Klebes et al., 2022). Furthermore, the simultaneous detection of different classes of biomolecules (i) allows the detection of multiple biomarkers from a limited sample volume, (ii) increases the density of

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information per sample volume, and (iii) helps to save resources and time (Falconnet et al., 2015; Wang and Walt, 2020).

In this review, we focus on multianalyte biosensors for the simultaneous detection of proteins and nucleic acids in terms of medical diagnostics (Fig. S1 and Table S1). First, we provide an overview on fundamentals for the development of multianalyte biosensors like strategies for biorecognition and sensing of multiple analytes. Second, multianalyte biosensors with a focus on different diagnostic applications (such as cancer and infectious diseases) are highlighted, along with a critical perspective on the performance and translation potential of these multianalyte biosensors into the market.

2. Designing multianalyte biosensors for the simultaneous detection of proteins and nucleic acids

The development of multianalyte biosensors is a very challenging task. Possible concentration differences in biological samples between nucleic acid and protein biomarkers need to be considered. Typically, proteins and nucleic acids are recognized by analyte-specific methods (such as PCR for nucleic acid detection), which often require specific and not compatible reaction conditions (for instance, thermal cycling leads to protein denaturation).

One particular aspect for the development of multianalyte biosensors is the selection of a biorecognition strategy that facilitates simultaneous detection of both biomolecule classes. Furthermore, a suitable multianalyte detection strategy needs to be selected to distinguish between signals from different targets.

2.1. Biorecognition strategies for the simultaneous detection of proteins and nucleic acids

Biorecognition elements like antibodies or aptamers are typically used for the detection of proteins (Fig. 1A), whereas oligo reporter probes are typically used for the detection of nucleic acids (Fig. 1B). Protein-binding biorecognition elements can also be linked to reporter oligos. Thus, the abundance of a target protein is encoded into a nucleic acid-based recognition (protein-to-nucleic acid transformation) and can be detected via nucleic acid hybridization (Fig. 1C). Similarly, reporter oligo probes can be linked to antigenic tags (such as digoxigenin). Using amplification, antigenic tags can be incorporated into the target nucleic acid (nucleic acid-to-protein transformation). Thus, the abundance of a target nucleic acid is encoded into a protein-based recognition and can be detected via antibody-antigen interaction (Fig. 1D).

To simultaneously detect both biomolecule classes, two different biorecognition strategies can be used (Fig. S1E). The first strategy combines antibody-antigen interaction (Fig. 1A) and nucleic acid hybridization (Fig. 1B). Therefore, such a biosensor needs to combine two different biorecognition methods for the simultaneous detection of both biomolecule classes. For example, Wang et al. developed a multi-analyte Simo® assay that employs specific antibodies and probes conjugated to dye-encoded paramagnetic beads to detect the corresponding protein and nucleic acid biomarkers (Wang and Walt, 2020).

The second strategy uses transformative biorecognition to encode the abundance of one biomolecule class into the other biomolecule class. Subsequently, both biomolecule classes can be detected with the same biorecognition method using a nucleic acid-based or protein-based recognition. Thus, this biorecognition strategy can be further broken down into the following strategies: (i) Multianalyte biosensors that combine protein-to-nucleic acid transformation (Fig. 1C) and nucleic acid hybridization (Fig. 1B) to detect both biomolecule classes via nucleic acid-based recognition. For example, Dong et al. used primer-ligated aptamers to capture target proteins. Employing multiplex-PCR target RNA and primer-ligated aptamer captured target proteins are detected simultaneously (Dong et l. 2020). (ii) Multianalyte biosensors that combine nucleic acid-to-protein transformation (Fig. 1D) and antibody-antigen interaction (Fig. 1A) to detect both biomolecule classes via protein-based recognition. For example, Klebes et al. introduced antigenic-labels via isothermal amplification into target DNA.

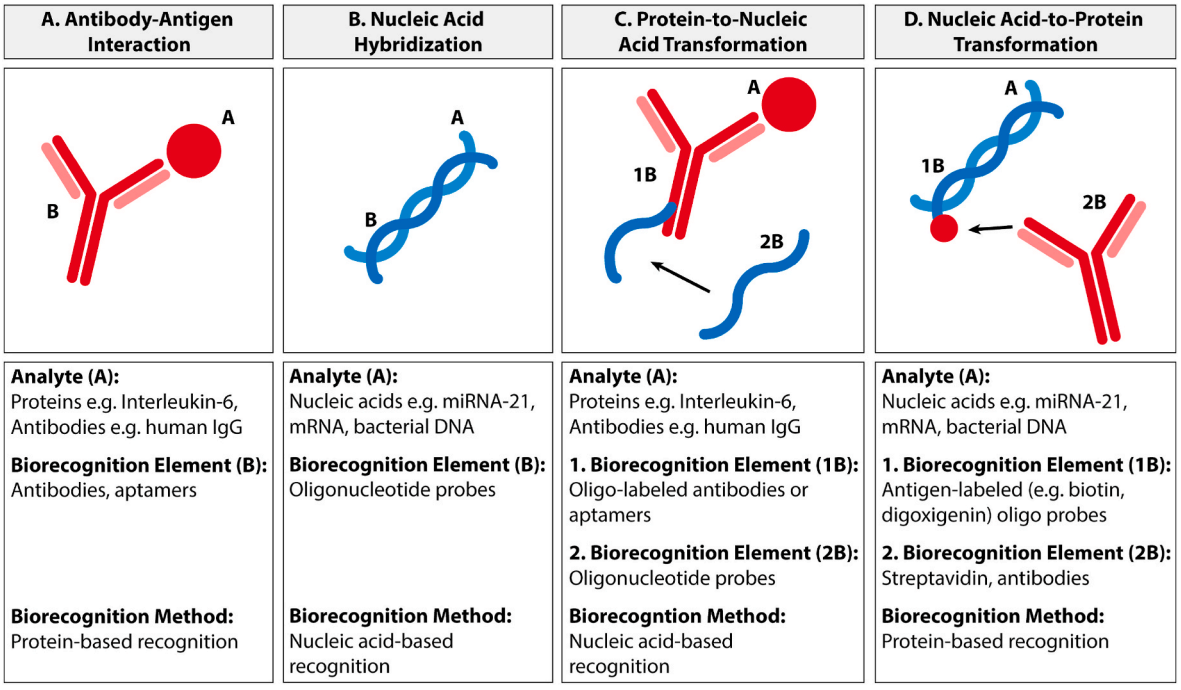

Fig. 1. Biorecognition of proteins and nucleic acids. (A) Detection of proteins via antibody-antigen interaction. **(B)** Detection of nucleic acids via hybridization of oligo probes. **(C)** Protein-to-nucleic acid transformation to encode the abundance of a protein into a nucleic acid-based recognition. This is realized by using oligo labeled protein-binding molecules (first biorecognition element) and oligo probes (second biorecognition element). **(D)** Nucleic acid-to-protein transformation to encode the abundance of a nucleic acid into a protein-based recognition. This is realized by using antigen-labeled oligo probes (first biorecognition element) and protein-binding molecules (second biorecognition element).

Subsequently, an immunoassay was used to detect target proteins and antigen-labeled amplicons (Klebes et al., 2022).

2.2. Signal transduction strategies for multianalyte sensing

Transducers convert biorecognition events into measurable signals (Fig. S1F) (Bhalla et al., 2016; Dincer et al., 2019). To distinguish between signals for multiple target analytes, three different detection strategies can be used (Fig. S1G).

The first strategy spatially separates biorecognition elements by different wells or spots. Thereby, the identity of the biorecognition element is determined by its spatial location (Cohen and Walt, 2019; Dincer et al., 2017). For example, Scott et al. developed a microarray biosensor that detects microRNAs (miRNAs) and proteins. The corresponding biorecognition elements are spotted onto the array surface with specific x-y-coordinates (Scott et al., 2017).

The second strategy regionally separates the biorecognition elements by using discrete regions (Dincer et al., 2017). This strategy is for example used by lateral flow assays (LFAs). The sample moves via capillary forces through discrete regions on the nitrocellulose and interacts with the corresponding biorecognition elements. For example, Klebes et al. developed a LFA, which simultaneously detects proteins and antigen-labeled amplicons on two regionally separated test lines (Klebes et al., 2022).

The third strategy uses encoded labels. Each uniquely encoded population of labels is functionalized with an individual biorecognition element. Bead-based technologies frequently use optical encoding with different fluorescent dyes or quantum dots. Another option is digital encoding. Falconnet et al. developed digitally encoded disc-shaped silicon microparticles which contain 10 binary bits represented by the presence or absence of small holes (Falconnet et al., 2015). Biorecognition elements linked to barcodes, such as color-coded molecular barcodes (nanoString Technologies, Inc.; Warren, 2018) or nucleic acid barcodes (Peterson et al., 2017), offer another way of encoding.

3. Diagnostic applications of multianalyte biosensors

Biological processes such as disease development and progression are regulated in a highly interactive way by different biomolecule classes. Therefore, the simultaneous detection of protein and nucleic acid biomarkers can provide, for example, more comprehensive information on tumors. Numerous types of cancer are for example associated with the upregulation of miRNA-21 (Feng and Tsao, 2016) and increased telomerase levels (Kim et al., 1994). A study determining the correlation between protein and gene expression of cancer tissue observed changes within the correlation compared to healthy tissue. Therefore, they concluded that it would be beneficial to test a patient for highly correlated mRNA transcripts before treating them with a drug against the corresponding protein. This strategy could improve the treatment outcomes by increasing the treatment target specificity (Kosti et al., 2016). Furthermore, the cross-validation of a cancer biomarker by its different molecular forms can also increase the accuracy of a diagnostic test. Single-point mutations, for example, often face poor detection specificity due to cross-reactivity with wild type or other mutation sequences (Dey et al., 2019). In terms of infectious diseases, it is important to enable an early detection of an acute infection and a target specific therapy. This can be facilitated via simultaneous detection of the pathogenic DNA and host immune response markers such as interleukins (Klebes et al., 2022) or virus-specific antibodies (Najjar et al., 2022). Furthermore, for infectious diseases such as HIV, the simultaneous detection of pathogenic nucleic acids and pathogen-specific antibodies can help to reduce the diagnostic window between infection and seroconversion (Chen et al., 2013).

The majority of the existing multianalyte biosensors address the simultaneous detection of protein and nucleic acid biomarkers associated with cancer or infectious diseases. However, these biosensors are at the early development status (TRL 3–4) and there is currently no commercial device for routine. Here, we provide a detailed overview of emerging multianalyte biosensors for various diagnostic applications. In addition, we summarize multiomic approaches for single-cell analysis, which can provide a comprehensive overview of cellular functions associated with diseases and thereby offer a valuable tool for biomarker discovery, evaluation, and drug discovery.

3.1. Multianalyte biosensors for cancer

To implement personalized medicine, cancer research aims to develop sensitive, specific, and cost-effective diagnostic strategies for clinical use. In this regard, liquid biopsy offers, in contrast to the traditional surgical tumor biopsy, an attractive sampling approach. Multiple biomarkers are described in the context of liquid biopsy including circulating tumor cells (CTCs), cell-free nucleic acids, extracellular vesicles (EVs), or circulating proteins (Arechederra et al., 2020; Bratulic et al., 2021; Hofman et al., 2019; Zhou et al., 2021b). However, the requirements (accuracy and cost-effectiveness) of current biosensors and the technical variability in the pre-analytical steps are hampering the transition into clinical practice (Alix-Panabières, 2020; Hofman et al., 2019). In order to overcome these issues, approaches assessing multiple targets at the same time can help to enhance diagnostic tests by enabling a more comprehensive analysis or the cross-validation of a biomarker by its different molecular forms (such as messenger RNA (mRNA) and protein levels).

CTCs can provide an alternative source of proteomic and transcriptomic information. However, the greatest challenge for CTC detection is their low quantity. Therefore, a variety of CTC enrichment methods have been developed, reaching from affinity-based methods (such as antibody-conjugated magnetic beads), density-based methods (like Ficoll–Hypaque separation), or size-based microfilters (Agarwal et al., 2018).

The integrated multi-molecular sensor (IMMS) was developed to detect both, B-type *Raf* Kinase $(BRAF)^{V660E}$ DNA and protein from CTCs. The IMMS integrates three different zones for the capture and release of CTCs, lysis, and electrochemical detection of DNA and proteins. Anti-melanoma associated chondroitin sulfate proteoglycan antibodies are used to capture CTCs within a long serpentine microchannel with an array of parallel electrode pairs along the channel. A direct current field is applied to release the captured CTCs. Subsequently, an electrical field is used to lyse the CTCs. Individual detection zones on the IMMS are used to detect target DNA via ligase-mediated amplification and the corresponding proteins via antibodies (Dey et al., 2019).

Surface-enhanced Raman scattering (SERS) was utilized for the *in situ* detection of miRNA-21 and telomerase in cancer cells. DNA-programmed gold nanorods dimer-upconversion nanoparticles core-satellite (Au NR Dimer-UCNP-CS) nanostructures are used as SERS- and luminescence-based probes. Hybridization of miRNA-21 to molecular beacons of the Au NR Dimer-UCNP-CS nanostructures causes the separation of the dimers, resulting in a decrease of the Raman signal. In the presence of telomerase, the specific telomerase primer strands are elongated, resulting in substitutional hybridization and release of upconversion nanoparticles of the Au NR Dimer-UCNP-CS nanostructure. This leads to an increase in luminescence. Therefore, the authors used a two-signal approach to realize the quantitative detection of different target biomarkers within cells without cell extraction (Ma et al., 2017).

Apart from CTCs, EVs such as exosomes can be used for cancer diagnostics. EVs are released by all living cells and have a subcellular structure formed from bilayer lipid membranes. They contain diverse biomolecules from the cell of origin including proteins and nucleic acids. Studies showed that cancer processes are mediated via EVs (Yokoi and Ochiya, 2021; Zhou et al., 2021a). Therefore, the analysis of tumor-derived exosomes can provide crucial information for cancer diagnosis and prognosis (Dong et al., 2020; Lim et al., 2019; Zhou et al.,

2020). However, the isolation and purification of exosomes from serum is a critical step and prone to several challenges like the high abundance of serum proteins and non-EV lipid particles. A variety of methods for the isolation of exosomes are described in the literature including ultracentrifugation, polymer-based precipitation, size exclusion chromatography, and density gradient centrifugation (Brennan et al., 2020; Lim et al., 2019). To capture exosomes, antibody- (Lim et al., 2019) or phospholipid- (Dong et al., 2020) conjugated magnetic particles can be used and combined with a diethiothreitol-mediated release. Another option is the application of cationic lipoplex nanoparticles that capture EVs through electrostatic interaction (Zhou et al., 2020).

The simultaneous detection of surface proteins and nucleic acids from the lumen of exosomes can be achieved, for example, via aptamer assisted multiplex-PCR (Fig. 2A). Here primer-ligated aptamers that bind to surface proteins are used for protein-to-nucleic acid transformation. Subsequently, PCR amplification is performed to detect both biomarkers (Dong et al., 2020). Another study showed the simultaneous detection of miRNA-21, Programmed Cell Death Ligand 1 (PD-L1) mRNA and protein at a single-vesicle level using a High-throughput Nano-Biochip Integrated System for Liquid Biopsy (HNCIB). The HNCIB biosensor consists of (i) a nano-biochip with immobilized lipoplex nanoparticles for EV enrichment, (ii) a total internal reflection fluorescence microscopy imaging system, and (iii) a deep learning algorithm for automated imaging analysis. Proteins are detected via immunostaining using fluorescent antibodies, whereas RNAs are detected via molecular beacons. They are transferred to the lumen by the fusion of EV particles with lipoplex nanoparticles (containing molecular beacons). Hybridization of the molecular beacon to its target leads to the separation of fluorophore and quencher resulting in the generation of fluorescence signal (Zhou et al., 2020).

Besides EV, nucleic acids and proteins are released from cancer cells or supporting tissue into the blood circulation. Therefore, they can serve as cancer biomarkers to determine molecular alteration in a tumor or measure the tumor load and metastatic potential (Lokshin et al., 2021). Xu et al. developed a superwettable microchip that integrates nanodendritic structures for electrochemical detection and superhydrophobic-superhydrophilic micropatterns which are able to confine microdroplets in microwells (Fig. 2C). Thus, using spatial separation, they were able to detect multiple circulating prostate cancer biomarkers (miRNA-375, miRNA-141, and prostate-specific antigen (PSA)). Redox-reporter modified DNA probes are applied for the detection of miRNA-375 and miRNA-141, while PSA detection is carried out using reporter-modified aptamers. Binding of the analyte leads to a conformational change of the biorecognition element and thus, alters

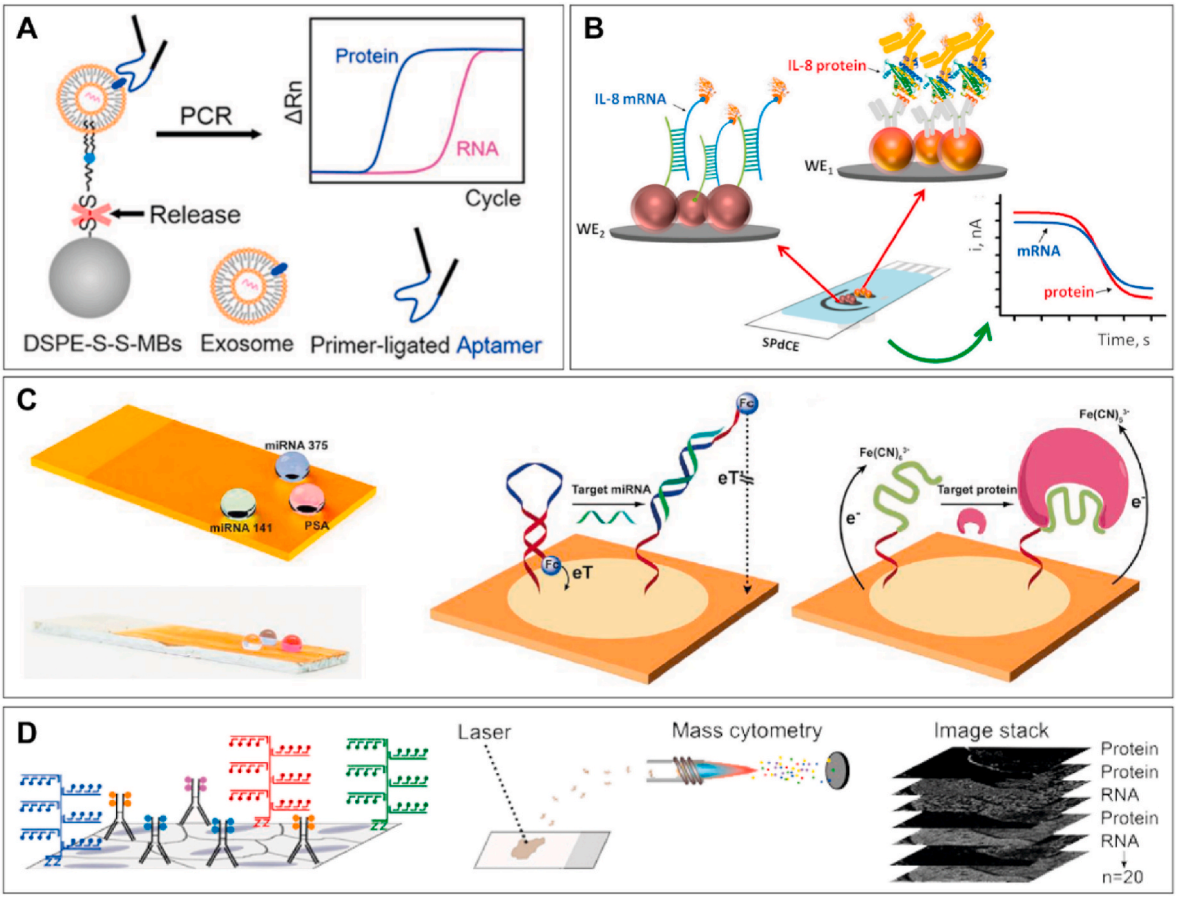

Fig. 2. Multianalyte biosensors for the simultaneous detection of nucleic acids and proteins associated with cancer. (A) Simultaneous detection of surface proteins and nucleic acids from the lumen of exosomes via aptamer assisted multiplex-PCR. Protein-to-nucleic acid transformation is implemented by using primer-ligated aptamers. Reprinted with permission from (Dong et al., 2020). Copyright 2020 American Chemical Society. **(B)** Dual amperometric magneto-biosensor for the simultaneous detection of IL-8 protein and mRNA from saliva. Magnetic beads functionalized with oligo probes and antibodies are used for biomarker isolation and detection on the working electrodes. Reprinted with permission from (Torrente-Rodríguez et al., 2016). Copyright 2016 Elsevier. **(C)** Superwettable microchip utilizing spatial separation for the detection of prostate cancer biomarkers. miRNAs are detected via redox-reporter modified DNA probes. Protein detection was carried out using aptamers. Reprinted with permission from (Xu et al., 2018). Copyright 2018 American Chemical Society. **(D)** Simultaneous detection of proteins, protein phosphorylations, and transcripts from tissue samples. Proteins are labeled via immunostaining, whereas *in situ* hybridization is utilized for mRNA labeling. Detection is enabled via laser ablation and mass-cytometric analysis. Reprinted with permission from (Schulz et al., 2018). Copyright 2018 Elsevier (CC BY-NC-ND 4.0).

the position of the reporter relative to the electrode surface. This results in a target dependent change in current leading to a signal decrease (Xu et al., 2018).

Another option for the detection of circulating proteins and RNAs is gold nanoparticles. For example, biotin-polyethylene glycol (PEG)-gold nanoparticles can be used to capture biotinylated target RNA and biotinylated detection antibodies binding to the target protein. Using capture antibodies and specific probes spotted onto an array-based multianalyte biosensor, the complexes can be detected via light-scattering (Scott et al., 2017). Gold nanoparticles were also used for a dual-functional DNA tweezer (DFDT) approach detecting miRNA-21 and Mucin-1 (MUC1). This "W-type" DFDT consists of three single-stranded oligos (two gold nanoparticle attached central stands and an arm strand dually labeled with fluorophores). In the presence of miRNA-21 and MUC1, two dependently displaced fuel strands hybridize to unpaired segment overhangs of the DFDT. This leads to a conformational change of the DFDT from an open to a closed state and the fluorescence emission is quenched (Yang et al., 2019). Similarly, a label-free approach uses a single substrate probe for the simultaneous detection of telomerase protein and RNA. The multianalyte biosensor uses a deoxyuridine/biotin-modified molecular beacon (dU-BIO-HP) that contains (i) a deoxyuridine/biotin in the side arm, (ii) a telomerase RNA recognition sequence in the loop, and (iii) a telomerase substrate primer at the stem end. In the presence of telomerase RNA, the dU-BIO-HP hybridizes with the target and opens its stem. This leads to the initiation of a DNA toehold strand displacement amplification (SDA) reaction, leading to single-stranded DNAs (ssDNA). Similarly, in the presence of telomerase protein, the stem of the dU-BIO-HP is elongated using an assistant DNA and complementary telomere repeats. This initiates a SDA reaction, leading to G-quadruplex monomers. Sybr green is used for the detection of short ssDNAs, whereas zinc protoporphyrin IX forms a duplex with the G-quadruplexes (Yin et al., 2019).

Simultaneous detection of circulating cancer markers can also be achieved by using resonance light scattering. The authors designed two complementary DNAs (cDNAs) which target miRNA-122 and alpha-fetoprotein (AFP). cDNA1 contains an AFP aptamer and segments that is partially the sequence of miRNA-122. cDNA2 consists of a miRNA-122 complementary sequence and a segment that is partially complementary to the aptamer. The resonance light scattering is constructed via the electronic interaction between dsDNA (hybridized cDNA1 and cDNA2) and methyl violet. The binding of miRNA-122 leads to the release of methyl violet, resulting in a signal decrease. Whereas binding of AFP leads to the formation of a new and bigger complex thus resulting in signal increase (Chen et al., 2018).

Cancer-related nucleic acids and proteins have also been identified in body fluids like saliva. For example, a concordance between the chemokine Interleukin-8 (IL-8) protein and mRNA levels in the salvia of oral cancer patients. To isolate biomolecules from complex samples like saliva magnetic beads functionalized with probes or antibodies can be used. To detect both biomarkers via dual amperometric magneto-biosensor (Fig. 2B), the captured IL-8 protein is sandwiched with a second antibody, labeled with horseradish peroxidase (HRP), whereas the captured and biotinylated target DNA is labeled with a streptavidin-HRP conjugate. For signal readout, the magnetic beads are captured to the corresponding working electrodes of the amperometric sensor (Torrente-Rodríguez et al., 2016).

The ability to link transcript, protein, and signaling networks in tissues could enable the discovery of novel routes for intervention, identification of biomarkers, and the assessment of drug efficacy. Thus, the spatial resolution of biomarkers within tissue samples is an important factor for precision medicine.

To realize the simultaneous detection of proteins, protein phosphorylations, and transcripts, imaging mass cytometry has been used. This method relies on staining and subsequent detection of tags in tissue samples using a mass spectrometer (Fig. 2D). The approach uses serial steps of *in situ* hybridization to build a large DNA tree across the target RNA. Metal-labeled oligos and antibodies are used to stain the target analytes. Mass-cytometric analysis of metal abundances is deployed for the detection and assembly of the metal abundance per laser shot into a high-dimension image (Schulz et al., 2018). Also, the NanoString nCouter® analysis system combined with the 3D Biology™ technology can be used for solid tumor profiling, allowing a comprehensive coverage of canonical signaling pathways. Antibodies that are conjugated to a DNA oligos are used to transform the abundance of proteins into a nucleic acid-based recognition. Subsequently, the DNA oligos are released and captured and reporter probes hybridize to DNA oligos and target nucleic acids. An electric current is applied to align and stretch the complexes at the cartridge surface. Color-coded molecular barcodes are used for quantification (nanoString Technologies, Inc.; Warren, 2018).

The multianalyte biosensors for the detection of nucleic acid and protein biomarkers associated with cancer either employ a nucleic acid-based recognition (Chen et al., 2018; Dong et al., 2020; Ma et al., 2017; Yang et al., 2019; Yin et al., 2019) or combining antibody-antigen interaction and nucleic acid hybridization (Dey et al., 2019; Schulz et al., 2018; Scott et al., 2017; Torrente-Rodríguez et al., 2016; Zhou et al., 2020) (**Table S2**). The majority uses encoded labels to enable multianalyte detection. Several multianalyte biosensors (Chen et al., 2018; Dey et al., 2019; Schulz et al., 2018; Scott et al., 2017; Torrente-Rodríguez et al., 2016; Zhou et al., 2020) detect cancer biomarkers by their different molecular forms (for example PD-L1 protein and mRNA), which could be beneficial for medical diagnostics as it enhances the accuracy of the test result.

Several approaches have the potential to be integrated at the point-of-care (POC), thanks to their low time to result and/or low system complexity. However, the transition of diagnostics from centralized laboratories to POC has not been made yet. The first requirement for this transition is whether the POC test meets the REASSURED criteria (Real-time connectivity, Ease of specimen collection, Affordable, Sensitive, Specific, User-Friendly, Rapid and Robust, Equipment free or simple, Environmentally friendly, Deliverable to end-users) (Land et al., 2019; Otoo and Schlappi, 2022) or not. Second, the majority of the approaches do not integrate sample preparation and/or require several manual steps. Therefore, future developments should focus on the integration of sample preparation and processing. This also applies to laboratory-based tests to improve repeatability of the diagnostic test and decrease user-related errors.

3.2. *Multianalyte biosensors for infectious diseases*

Emerging infectious diseases play a public health problem, either due to newly appearing or rapidly spreading infectious diseases (World Health Organization. Regional Office for South-East Asia. 2014). In extreme cases, emerging infectious diseases may cause pandemics such as the recent outbreak of COVID-19 (Morens and Fauci, 2020), which underlines the importance of rapid and accurate detection of pathogens.

Gold standard methods for pathogen identification are culture and microscopy-based techniques, laboratory-based immunoassays and nucleic acid amplification tests (NAATs). Thereby, the processing time varies from several hours (NAATs) to several days (culture-based detection). In addition, these methods require a specific infrastructure and trained laboratory personnel (Phaneuf et al., 2018). Therefore, rapid antigen tests have become (due to their short turnaround time, POC feasibility, and affordability) a widely used tool for SARS-CoV-2 diagnostics (Loeffelholz and Tang, 2020; Wagenhäuser et al., 2021). Both biorecognition methods, rapid antigen tests and PCR, have their advantages (speed or sensitivity), but neither meets all desirable criteria (speed and sensitivity). A recent study showed, that combining rapid antigen tests with a PCR-based test strategy can help to detect patients with a high SARS-CoV-2 viral load (Wagenhäuser et al., 2021).

A combined detection of SARS-CoV-2 antigen and RNA from nasopharyngeal swab samples is enabled via an anti-resonant-optical-waveguides (ARROW) photonic biosensor. Magnetic beads

functionalized with nucleic acid probes or antibodies are used to capture the target biomarkers. Antigen-to-nucleic acid transformation is achieved by using a dibenzocyclooctyne labeled antibody, which is tagged with fluorescently labeled DNA reporter probes. The DNA reporter probes are cleaved off by ultraviolet exposure, while the target RNA is thermally eluted from the magnetic beads and fluorescently tagged with a POPO-3 nucleic acid staining dye. Simultaneous quantification of both biomarkers is performed via a multi-channel optofluidic waveguide chip (Meena et al., 2021). This approach was also implemented for the detection of Zika-virus related nucleic acid and protein biomarkers (Stambaugh et al., 2018).

In addition to the pathogen itself, pathogen-specific antibodies or host inflammation biomarkers can be detected in body fluids. For example, the simultaneous detection of pathogens and host immune response biomarkers like IL-6 can facilitate the early detection of an acute infection (Klebes et al., 2022). This could also help to reduce antibiotic misuse, as the current therapy is mostly based on empiric antimicrobial treatment (Llor and Bjerrum, 2014). Similarly, the combined detection of viral RNA and virus-specific antibodies could close or reduce the diagnostic window between infection and seroconversion and can provide insight into disease progression and severity (Meena et al., 2021; Najjar et al., 2022).

The simultaneous detection of viral RNA and IgG antibodies against the S1, S1-RBD, and N protein of SARS-CoV-2 from saliva samples is enabled via a 3D-printed lab-on-a-chip (LOC) platform containing an electrochemical biosensor (Fig. 3A). All relevant steps from sample preparation to readout are integrated (Fig. 3B). The sample is split and transferred into (i) an antibody detection reservoir or (ii) a sample preparation reservoir for RNA extraction. RNA is amplified by a loop-mediated isothermal amplification (LAMP)-CRISPR-based assay and detected on the biosensor electrodes. For the simultaneous detection, four individual electrodes are used. The electrochemical readout is performed by using HRP and 3,3′,5,5′-Tetramethylbenzidine (TMB) (Najjar et al., 2022). Johnston et al. introduced a microfluidic multiplexed biosensor (BiosensorX) which comprises a single-cannel with sequentially arranged incubation areas (Fig. 3C). The approach combines the detection of multiple viral RNAs with the detection of β-lactam and thus allows therapy monitoring of bacterial co- or superinfection in COVID-19 patients. COVID-19-specific RNA is detected from nasal swabs via CRISPR Cas13a-powered assay. The reaction mix contains Leptotrichia buccalis (Lbu)Cas13a, a target-complementary CRISPR RNA (crRNA), and the reporter RNA (reRNA) labeled with biotin and 6-FAM. In the presence of the target RNA, reRNAs are trans-cleaved by the Lbu-Cas13a-crRNA complex. Non-cleaved reRNAs are binding to the immobilization area and are detected via enzyme-labeled antibodies. β-lactam detection from serum was achieved using the competitive binding of piperacillin-tazobactam and/or biotinylated ampicillin to the penicillin binding protein 3. The enzyme glucose oxidase is applied for amperometric signal generation, which is inversely proportional to the amount of detected analyte (Johnston et al., 2022).

Phaneuf et al. developed a centrifugal microfluidic-based multianalyte biosensor for the detection of three enterotoxins (cholera toxin, Staphylococcal enterotoxin B, and Shiga-like toxin 1) and three enteric bacteria (*Campylobacter jejuni*, *Escherichia coli*, and *Salmonella typhimurium*), associated with diarrheal disease. Nucleic acid detection is enabled via LAMP, whereas proteins are detected via immunoassay. The disk comprises two sample inlets, nine immunoassay chambers, and nine amplification chambers, which are connected with a zigzag-style aliquoting channel. A non-contact heating system generates the required temperature of 65 °C for the LAMP reaction. To protect the immunoassay reagents from high temperatures, a reflective mask is implemented (Phaneuf et al., 2018).

Another microfluidic technology, named Evalution®, uses digitally encoded microparticles. The microfluidic cartridge consists of 16 microscale channels containing the microparticle mixes. Each channel connects a waste reservoir and an inlet well, thus 1 to 16 samples can be processed sequentially or simultaneously. The target cytokines or antibodies are premixed with fluorescently labeled antibodies and subsequently bind on the flow to the correspondingly encoded microparticles. For the detection of pathogenic DNA (respiratory syncytial virus A and B), labeled dsDNA amplicons are added to the cartridge. The dsDNA is denatured on the flow using a temperature-controlled transit zone and subsequently can hybridize to corresponding encoded microparticles (Falconnet et al., 2015).

The Rheonix CARD® is a microfluidic platform that can be used for the detection of antigens or antibodies together with nucleic acids from HIV. Thus, aiming to decrease the diagnostic window between infection and seroconversion. The multianalyte biosensor integrates sample dilution, lysis, nucleic acid purification, amplification, and detection via lateral flow immunoassay (LFIA). Since the protocols for nucleic acid and antibody detection are not compatible, the sample is split. The portion for antibody detection is directly transferred to the LFIA, whereas the other part for nucleic acid analysis is further processed (i.e., lysis, extraction, amplification, and labeling) before the labeled target amplicons are detected on a second LFIA (Chen et al., 2013). A LFIA was also implemented for the simultaneous detection of pathogenic DNA and inflammation markers (Fig. 3D). This approach employs similar to the Rheonix CARD® platform a nucleic acid-to-protein transformation-based biorecognition strategy. However, in contrast to the above-described approach the sample is not split. Here, protein-compatible isothermal amplification and labeling of target DNA in the presence of the target protein is performed. Subsequently, the double-labeled target amplicon and the target protein are detected via LFIA (Klebes et al., 2022).

The simultaneous detection of pathogens along with quantitative detection of lactoferrin, provides relevant information regarding the diagnosis of urinary tract infections. Mohan et al. developed a multianalyte biosensor consisting of an array of 16 sensors, functionalized with capture probes or capture antibodies. For the detection of urinary tract infection-associated pathogens, hybridization of the target 16S ribosomal RNA to the corresponding capture and detection probe is used. In the case of target protein detection, a sandwich assay is formed by capture and detection antibodies. Both assays are coupled to an HRP-based redox reaction, resulting in a quantifiable electrical signal. To enable simultaneous detection via different biorecognition methods, sets of oligo probes are optimized for hybridization at 37 °C (Mohan et al., 2011).

Functional nucleic acids like aptamers and DNAzymes, have become increasingly popular over the last years. Furthermore, they facilitate new developments in terms of multianalyte detection of proteins and nucleic acids. Montserrat Pagés et al. developed a DNA-only bioassay for the simultaneous detection of thrombin and target nucleic acids (Fig. 3E). The multianalyte biosensor employs two aptazymes (a group of functional nucleic acid that merges aptamers and DNAzymes) for the detection of protein and nucleic acid targets. The first aptazyme (Fig. 3E, top) contains a thrombin binding aptamer for target capturing and an RNA-cleaving DNAzyme for signal generation. In contrast to the standard DNAzymes, the authors used a DNAzyme that is optimized to work at room temperature. The second aptazyme (Fig. 3E, bottom) comprises a probe for nucleic acid target capturing and a RNA-cleaving DNAzyme for signal generation. Furthermore, both aptazymes are blocked with a complementary inhibitory sequence. The addition of target biomarkers removes the inhibitory sequence and activates the DNAzyme which cleaves a reporter nucleic acid, leading to a fluorescence signal increase (Montserrat Pagès et al., 2021).

Table S3 summarizes multianalyte biosensors for the simultaneous detection of nucleic acid and protein biomarkers associated with infectious diseases. Most biosensors combine antibody-antigen interaction and nucleic acid hybridization (Falconnet et al., 2015; Mohan et al., 2011; Phaneuf et al., 2018; Stambaugh et al., 2018). However, there are also multianalyte biosensors using the same biorecognition method, by combining protein-to-nucleic acid transformation and nucleic acid

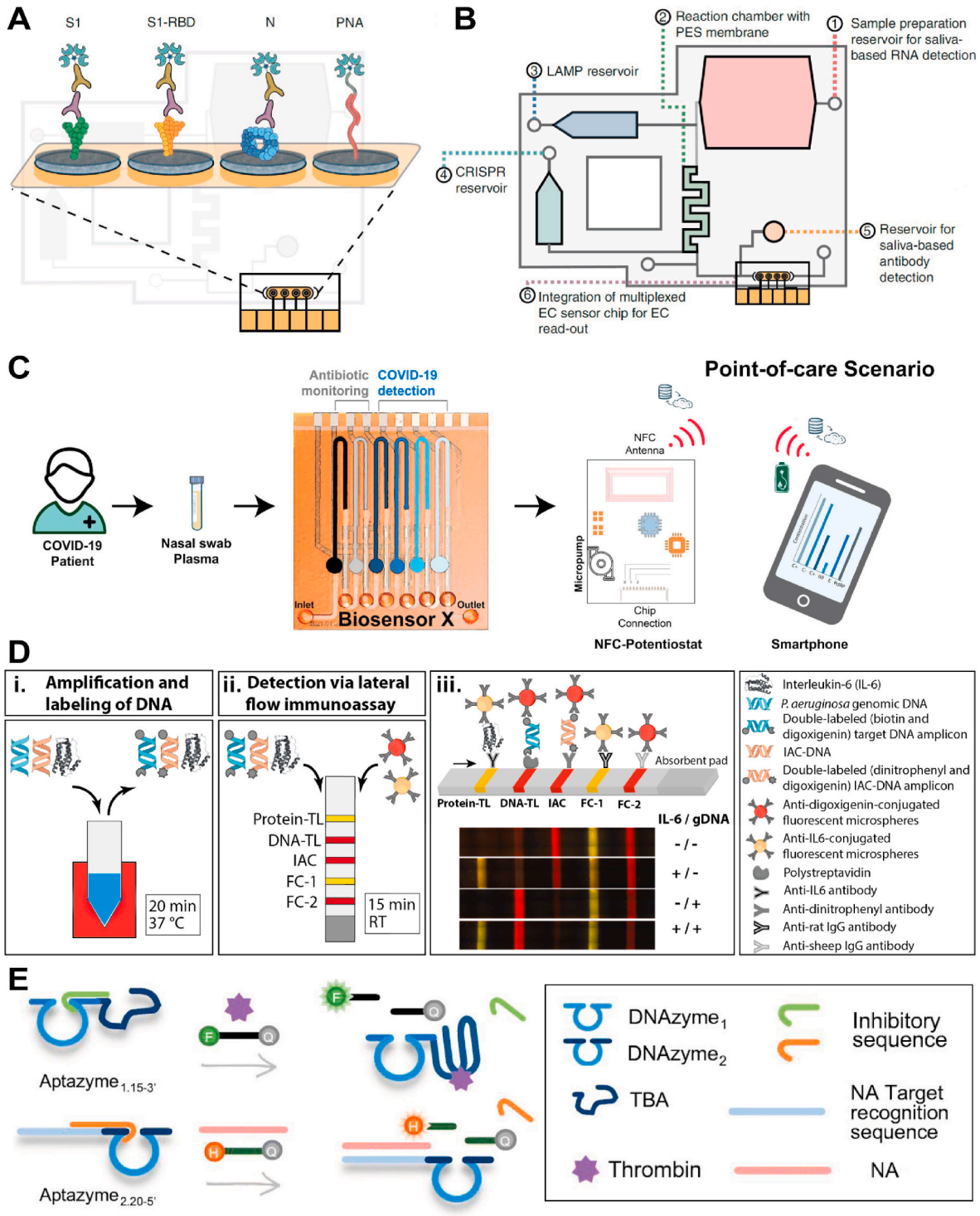

Fig. 3. Multianalyte biosensors for the detection of nucleic acid and protein biomarkers for infectious diseases (A) Electrochemical LOC-based biosensing platform for the simultaneous detection of SARS-CoV-2 RNA and host IgG antibodies. Working electrodes are functionalized with SARS-CoV-2 antigens for the detection of host antibodies and PNA for the detection of RNA. **(B)** Structure of the platform integrating sample preparation, LAMP-CRISPR-based assays, and electrochemical biosensor. Reprinted with permission from (Najjar et al., 2022). Copyright 2022 The Author(s), under exclusive license to Springer Nature Limited. **(C)** BiosensorX with sequentially arranged incubation areas for the combined detection of SARS-CoV-2 viral RNA from nasal swab samples and β-lactam from plasma samples. To enable on-site detection the biosensor is combined with a near field communication (NFC) potentiostat and a micro peristaltic pump. Reprinted with permission from (Johnston et al., 2022). Copyright 2022 Elsevier (CC BY 4.0). **(D)** Multianalyte LFIA for the simultaneous detection of bacterial DNA and host inflammatory biomarkers. (i.) Recombinase polymerase amplification is used to amplify and label DNA with antigenic tags. (ii.) Both biomolecules are detected via LFIA using antibody-conjugated beads. (iii.) Schema of the LFIA. Reproduced with permission from (Klebes et al., 2022). Copyright 2022 Elsevier (CC BY-NC-ND 4.0). **(E)** DNA-only multianalyte biosensor using aptazymes for the simultaneous detection of thrombin and nucleic acids. $Aptazyme_{1.15\text{-}3'}$ contains a thrombin binding aptamer to capture the corresponding protein and an RNA-cleaving DNAzyme for signal generation. $Aptazyme_{2.20\text{-}5'}$ contains instead of the aptamer a probe that captures the target nucleic acid. Binding of the biomarkers removes the inhibitory sequence and activates the DNAzyme, which cleaves a reporter nucleic acid, leading to a fluorescence signal increase. Adapted with permission from (Montserrat Pagès et al., 2021). Copyright 2021 Springer Nature.

hybridization (Meena et al., 2021; Montserrat Pagès et al., 2021) or by combining nucleic acid-to-protein transformation and antibody-antigen interaction (Chen et al., 2013; Klebes et al., 2022). In contrast to multianalyte biosensors for cancer diagnostics, various strategies for the sensing of multiple analytes are used. All approaches detect bacterial or viral nucleic acids to identify the corresponding pathogen within bodily fluids. Pathogens can also be measured via pathogen-specific proteins like the nucleocapsid antigen of SARS-CoV-2. To combine the benefits of both tests, some examples detect pathogen specific DNA/RNA and antigen (Meena et al., 2021; Stambaugh et al., 2018). Another interesting combination is the simultaneous detection of bacterial DNA and toxins. Bacterial toxins are, for example, determined in stool samples for diarrheal disease (Phaneuf et al., 2018). Several multianalyte biosensors combine the detection of pathogens and protein biomarkers of the host, including inflammation markers (Falconnet et al., 2015; Klebes et al., 2022), antibodies (Chen et al., 2013) or other markers like lactoferrin (Mohan et al., 2011) and thrombin (Montserrat Pagès et al., 2021). This combination is very beneficial to determining clinically relevant infections, monitoring infections, or closing the diagnostic window.

We observed, that several approaches have the potential to be used at the POC. The current SARS-CoV-2 pandemic once more has emphasized the importance of POC diagnostics for prevention and infection control. Despite the already promising advances in the multianalyte diagnostic tests for POC detection of infectious diseases, the development of devices meeting the REASSURED criteria is very challenging and not fully met yet. To achieve these goals, highly integrated devices need to be developed, which can perform sample-to-result detection (Wang et al., 2021). However, currently, the majority of multianalyte approaches still require manual pipetting steps and/or extensive sample preparation. The evaluation of a suspected infectious disease is often a time critical event therefore, diagnostics would benefit significantly from a fast and reliable multianalyte POC platform.

3.3. *Multianalyte biosensors for further diseases*

Besides cancer and infectious diseases, biomarkers can be used for the diagnosis of other diseases including Alzheimer's, cardiovascular, and liver diseases. Table S4 summarizes multianalyte biosensors that target biomarkers for diseases other than cancer and infectious diseases.

In cardiovascular diseases, several biomarkers have been identified including protein biomarkers such as C-reactive protein (CRP), brain natriuretic peptide (BNP), and Low-density Lipoprotein (LDL), as well as nucleic acid biomarkers such as cell-free mitochondrial DNA (cfmDNA). Dinter et al. developed a microfluidic platform that detects these

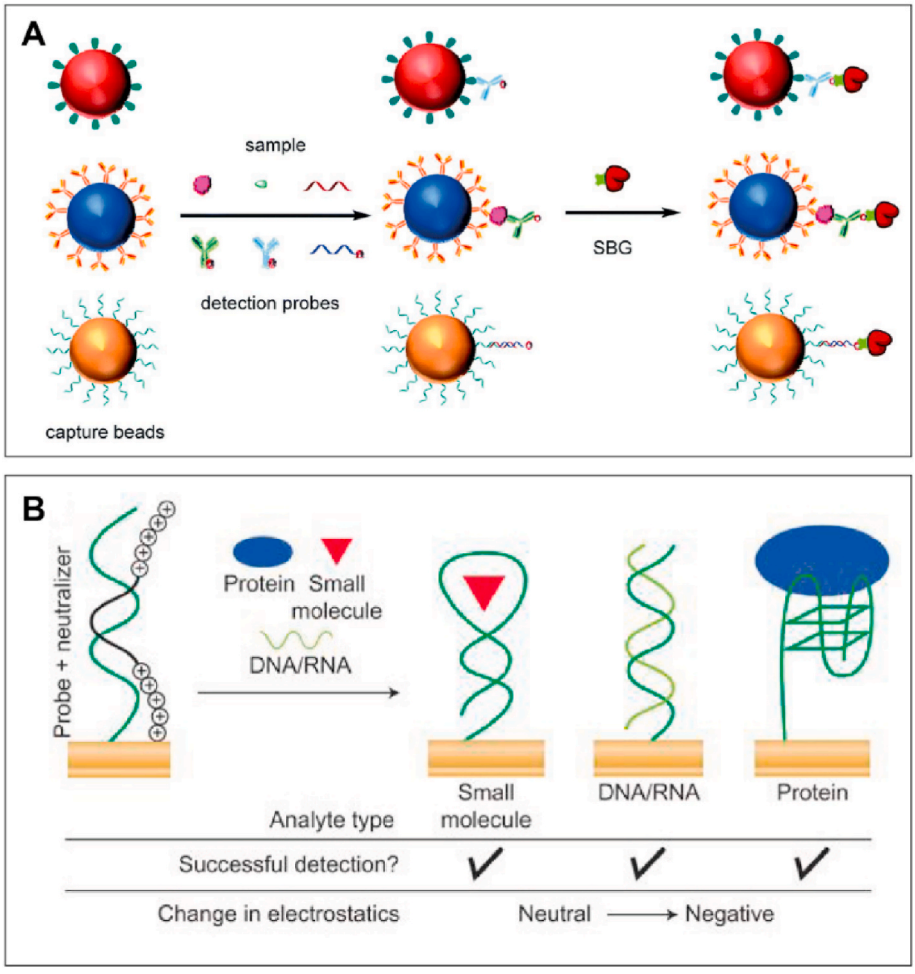

Fig. 4. Multianalyte biosensors for the detection of nucleic acid and protein biomarkers for further diseases. (A) The multi-analyte Simoa® assay uses functionalized dye-encoded paramagnetic beads to capture different biomarkers like cortisol, interleukin-6, and miRNA-141. Excess of beads ensures that only one or zero analyte is captured by each bead. For fluorescence detection, a second antibody or probe conjugated to β-galactosidase is added and the beads are loaded onto an array of microwells (one bead per well). Reproduced with permission from (Wang and Walt, 2020). Copyright 2020 Royal Society of Chemistry (CC BY-NC 3.0). **(B)** Electrochemical detection of proteins and nucleic acids using a neutralizer displacement assay. The neutralizer binds to the probe on the electrode surface and neutralizes its charge. In the presence of the biomarker, the neutralizer is displaced, which leads to a change in the surface charge. Reproduced with permission from (Das et al., 2012). Copyright 2012 Springer Nature.

biomarkers simultaneously for a quick diagnostic statement. Dye-encoded microbeads conjugated to the capture antigens or nucleic acid probes are immobilized as a planar layer in the flow cell of the microfluidic chip. Fluorescence labeled autoantibodies are employed for the detection of the corresponding antigens. The target cfmDNA binds to the immobilized nucleic acid probe and a second fluorescence labeled probe is used for the detection (Dinter et al., 2019).

The single molecule array (Simoa®) assay tries to overcome assay limitations like low analytical sensitivity by using a digital detection platform. This employs dye-encoded paramagnetic beads which are conjugated to capture antigens, antibodies, or nucleic acid probes (Fig. 4A). For a proof-of-concept, the simultaneous detection of cortisol, interleukin-6, and miRNA-141 is successfully demonstrated. An excess of beads is used to ensure that only one or zero target analyte binds. Subsequently, biotinylated detection antibodies or probes and streptavidin conjugated β-galactosidase are added. For signal readout, the complexes are resuspended in a fluorogenic substrate solution and loaded onto an array of microwells (each microwell can only hold one bead). The enzymatic reaction is producing a fluorescent product within the microwell, which can be detected by a charge coupled device camera (Wang and Walt, 2020). Wu et al. developed a similar approach that uses nanoparticles for the simultaneous quantification of Amyloid β 1–42, tau protein, miRNA-146a, and miRNA-138, which are associated with Alzheimer's disease. Antibody or probe functionalized magnetic nanoparticles are used to target the corresponding biomarkers in cerebrospinal fluid. Functionalized quantum dot-doped nanoparticles are used for signal detection. After a washing and denaturation step, the quantum dot-doped nanoparticles are released and a microscope is employed for quantification (Wu et al., 2021).

seqCOMBO is another bead-based approach that can be applied to the Luminex MAGPIX system. It was developed to detect liver-type arginase 1 and miRNA-122 from serum to enable an early diagnosis of liver injury. To capture target proteins, antibody conjugated beads are added to the sample. The supernatant is separated from the antigen-antibody-bead complex to capture the miRNA using probe conjugated beads (requires different reaction buffers). For the signal detection, both sets of beads are merged and biotinylated detection antibodies, SMART-C biotin, and phycoerythrin-labeled streptavidin are added to the reaction. Using the Luminex MAGPIX system the mean fluorescence intensity is determined (Marín-Romero et al., 2021).

Another multianalyte approach uses graphene oxide, which can be used as an effective fluorescence quencher and can spontaneously absorb ssDNA. In the absence of the target DNAs or proteins, the labeled reporter probes (ssDNA or aptamer) are absorbed and quenched by the graphene oxide – representing the "off" state. In the presence of the biomarkers, the reporter probe detaches from the surface and binds to its target analyte, resulting in a fluorescence signal increase. For a proof-of-principle, the authors used thrombin and a not further specified sequence-specific DNA (Zhang et al., 2011).

Das et al. developed a neutralizer displacement biosensor that allows electrochemical detection of different classes of biomolecules. Herein, a target probe (DNA or aptamer) is immobilized on the electrode surface and bound by a neutralizer (Fig. 4B). The neutralizer is a conjugate of cationic amino acids and peptide nucleic acids which neutralizes the charge. In the presence of the target analyte, the neutralizer is displaced, which leads to a significant change in the surface charge. Therefore, the presence of an analyte is not only determined by its molecular charge but also by the probe, which is unmasked on binding (Das et al., 2012).

Huang et al. reported a multianalyte approach based on a target-induced molecular-switch on triple-helix DNA-functionalized carbon nanotubes. The triple-helix DNA-functionalized carbon nanotubes are generated by the hybridization of assistant DNA probes and signal DNA probes on the carbon nanotube surface. The assistant DNA probe contains a central target-specific sequence (aptamer targeting the protein or DNA probe targeting DNA), which is flanked by two segments that hybridize with the signal DNA probe to form the triple-helix DNA. The binding of the target analytes to the corresponding assistant DNA probes induces structural changes within the triple-helix DNA. This results in an unprotected single-strand signal DNA probe that can bind to capture probes, which are immobilized on a nitrocellulose membrane of a lateral flow strip. In the presence of thrombin and/or tDNA, a visible signal was generated at the corresponding test line (Huang et al., 2020).

3.4. Single-cell multiomics in diagnostics

High-throughput approaches, such as sequencing, offer an attractive tool for biomarker discovery and validation and, therefore, have a significant impact on clinical diagnostics. In this regard, next-generation sequencing can be used to examine for example the genome of a cell population. However, these methods do not register the heterogeneity within the cell population like it is for example observed within tumor tissue (Tang et al., 2019). This drawback is addressed by single-cell technologies. The simultaneous measurement of DNA, mRNA, and protein levels at a single-cell resolution is enabled by recent advances in single-cell isolation and barcoding technologies (for a comprehensive overview please see (Hu et al., 2018; Lee et al., 2020; Nassar et al., 2021; Subramanian et al., 2020)).

CITE-seq (cellular indexing of transcriptomes and epitopes by sequencing) and REAP-seq (RNA expression and protein sequencing assay) (Fig. 5A) are both using DNA-labeled antibodies for the detection of cell surface proteins combined with single-cell RNA-sequencing to measure the expression levels of genes and cell surface proteins in single-cells. The antibody-conjugated DNA oligos contain a PCR handle, an antibody-identifying barcode, and a poly-A tail. Using microfluidics, each antibody-labeled cell is encapsulated into a droplet together with a bead functionalized with poly-dT primers. The cells are lysed within the droplet and mRNAs and DNA-labeled antibodies bind to the poly-dT primer conjugated beads. With the help of reverse transcriptase and PCR, a library is generated and sequencing is carried out to quantify mRNA and protein levels (Peterson et al., 2017; Stoeckius et al., 2017; Todorovic, 2017).

RAID (single-cell RNA and Immuno-detection) is a single-cell multiomic approach that can also detect intracellular proteins. Immunostaining of intracellular proteins requires permeabilization of the cells, which would lead to loss of mRNAs. Thus, Gerlach et al. implemented chemically reversible crosslinking to permeabilize cells and stain them with RNA-barcode-labeled antibodies. The RNA-barcode has an antibody-specific barcode, a unique molecular identifier, and a poly-A tail. The cells are sorted into 384-well plates and the crosslinking is reversed to perform cDNA synthesis using CEL-seq2-compatible poly-dT primers. To generate a sequencing library and quantify protein and mRNA levels the single-cell samples are pooled (Gerlach et al., 2019).

Another example called PLAYR (Proximity Ligation Assay for RNA) enables the detection of mRNAs in single cells and is compatible with standard antibody staining of proteins (**Fig. 5B**). This approach also requires fixed and permeabilized cells to first label target proteins. Subsequently, PLAYR probe pairs are employed to hybridize to two adjacent regions of the target mRNAs and provide a docking site for RNA-specific insert-backbone oligos, which form an ssDNA circle that can be ligated. Using rolling circle amplification, the ligated ssDNA circle is amplified. For signal detection, labeled detection probes are hybridized to the complementary regions of the amplicon. PLAYR can be used with metal or fluorescently labeled reporters to implement analysis via mass- or flow cytometry (Frei et al., 2016). Another strategy for single-cell analysis combines proximity extension assay (PEA) and specific RNA target amplification (STA) (Fig. 5C). In PEA, pairs of antibodies are functionalized with ssDNA oligos with complementary 3' ends. Binding of the antibody pair in close proximity on the target protein allows hybridization of the oligos – converting proteins into a DNA-based recognition format. Using reverse transcription and random primers, mRNA is converted into cDNA. Finally, cDNA and DNA reporter are co-amplified and co-detected by quantitative PCR or sequencing

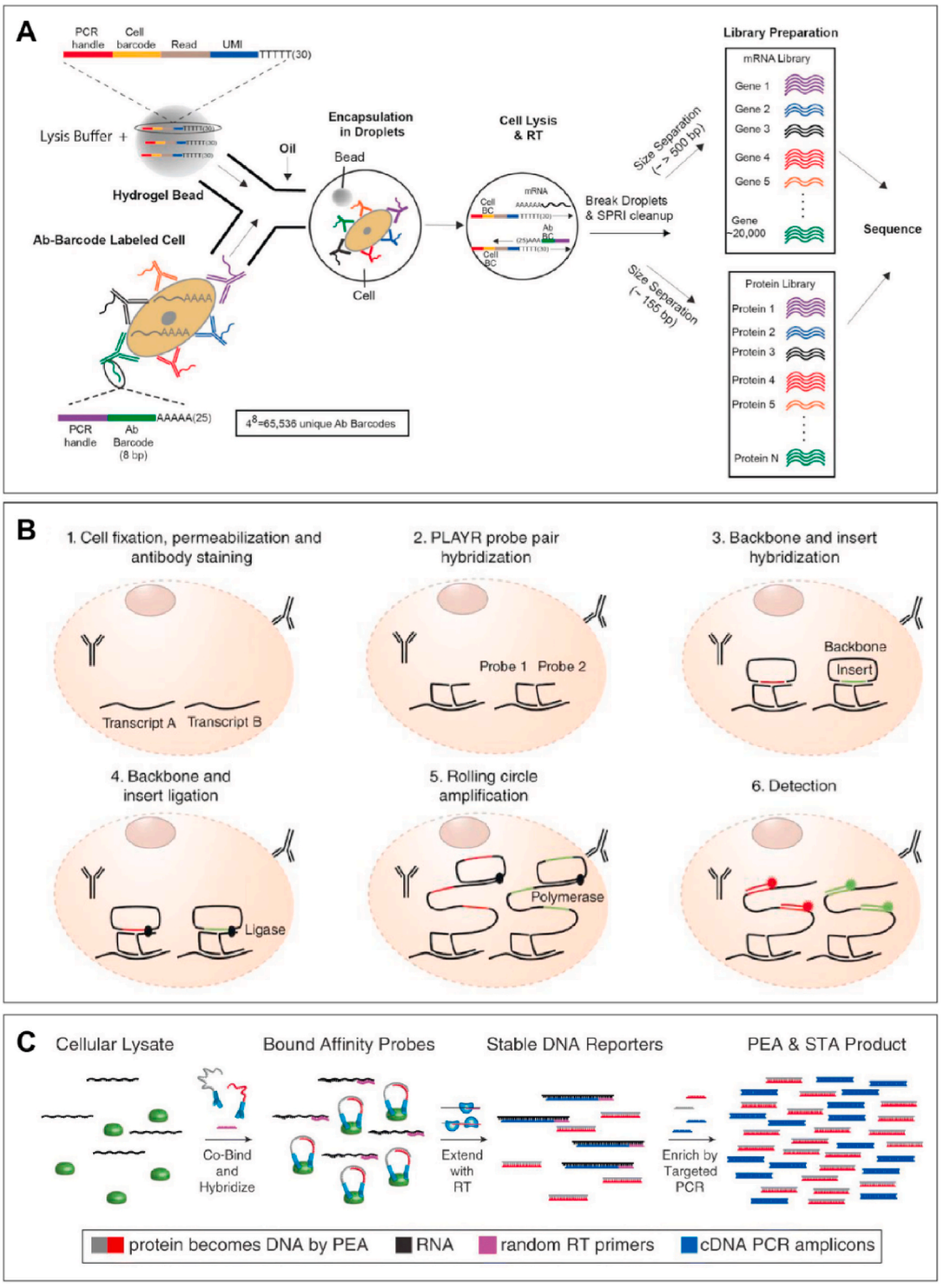

Fig. 5. Single-cell multiomic approaches for the simultaneous detection of proteins and nucleic acids. (A) REAP-Seq enables the simultaneous measurement of expression levels of genes and cell surface proteins in single-cells. Proteins are targeted by DNA-labeled antibodies before each cell is encapsulated together with a poly-dT primer-functionalized bead into a droplet. The cells are lysed within the droplet and mRNAs and the DNA-labeled antibodies are binding to the bead. Finally, a sequencing library is generated and mRNA and protein levels are determined. Reproduced with permission from (Peterson et al., 2017). Copyright 2018 Springer Nature. **(B)** PLAYR enables the detection of mRNAs and (intracellular) proteins of single cells. The cells are fixed and permeabilized before target proteins are labeled with antibodies. PLAYR probes are used to hybridize to mRNA. They provide a docking site for RNA-specific insert-backbone oligo, which are ligated via Rolling circle amplification to form an ssDNA circle. For signal detection, labeled detection probes are hybridized to the amplicons. Detection is performed via mass- or flow cytometry. Reproduced with permission from (Frei et al., 2016). Copyright 2016 Springer Nature. **(C)** Simultaneous detection of RNAs and proteins from single cells by combining PEA and STA. Using PEA, pairs of oligo-labeled antibodies bind in close proximity to the target protein, which allows the hybridization of the oligos. mRNA is converted into cDNA by using reverse transcription and random primers. Finally, the cDNA and DNA reporters (STA and PEA products) are co-amplified and co-detected by quantitative PCR or sequencing. Reproduced with permission from (Genshaft et al., 2016). Copyright 2016 Springer Nature (CC BY 4.0).

(Genshaft et al., 2016).

Similarly SPARC (Single-Cell Protein And RNA Co-profiling) combines single-cell RNA sequencing and PEA to measure intracellular proteins and mRNA. This approach uses isolation and lysis of single cells in the presence of oligo-dT conjugated magnetic beads. Thus, mRNA can hybridize to the magnetic beads whereas the target proteins are present in the supernatant. The supernatant is analyzed via PEA as described above, whereas, the mRNA is processed using a Smart-seq2 approach for sequencing library preparation. This means a reverse transcriptase with terminal transferase activity and a second template-switch primer are applied to generate cDNA with two universal priming sequences. Subsequently, the cDNA is pre-amplified via PCR and sequencing library preparation is carried out (Reimegård et al., 2021). Kochan et al. developed a protocol that combines immunofluorescence with single-molecule RNA fluorescence *in situ* hybridization (smRNA FISH). smRNA FISH uses transcript-specific probes consisting of complementary DNA oligos labeled with a fluorescence dye. This combination seems obvious, but the reaction conditions differ and the staining protocols are often insufficient regarding signal intensity and staining patterns. The authors were able to successfully implement a protocol by using an RNase-free modification of the immunofluorescence staining (Kochan et al., 2015).

The visualization of viral nucleic acids and protein in the course of infection helps to understand the fundamental processes of replication and virus assembly, which is essential for disease control. This can be realized by simultaneous single-cell visualization of DNA, RNA, and protein biomarkers. In comparison to conventional DNA fluorescence *in situ* hybridization (FISH), this approach uses branched DNA *in situ* technology and utilizes paired probes. This enhances the sensitivity and specificity. The proteins are detected via antibody-based immunostaining (Puray-Chavez et al., 2017; Shah et al., 2020). Similarly, Popovic et al. employ a combination of branched DNA FISH and antibody staining to measure mRNA and protein abundance in single human cells (Popovic et al., 2018).

Table S5 summarizes single-cell multiomic approaches for the simultaneous detection of nucleic acid and protein biomarkers. In this regard, sequencing is a powerful tool realized by combining protein-to-nucleic acid transformation and nucleic acid hybridization (Genshaft et al., 2016; Gerlach et al., 2019; Peterson et al., 2017; Reimegård et al., 2021; Stoeckius et al., 2017). Other single-cell multiomic approaches combine classical immunostaining using labeled antibodies with nucleic acid detection via FISH (Frei et al., 2016; Kochan et al., 2015; Popovic et al., 2018; Puray-Chavez et al., 2017; Shah et al., 2020). Despite the advantages that single-cell multiomics offers and the promising advances that have been made, there are still challenges that need to be overcome such as single-cell isolation and data analysis. The current protocols for single-cell multiomics are time-consuming and complex and therefore, currently rather used for research than diagnostic applications. However, the high-throughput detection capability and the possibility to link proteomic and transcriptomic information at the single-cell level will play a significant role in the detection of new diagnostic biomarkers.

4. Future perspective and conclusion

There exists a pool of fundamental research in the area of multianalyte sensing (TRL 1–2, basic principles that begin to be translated into applied research environment and development of research plans/protocols addressing the hypothesis) that has the potential of reaching TRL 3 (proof-of-concept of a laboratory model with spiked and/or artificial samples) or higher (Ates et al., 2021). In general, the success rate of technologies reaching TRL 7 and higher is low. Ates et al. described that the main influencing factors are the analyte of interest, the used sensing technology, and its complexity (Ates et al., 2021). Technologies at TRL 1–2 are not reviewed here, but we want to highlight the emerging field of single molecule sensing, which has the potential to enable multianalyte sensing at single molecule level. Herein, nanopore sensing offers a platform that can discriminate various classes of biomolecules and even resolve structural modification of biomolecules (Ensslen et al., 2022; Wu and Gooding, 2022; Ying et al., 2022). Another novel development, called ProtSeq, is the recognition of amino acids via DNA barcodes for high-throughput identification of single peptide sequences (Hong et al., 2022).

Here, we analyzed multianalyte biosensors for the detection of protein and nucleic acid biomarkers for medical diagnostics. Multianalyte biosensors for cancer diagnostics are aiming to enable a sensitive, specific, and cost-effective detection of biomarkers for clinical use. Liquid biopsy offers an attractive approach for the detection of various biomarkers including CTC, cell-free DNA, or EVs (Arechederra et al., 2020; Bratulic et al., 2021; Hofman et al., 2019). However, these biosensors are mainly either testing the hypothesis (TRL 3) or "validation in the lab" phase (TRL 4) (Ates et al., 2021) and have not been used as a cancer diagnostic tool. In particular, the assay requirements and the technical variability in the pre-analytical steps are hampering the transition into clinical practice (Alix-Panabières, 2020; Hofman et al., 2019). Furthermore, the majority require several manual steps for the sensing of multiple analytes or exclude the sample preparation. Nevertheless, the detection of multiple cancer biomarkers will have a significant impact on clinical diagnostics, especially in early cancer biomarker detection, personalized therapy and therapy monitoring. Therefore, future research should focus on (i) the identification of specific biomarker combinations that can determine cancer's origin, status, and progression, (ii) the development of sample-to-answer biosensors that allow the simultaneous detection of various cancer biomarkers and (iii) converting the information to a clinically relevant format (Alix-Panabières, 2020).

Several arguments demonstrate the advantage of multianalyte detection in terms of infectious diseases. For example, the combined detection of inflammation markers and pathogenic nucleic acids is very beneficial for the early detection of infections combined with a target-oriented therapy (Klebes et al., 2022). However, these biosensors are mainly in the research or integration phase and there are currently no commercial products available for the clinical routine (Ates et al., 2021). Nevertheless, microfluidic platforms like Evalution® (Falconnet et al., 2015) or Rheonix CARD® (Chen et al., 2013) can already be used by researchers and have the potential to be commercialized for IVD. The importance of POC diagnostics for the control of infectious diseases was once more highlighted by the current SARS-CoV-2 pandemic (Wang et al., 2021). In this regard, LFAs are a highly attractive tool for POC diagnostics, because they are widely accepted by users and cheap in production (Brunauer et al., 2020). Thus, the combination of isothermal amplification and LFIA can offer a fast and simple way for the detection of nucleic acid and protein biomarkers at the POC (Kaur and Toley, 2018; Klebes et al., 2022). Nevertheless, pathogens need to be lysed for nucleic acid analysis. The current lysis protocols are not compatible with simultaneous protein analysis. Thus, current multianalyte approaches split the sample or only show the proof-of-principle by using purified target nucleic acids.

For a successful transfer into the application, future research needs to focus on (i) suitable sample preparation techniques such as pathogen lysis protocols, and (ii) sample-to-answer workflows that allow the simultaneous detection of pathogens and infection biomarkers at the POC.

In the near future, single-cell multiomics will have a significant impact on clinical diagnostics, especially on the discovery and validation of biomarkers. The simultaneous detection of transcriptome and proteome is mainly possible due to recent advances in single-cell isolation and barcoding technologies. Currently, these single-cell approaches are rather complex and their development for applications is at an early stage. Nevertheless, single-cell multiomics can provide insight into factors that regulate cellular states and thus offer a comprehensive understanding of cellular processes (Hu et al., 2018; Lee et al., 2020).

Current multianalyte biosensors reflect the undisputed advantages of multianalyte detection for medical diagnostics. Thus, the development of generic biosensors detecting different classes of biomolecules and their transition into clinical practice is highly desirable and will revolutionize medical diagnostics.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2023.115800.

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