Review
Human Sputum Proteomics: Advancing Non-Invasive Diagnosis of Respiratory Diseases with Enhanced Biomarker Analysis Methods

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Abstract: Many ailments can be diagnosed while they are asymptomatic, meaning that the patient has no signs or symptoms of a progressing disease. If caught in their initial stage of formation, these disorders can be effectively treated, leading to successful outcomes; curative therapies can halt illnesses from advancing, thus improving the quality of life and long-term survival of the patient. Still, cutting-edge upgrades in precision technologies are necessary for early, reliable, affordable, and rapid disease detection, but also vital for the well-being of people and the future of global public health.

The emerging role and utility of non-invasive and repeatable diagnostic test approaches for the detection of health conditions have been exemplified by liquid biopsies based on genomic biomarkers. As such, biological fluids permit any measurable molecular indicator or signature (e.g., proteins) to provide valuable information on an individual’s wellness and/or disease. Among the bodily secretions used for non-invasive diagnostics is sputum, a complex viscous gel-like biopolymeric network that has gained growing recognition as a rich source of biomarkers of airway infections and pulmonary diseases, and serves as a determinant to reveal other illnesses. As per the World Health Organization, the burden of respiratory conditions is exacerbated by factors ranging from considerable subjection to air pollution and occupational contaminants to tobacco smoking and second-hand smoke, in addition to poor socio-economic status. Due to the likely increase in these determinants, respiratory tract ailments are on the rise, affecting the health of many individuals, in addition to putting stress on healthcare facilities and services worldwide. The aim of this study was to perform a narrative review of sputum constituents with an emphasis on proteins and glycoproteins assessed as possible biomarkers of lung and other organ diseases. A search was conducted using mucus, sputum proteomics, sputum biomarkers, and point-of-care testing as keywords employing Google, PubMed (MEDLINE), and Web of Science, selecting the most referenced and related papers of the last decade. We, therefore, highlight the need to use expectorated or induced sputum specimens as a routine sample source for testing valuable protein biomarkers to diagnose these chronic disorders, predict inflammation and disease progression, as well as monitor the effectiveness of treatments. Further, we discuss the urgent need for fast and reliable point-of-care methods to detect and quantify crucial protein biomarkers in sputum specimens, and the limitations faced when dealing with their complex matrices.

Keywords: COVID-19; immunoaffinity capillary electrophoresis; lateral flow immunoassay; point-of-care testing; respiratory disease biomarkers; SARS-CoV-2 virus; sputome; sputum proteomics

1. Introduction

Pivotal studies on sputum began in the 1950s–1970s and research has progressed significantly over the past decades. Initially, investigators and clinicians examined sputum cells on stained smears and mycobacterial cultures [1–4]; today, advanced imaging techniques coupled with newly found biomarkers have the potential to evaluate the efficacy...
of therapies in patients with pulmonary illnesses, specifically tuberculosis (TB) [5]. As more biomarkers are identified, there is a greater probability of boosting research and development of enhanced pharmaceuticals, methods to measure disease activity, and the impact of various therapeutics. However, many conditions are still diagnosed as if they are homogeneous entities, based on criteria that have not changed in a century, including complex and heterogeneous ones, such as chronic airway diseases [6,7].

Technically speaking, phlegm is the mucus secreted in the respiratory system, whereas sputum is considered phlegm that contains a mix of saliva, cellular debris, and other substances that are coughed up from the lungs and bronchial tubes. Saliva is one of the body fluids secreted by salivary glands, which has several functions, including digestion, protection of the teeth, and lubrication of the mouth in the oral cavity. Saliva is typically clear, watery, and thin in consistency. Sputum can vary in color depending on the underlying conditions and is thicker and more viscous when compared with saliva. The use of salivary and sputum samples is gaining importance for both point-of-care testing and to assess the state of health or disease. Small-molecular-mass substances and biomolecules present in saliva and sputum can be potential predictor biomarkers of wellness and disease states. Nonetheless, in this narrative review, we will primarily discuss protein biomarkers derived from sputum specimens. Additional information on biomarkers found in saliva and sputum that is not presented in this paper can be found in refs. [8–15].

Recent milestones in drug product development are now being used to manage many diseases. Still, it is essential to note that not all chronic respiratory ailments respond favorably to existing remedies, including biological therapies. Focus has therefore shifted towards personalized medicine, highlighting the importance of biomarkers to redirect costs for alternative treatment strategies so that patients can benefit the most from them. The finding of new biological indicators has not only made it possible to identify specific sick subgroups but also offers targeted biologic therapies to individuals suffering from severe respiratory conditions [16]. As such, proteomics can reveal molecular pathways of diseases and provide translational perspectives to inform clinical decision-making. In fact, studies in three types of asthma—classic, cough-variant, and chest tightness variant—found that from the analysis of more than 1000 induced-sputum proteins, 23 secreted proteins were higher in experimental groups versus control groups [17].

Experiments performed in the last decade on an array of proteins located in the sputum of patients affected with severe pulmonary disorders have allowed for the pinpointing of important proteins and peptides whose concentration levels alter in response to the person’s state of health [18]. Certain afflictions, such as nontuberculous mycobacterial (NTM) lung disease, are hard to diagnose and cure; preliminary investigations have revealed that two proteins correlated with iron chelation were significantly downregulated in critical cases of NTM lung disease, and its treatment was associated with heterogeneous changes in its sputum protein profile [19]. In another study, the identification and characterization of specific sputum proteins, such as beta-integrin, vitamin-D binding protein, uteroglobin, profilin, and cathelicidin, used as biomarker signatures, were sufficient to differentiate active tuberculosis from non-active TB patients [20].

Oligomeric mucins, the major gel-forming constituents of extracellular mucus that includes the airways’ mucus gel or sputum, are complicated to handle when trying to discern their protein constituents with proteomic approaches due to their heavily glycosylated nature (up to 90%), high molecular weight (200 kDa–200 MDa), and size (Rg 10–300 nm). Their core or interior proteins are very large and highly substituted with oligosaccharides, which only permit access to a limited and restricted portion of their proteins [21]. For instance, a large-scale label-free quantitative mapping of the sputum proteomics showed that the “core” sputum proteome was composed of 284 proteins, and the “extended” proteome contained 1666 proteins [22]. Specific studies performed on the sputum proteome of cystic fibrosis patients have shown associations with progressive lung function impairment, some of which might have value as biomarkers of the severity of this illness [23]. Other phlegm proteins, such as eotaxin-1, interleukin-5 (IL-5), and eosinophil peroxidase (EPX), are po-
tential predictors of remission in patients afflicted with severe eosinophilic asthma. The confirmation of sputum biomarker values for certain airway maladies in clinical settings is now recommended by the American Thoracic Society and the European Respiratory Society, including guidelines for the management of severe asthma [24]. Still, much remains to be done, as the currently validated biomarkers only anticipate responses in a subset of cases [25].

While sputum supernatant extract is a viable alternative to liquid biopsy specimens for the diagnosis of lung cancers [26], the study of biopsy technologies for omics (genomics, transcriptomics, proteomics, metabolomics, and others) continues to evolve. This is particularly true in the field of sputum proteomics, or the entire complement of proteins in sputum, known as the sputome. Although liquid biopsies are not yet recognized as the gold standard tool to confirm different ailments, they are primarily used as a complementary test for tissue cultures [27]. Obstacles, such as the lack of sensitivity and precision in identifying some particular diseases, still remain, yet trends in non-invasive tests indicate that the future of screening and monitoring of chronic illnesses is promising; new sources of sputum molecular biomarkers like extracellular vesicles have the potential to become a non-invasive liquid biopsy diagnostic tool for pulmonary illnesses [28].

Breakthrough technological advances allow for the simultaneous confirmation and classification of hundreds of proteins, creating optimism that an abundance of novel protein biomarkers can be validated, but progress has been slower than anticipated. While proteomics has the potential to improve diagnostic, prognostic, and predictive tests, its applications in the medical field have yet to be fully realized [29,30]. Also, the utilization of protein biomarker panels, rather than individual protein biomarkers, offers a more comprehensive representation of human physiology, especially in situations with elaborate differential diagnoses and in patients with multiple comorbidities [30]. In infectious diseases, such as the coronavirus disease (COVID-19) caused by severe acute respiratory syndrome (SARS-CoV-2 virus), reports indicate that proteomic signatures track symptom severity and antibody responses that may identify individuals who are more likely to suffer from persisting symptoms [31].

In this narrative review article, we discuss the various procedures to collect sputum, methods and systems that disrupt/dissolve the intricate, viscous, and variable sputum/phlegm meshwork structures to release their contents, techniques to identify and characterize crucial sputum protein biomarkers, as well as the advantages and disadvantages of each approach. We also explore how innovative healthcare technologies such as point-of-care testing (POCT) can improve patient outcomes when successfully implemented.

2. Methods and Systems Utilized to Study Sputum Proteomics

Approximately 95% of epithelial surfaces are mucosal and comprise a single layer of epithelial cells mostly covered by a gel-like layer of mucus. They form semipermeable barriers enabling nutrient absorption and waste secretion and provide a major route of entry and release for pathogens, including viruses [32]. The mucus layer contains a range of mucin glycoproteins as its main components. The mucin family of glycoproteins is classified into those that are secreted by specialized epithelial cells and form the mucus gel and those that are embedded in the epithelial cell membrane. Specifically, mucus provides protection against dehydration, abrasion, toxins, and pathogens and is a reservoir for antimicrobial molecules [32].

Mucus hyperproduction, hypersecretion, and/or hyperconcentration are primary symptoms of obstructive lung and other airway afflictions. Inflammation of the respiratory tract mucosa due to infectious or noninfectious conditions results in hyperreactivity of the cough receptors [33,34]; mucus regulation is therefore valuable from a therapeutics perspective as it contributes to improved airflow, reduces symptom and disease severity, and to an extent, helps with illness prognosis [34]. Due to the complexity and viscosity of sputum/phlegm, a mix of protocols has been developed for specimen collection and dissolution, followed by the evaluation of sputum protein constituents. Generally, measur-
ing certain proteins in sputum can vary widely between repeat analyses, is susceptible to sample processing effects and changes in response to the organism’s conditions, and can be hard to quantify accurately and precisely by mass spectrometry, despite other proteins having high inter-individual variance [18,22]. It is thus preferable to use additional testing to obtain an accurate conclusion of a respiratory illness. For example, sputum analysis of proteins coupled with microscopic examination of mycobacteria can be applied as a screening tool for diagnosing pulmonary tuberculosis [35]. Equally, samples of proteomic signatures from other biological fluids that reflect differences in the inflammatory responses and can be linked to symptoms that vary in severity and duration may be utilized [31].

The production of phlegm is usually minimal in a healthy person, but a diseased state can increase the amount or alter the nature of a patient’s sputum. Airway mucus is composed of water (98%), salt (0.9%), globular proteins (0.8%), and high-molecular-weight polymers (0.3%) in subjects that are well. In “muco-obstructive lung diseases” the values of the MUC5B and MUC5AC, the predominant gel-forming mucins secreted in the respiratory tract, can be up to 10 times higher or more in concentration; MUC5AC, in particular, increases disproportionately [34,36]. Mucins, the key component of mucus, are large glycoproteins containing regions rich in serine and threonine residues that can bind by O-glycosylation to glycan chains [32]; mucin glycoproteins polymerize covalently via disulfide bonds and can aggregate, forming plugs and blocking airflow. In some critically ill COVID-19 patients with low survival outcomes, the formation of sticky sputum or sticky plugs (viscous secretions) builds up in their air tracts, suggesting that alterations in their mucus attributes are an early warning of likely advances in their pathogenesis [37].

First introduced in the 1960s by Japanese thoracic surgeon Shigeto Ikeda, flexible bronchial fibroscopy, or bronchoscopy [38,39], is the indispensable procedure applied by respiratory physicians to investigate the throat and airway or respiratory tract. Bronchoscopies can also be used to aspirate mucus, mucus plugs, and/or to perform bronchoalveolar lavage; however, the invasive nature of fibroscopy is still a primary limitation of the exam in gravely ill patients [33,39]. Today, sputum specimens are collected by one of the two non-invasive methods—either the expectorated or the induced procedure. The expectorated or spontaneous mucous secretion is taken by coughing, following standardized protocols to collect the best sample and avoid contaminating the specimen [40,41]. The feasibility for patients with severe airflow obstruction to expel phlegm is low; consequently, an induced sputum sample is required with the aid of an ultrasonic wave nebulizer [42]. A specimen is taken after hypertonic saline in concentrations of 0.9–7% is inhaled for 15–30 min [43,44]; this helps the production of phlegm in children who cannot do so naturally and is an ideal procedure to diagnose airway illnesses [45–47]. To prevent bronchoconstriction that would be induced by hypertonic saline inhalation, it is recommended to inhale salbutamol before sampling [48,49]. Since sputum induction and its processing has a high success rate and is considered safe for children 6 years older, it has become popular as a non-invasive clinical tool to assess airway inflammation [49,50].

After collecting the specimen, the sputum content must be released, which is a challenging process. The extraction protocol applied to obtain proteins from sputum specimens varies significantly, especially when used with lateral flow immunochromatographic assays, as the viscosity of phlegm interferes with the migration of the antigens on the test strip [51]. Investigators have sought to extract proteins by homogenization of the heterogeneous material, primarily for its plug-containing sputum [52], while others have obtained proteins by acetone sedimentation. Acetone-precipitated samples are then dissolved in 8M urea, reduced with Tris-(2-carboxyethyl)phosphine, and alkylated with iodoacetamide [17]. A summary of protocols for extracting proteins from sputum is found in Table 1 [17,34,41,51–56]. Regardless of the procedure used, fluctuations may occur in all methods due to the non-standard selective approach to sample preparation.
Table 1. Overview of types of procedures applied to release bacterial, viral, and diverse specimen constituents from the complex sputum meshwork.

<table>
<thead>
<tr>
<th>Procedure Performed or Substances Added to Sputum or Bronchial Aspirate Samples</th>
<th>Rationale</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical homogenization.</td>
<td>To macerate or crush the sputum in the presence of a suitable buffer, to obtain viable single-cell suspensions, or to disperse fragments of the sputum gathered evenly throughout the mixture.</td>
<td>[34,52]</td>
</tr>
<tr>
<td>Acetone sedimentation.</td>
<td>Acetone-precipitated samples are dissolved in 8M urea, reduced with Tris-(2-carboxyethyl)phosphine, and alkylated with iodoacetamide.</td>
<td>[17]</td>
</tr>
<tr>
<td>Tris(2-carboxyethyl)phosphine (TCEP), N-acetyl-L-cysteine (NALC), bovine serum albumin (BSA), and protease inhibitor cocktail.</td>
<td>TCEP and NALC (reducing agents) are used to break the disulfide bonds of sputum. BSA is used as a blocking agent.</td>
<td>[51]</td>
</tr>
<tr>
<td>Hydrogen peroxide (H$_2$O$_2$).</td>
<td>Enzymatic liquefaction. The peroxide solution, in the presence of endogenous catalase, is used to trigger the formation of an oxygen bubble.</td>
<td>[53]</td>
</tr>
<tr>
<td>NaOH, sodium dodecyl sulfate (SDS), glass beads, temperature (60 °C).</td>
<td>Breakdown of sample mixture.</td>
<td>[54]</td>
</tr>
<tr>
<td>PureLyse® bead blender, solid phase extraction, which does not require chaotropic salts or organic solvents.</td>
<td>A miniaturized bead beating system for mechanical pathogen lysis (effectively disrupts tough-walled microorganisms). This is a disinfection and liquefaction method.</td>
<td>[55]</td>
</tr>
<tr>
<td>Dithiothreitol (DTT), homogenization, centrifugation, and supernatant desalting.</td>
<td>Release of proteins from sputum.</td>
<td>[56]</td>
</tr>
</tbody>
</table>

3. Considerations for the Selection of a Safe and Cost-Effective Lateral Flow Assay

Each method described in Table 1 can free the molecular constituents of sputum and is a valuable technique for use in a laboratory designed to perform experiments under a chemical fume hood—this effective ventilation system reduces unpleasant odors and protects against exposure to some hazardous chemicals. However, choosing the procedure that is best suited for home or point-of-care testing and diagnosing of COVID-19 and other infectious diseases is not straightforward. Multiple COVID-19 rapid home lateral flow immunochromatography test kits are now available in local pharmacies; these assays contain some solutions, such as detergents, inorganic phosphate, sodium azide, and other microorganism preservatives. It is important to state that the substances in these kits are present in very low concentrations and are therefore unlikely to cause human toxicity [57]. Nonetheless, as the market for these diagnostic products expands and access to them grows, the likely outcome will be an increase in subjection to liquid agents, possibly causing allergic reactions or topical irritations. Users should be aware of hazards associated with contact with these reagent fluids [57].

Diagnostic tests for respiratory viral infections traditionally use nasopharyngeal and oropharyngeal samples. In the case of ascertaining COVID-19, the most popular methods to detect SARS-CoV-2 are via antigen-based lateral flow immunochromatography assays or lateral flow immunoassays (LFIAs) and reverse transcriptase polymerase chain reaction (RT-PCR) assays with nasal and throat swab specimens. These techniques use a sampling protocol that can be stressful, especially in children, particularly when repeated testing is
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The extraction and enrichment of the target molecule in a sample is crucial to obtain a high rate of analytical sensitivity and detection limit in LFIAAs. In complex matrices, it is necessary to acquire the maximum quantity of the specific analyte, especially if the substance of interest is found in low concentrations. Recently, a rapid and affordable pretreatment protocol for releasing constituents of sputum was published. As depicted in Figure 1, this process consists of adding Triton X-100 and/or another detergent, Alcalase (subtilisin), and/or another proteolytic or digestion enzyme to a sputum sample, thereby exposing hidden or masked antigenic sites of viral specimens or lingering fragments of viral proteins present in the sputum meshwork [41].

![Figure 1](https://via.placeholder.com/150)

Figure 1. Representation of a sample preparation (disruption–extraction–digestion), whereby the non-ionic detergent Triton X-100 and the endoprotease Alcalase are applied to the sputum specimen [41]. As depicted in (panel A), the phlegm is a thick, rubbery, sticky, viscous, and gel-like biopolymeric network, which contains different types of cells, cell debris, microorganisms, and chemical-biochemical entities. After adding the detergent and the protease, some disruption occurs (panel B), influenced by the incubation time, quantity of the proteolytic enzyme, temperature, and the pH of the solution. The incubation temperature was set at 25 degrees Celsius, and the experiment resulted in a solution containing primarily soluble material and a precipitate of insoluble components (panel C). Following decantation or centrifugation, the supernatant was tested for the presence of SARS-CoV-2 virus, or virus components, on a lateral flow immunoassay (LIFA) platform or strip.

Cumbersome and multi-step procedures can be error-prone, require skilled users, as well as additional consumables and laboratory instrumentation, and are hard to obtain in low-resource settings [55]. For example, the RT-PCR test, considered the gold standard test, may take 1 to 2 days to produce results and is often expensive due to the need for trained technicians and costly reagents. The average price of an RT-PCR test in the United States of America ranges from USD 50 to USD 200; in low- and middle-income countries, this price can make RT-PCR screening for COVID-19 and other diseases prohibitive [58,59]. Conversely, the rapid antigen test cost is about USD 10 to USD 30 in the U.S. and is less than USD 10 in other developed nations [60]. The antigen tests do not require trained healthcare professionals and provide results in 15 to 20 min. Unfortunately, many LFIA tests that use
nasopharyngeal and oropharyngeal samples have poor analytical sensitivity unless there is a high viral load, but a sputum specimen can provide a positive COVID-19 diagnostic result with a rapid antigen test when nasopharyngeal and oropharyngeal samples yield a negative finding [41].

4. Advanced Technologies to Enhance Lateral Flow Assay Analytical Sensitivities and Specificities

It is not always possible to establish the intensity of the color band or even detect the positive color in an LFIA platform with the naked eye. Other criteria are also necessary to confirm an affirmative outcome with an antigen-based assay. Increasing the analytical sensitivity and specificity of LFIA tests are inevitable requirements to broaden its application areas, and both are essential for assay optimization and signal amplification techniques [61]. Most commercially available LFIA offer qualitative or semi-quantitative analyses and need dedicated tools for the quantitative measure of biomarkers. To improve the analytical sensitivity and accuracy of LFIA tests, efforts have been made to address these limitations. For example, a 20-min lateral flow antigen test was designed to be read by an artificial intelligence logarithm, known as lateral flow with artificial intelligence read (LFAIR), whose results can be accessed via a smartphone application. Not only does it provide results at home, but it can also digitally transmit these findings to healthcare workers and public health authorities in real time [58].

Supplemental components have been incorporated into these devices to allow for the use of external detectors. For instance, a compact optical spectroscopy apparatus was produced using a 3D printer, carrying a micro-spectral chip that can harness sophisticated light traces to estimate magnified detection signals and measure spectral results concurrently. This technique was created to significantly reduce misinterpretations of LFIA results [62]. Another system developed to enhance sensitivity in LFIA was realized by preventing the competition between specific and background antibodies that permit binding to nanosized labels. This blocking mechanism was activated electrokinetically to drive the delivery of the label once the antigen-antibody is in the test zone. The limit of detection using electrophoretically driven LFIA (eLFIA), has been proven to be 1000 times lower than that of conventional LFIA [63]. Various post-assay upgrades based on chemical reactions to facilitate high sensitivity for LFIA were presented in a critical review [64]. In addition, a centrifugation-assisted lateral flow immunoassay (CLFIA) has been recommended to increase sensitivity when compared to traditional LFIA based on strips [65], and magnetic enrichment applying multifunctional nanocomposite probes has also been proposed [66]. A recent publication highlights the importance of assay optimization and specificity of LFIA, which rely on the affinity, stability, and properties of the immobilized capture molecule [61].

5. Various Technologies used to Study Proteins in Sputum

Proteomics is the comprehensive study of the composition, function, interactions, and structures of all proteins contained in a cell, tissue, or individual to understand an organism’s nature. This area of research encompasses a broad range of technologies aimed at determining the identity and quantity of expressed proteins in cells, their three-dimensional structure, and interacting molecules [67–69]. It is estimated that there are at least one million human proteins, many of which contain alterations such as post-translational modifications (PTMs) [69–71]. In 2013, the proteomics community coined the word “proteoforms” to refer to all the unique protein molecules produced from a gene. The chemical diversity of proteins is expressed in their many proteoforms, derived from combinations of genetic polymorphisms, RNA splice variants, and post-translational modification [68–71].

While the first analyses of proteins in biological fluids date back to the early 1970s [72], trailblazing advances in evaluating proteomic sputum have only occurred in the last decade [17–20]. The rationale for looking at phlegm as a wealth of information on lung health is based on its composition, which encompasses mucus and microbial constituents.
Researchers can therefore colonize bacteria/viruses and particulate/inhaled matter derived from the external environment to inflammatory cell components, including cells that reside either in the airways’ lumen, tissue, or cell debris in these compartments [20]. Since precision medicine extends beyond a single biomarker/treatable trait where a patient may have multiple options for treatment [6], it is ideal to use multiple techniques capable of analyzing several biomarkers or one single platform that can identify and quantify numerous biomarkers. Table 2 summarizes the various options to study proteins in sputum that are discussed in other publications [20,73–79]. It is apparent that the protein composition of sputum best reflects the state of the lungs [56]. Additional information on sputum proteomics studies can be found in references [80–88].

Table 2. Overview of various techniques used to analyze proteins/peptides extracted from sputum.

<table>
<thead>
<tr>
<th>Procedure Performed to Analyze Proteins/Peptides in Sputum</th>
<th>Number of Proteins/Peptides Analyzed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-dimensional electrophoresis gel (2-DE), and one-dimensional gel electrophoresis followed by liquid chromatography–tandem mass spectrometry (GeLC-MS/MS)</td>
<td>191</td>
<td>[73]</td>
</tr>
<tr>
<td>Mesoporous silica beads and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF-MS)</td>
<td>&gt;400</td>
<td>[74]</td>
</tr>
<tr>
<td>Liquid chromatography–mass spectrometry (LC-MS)</td>
<td>192–1666</td>
<td>[23,24,75,76]</td>
</tr>
<tr>
<td>Capillary liquid chromatography (capLC-MS)</td>
<td>203</td>
<td>[77]</td>
</tr>
<tr>
<td>Multi-dimensional protein identification technology (MudPIT)</td>
<td>2210</td>
<td>[78]</td>
</tr>
<tr>
<td>Aptamer-based assay (SOMAscan)</td>
<td>1129</td>
<td>[79]</td>
</tr>
</tbody>
</table>

These references are selected illustrations of efforts to identify, quantify, and characterize proteins and peptides, including antibiotic peptides, present in sputum samples.

6. Potential Use of Point-of-Care Platforms for the Screening of Sputum Multi-Biomarker Proteins to Diagnose Infectious and Chronic Respiratory Diseases

The use of biomarkers as early warning systems in the evaluation of disease risk has increased markedly in the last decade. Making the distinction between a potential biomarker and a reliable biomarker that can be universally applied to guide critical clinical choices is a main obstacle in the field of biomarkers [89]. When referring to POCT platforms, which are designed to detect the presence of biomarkers with inexpensive and easy-to-use platforms, can they provide certainty in the detection of accurate and reliable biomarkers? Unfortunately, not always. There have been several critical roadblocks in the development of inexpensive, minimal-equipment diagnostics, and even more so in improving sample preparation, and biomarker analytical sensitivity and specificity. Quantitative measurement of biomarkers in complex samples is inherently difficult to perform robustly, particularly when constrained to a minimal-equipment framework [90]. There are other factors that are also important to consider challenging in the analysis of biomarkers, such as proteins. The profiling of proteins in biological fluids has been central for decision-making in numerous diseases since the introduction of many immunoassays in the 1970s [91]. However, obstacles to comprehensive proteomic profiling include the immense size and structural heterogeneity of the proteome, and in most cases, protein screens have been hampered by technical limitations, in particular limitations in sensitivity, specificity, multiplexing, and sample throughput [91]. Nonetheless, the bottleneck of sample analysis has always been sample preparation, which is often characterized by being time-consuming, labor-intensive, and error-prone [92]. This is particularly true when analyzing complex biological matrices such as sputum, which must be liquefied before analysis of its constituents [41,93]. Addi-
tionally, it is noteworthy to mention that many proteins are found at very low abundance in biological fluids, and the polyreactivity of affinity-capture agents may have the potential to generate false results [70]. Furthermore, as numerous diseases include various pathophysiological processes, no single biomarker can be regarded as ideal in fulfilling the necessary criteria for a comprehensive diagnostic or prognostic assessment revealing optimal clinical application [94]. Therefore, a multi-component biomarker approach with the aid of crucial biomarkers to create a detailed profile of a patient’s disease is vital to generate an accurate and reliable diagnosis and prognosis. Due to the challenges faced in collecting sputum and developing a simple method to dissolve the complex sputum matrix, emphasis should be placed on whether a POC testing device is intended to be manufactured for the analysis of sputum constituents under the guidelines of ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, Delivered) [41,94].

Currently, a gamut of technologies exists to extract and isolate proteins from intricate mixtures to further analyze them. One- and two-dimensional electrophoresis, or high-performance liquid chromatography (HPLC), are the most common uncoupling methods, but mass spectrometry (MS) is now seen as the benchmark [95–97]. Advances in quantitative MS through the application of stable isotope labeling and scanning techniques, as well as multiple reaction monitoring (MRM), have greatly enhanced the specificity and sensitivity of MS-based assays [98]. Factors including patient heterogeneity and variations in sample acquisition, storage, stability, and dynamic range of analytes, including their modification status and macromolecular interactions, all influence the ability to discern biomarkers. Despite the precision and automation of these bulky instruments in the discovery and corroboration of biomarkers, they are exclusively used in research and/or central clinical laboratory settings and do not serve the purposes of a point-of-care testing system in a doctor’s office or at home for self-testing. Still, these scientific innovations have been pivotal in the discovery of new biomarkers and corroborating the results of these biological indicators via POC tests. There is a hope that progress in the design and manufacturing of miniaturized instruments, including MS, will catalyze the transition of healthcare from bench to bedside [99].

LFIA, founded on an affinity-capture–migration technology, occasionally yield false-negative or false-positive misdiagnoses, yet they continue to be used as a screening tool [100–103]. Conversely, immunoaffinity capillary electrophoresis (IACE), based on the principle of an affinity-capture–separation or a two-dimensional technology, is a popular test due to its ability to confirm results [70,103–108]. Even if there are doubts about the biomarker(s) separated by capillary electrophoresis (CE) (electropherogram), the CE outlet terminal can be coupled to a mass spectrometer that also validates findings. This feature cannot be performed via lateral flow assays. Figure 2 depicts a scheme of disrupted/lysed sputum sample constituents subjected to lateral flow immunoassay and capillary zone electrophoresis.

The implementation of a CE platform, either in microchannels or fused silica glass as a POC device, began in the 1990s [109–114]; since then, several mini handheld prototypes have been manufactured [113–117]. One palmtop bioanalyzer has a laser-induced fluorescent detector—the authors affirm that this tiny device combines seamless design with low-cost construction, retailing for USD 500 [113]. The palmtop bioanalyzer consists of modules such as automated sample injection, CE separation, orthogonal LIF detection, instrument control and display, data acquisition, processing, storage, and battery power supply. Figure 3 presents the separation of standard proteins using this CE bioanalyzer, yielding results achieved in under 7 min.
Figure 2. Diagrammatic representation of (A) a disrupted/lysed sputum sample treated with a detergent and a proteolytic enzyme as described in ref. [41]. This processed specimen was centrifuged to remove cellular debris. An aliquot of the supernatant constituents was tested via an LFIA platform for the detection of the SARS-CoV-2 nucleocapsid protein antigen N (B). Another portion underwent capillary zone electrophoresis, and the separated components of the supernatant of the entire disrupted/lysed sample were monitored at the low nm detection range (C) as described in ref. [104]. The electropherograms can vary significantly if distinct samples and dissimilar pretreatment procedures are applied. Supplementary experiments are underway to enrich and separate specific components via immunoaffinity capillary electrophoresis.

Figure 3. A handheld capillary electrophoresis instrument (A), shown in ref. [113]. Panel (B) is an electropherogram of three protein standards derivatized with fluorescein isothiocyanate (FITC), monitored by laser-induced fluorescence detection (LIF). The separation of these protein standards was carried out in a 50 µm I.D. fused silica capillary, 360 µm O.D., and 3.8 cm in length; the electropherogram was adapted from ref. [113].
7. Future Evolution and Benefits of a Point-of-Care Platform for the Determination of Multiple Biomarkers to Improve Early Detection and Diagnosis of Diseases

As precision medicine enters the multi-omics era, technology also needs to evolve [118]. Therefore, strengthening the healthcare system is the most ideal way to keep the population healthy and maintain a productive workforce [119]. As such, we propose a miniaturized point-of-care system based on a two-dimensional capture–separation system capable of analyzing multiple biomarkers that can be identified and characterized to avoid false-positive and false-negative diagnostic results. This instrument is designed to be portable, cost-effective, generate data in a short time, and have high analytical sensitivity and specificity. It can be manufactured with modular components that are easy to replace and affinity-capture ligands (antibodies, antibody fragments, nanobodies, lectins, aptamers, phages, other affinity chemistry, or a combination of one or more ligands) that can be reused multiple times. The primary function of this miniaturized instrument is to serve as a POCT for early diagnosis and prognosis of ailments by determining protein biomarkers. Some chronic diseases have an infectious origin, such as cervical cancer (human papillomavirus—HPV) and liver cancer (hepatitis B and C viruses). Also, many patients with infectious diseases require long-term care; for example, human immunodeficiency virus (HIV) infection, recognized as a chronic disease, can now be successfully managed over time [120]. Although non-communicable diseases (NCDs) are considered silent killers, they attract less attention than infectious diseases and threaten to overwhelm global health systems with their rapid rise. NCDs cause nearly 74 percent of all deaths worldwide, a majority occurring in low- and middle-income countries [121–124]. The proposed portable POC model instrument with multidimensional capabilities and numerous applications is depicted in Figure 4.

Figure 4. Diagrammatic representation of a multidimensional CE system (panel (A) and panel (B)) equipped with an analyte concentrator-microreactor (ACM-1 and ACM-2) device at each transport capillary (TC) intersection with a separation capillary (SC-1 and SC-2). Here, the ACM device has a staggered or zigzag configuration. All small arrows represent the path of the sample introduction from the inlet (1) to the outlet (6) into a waste container. Arrows 7 and 8 show the direction of separation of the analytes captured by and released from ACM-1 and ACM-2 in a sequential and separated mode. The green circles illustrate the various microvalves (V-1 to V-6) that control the passing of fluid. When the microvalves V-2, V-3, V-5, and V-6 are closed, and microvalves V-1, V-4, and V-7 are opened, the specimen containing the target biomarkers having passed through the transport capillary (TC) from inlet 1 to outlet 6, and ACM-1 and ACM-2. After applying a cleaning buffer to remove unbound material, microvalves V-1, V-5, V-6, and V-7 are shut, and microvalves V-2 and V-3 are opened. A small volume or plug of an elution buffer or solution is introduced into separation capillary SC-1 from container C-1, and the targeted biomarkers captured by ACM-1 are released. The ACM-1 and ACM-2 devices are filled with a matrix containing specific affinity-capturing...
ligands immobilized to a monolith solid support or beads, or covalently attached directly to the inner wall of the interior of each ACM. The selective affinity-capturing ligands can be an antibody, antibody fragments, nanobodies, lectins, aptamers, other substances, or a combination of all these selective affinity ligands. Each ACM device captures one or more specific targeted analytes. Once the plug of an elution buffer is applied, an independent capillary electrophoresis process is initiated sequentially from the inlet side before the separation of capillary 1 (SC-1) in the direction of the flow. The process of separation of the biomarker is preferentially carried out using capillary zone electrophoresis (CZE), but other separation modes can be performed. The components released from ACM-1 and separated by CZE are detected/identified at the detection point where a detector (UV, fluorescence, or others) is positioned. A similar but independent and sequential protocol is applied to the elution process to release biomarkers captured by the ACM-2 device, using container C-2 (filled with an elution buffer-2 or separation buffer-2) and separation capillary SC-2. Panel (A) depicts an additional microvalve (V-8) where the two independent separation capillaries (SC-1 and SC-2) are merged to share a single detector. Panel (B) illustrates these capillaries (SC-1 and SC-2), each with its respective monitor. Panel (A) also shows two additional auxiliary capillaries (AC-1 and AC-2) used when a separation buffer requires a different chemical composition from the one of the binding buffer, as it is required when coupled to a mass spectrometer detection system. The figure was adapted from ref. [125–128].

8. What Are the Advantages of Affinity-Capture–Separation Analytical Techniques When Compared with Traditional Methods?

Traditional affinity-capture immunoassays, such as enzyme-linked immunoassay (ELISA), have become the gold standard clinical diagnostic tools for the detection and quantification of protein biomarkers [129,130], and efforts have been made to use them as a platform for a point-of-care detection system for the diagnosis of infectious diseases [129,131]. ELISA continues to serve as the benchmark for diagnosis by detecting specific antigens in biological fluids for multiple applications. However, the technique has disadvantages, such as the possibility of false-positive and false-negative results, costly instrumentation, the requirement for trained personnel, and taking longer to measure multiple analytes. In the classical double-antibody sandwich method, two different antibodies to capture and recognize the same protein antigen are needed to enhance analytical specificity, so only one target protein biomarker can be detected at a single time. Despite its limitations, ELISA remains the time-tested and best-validated method for measuring individual cytokines [132]. Presently, multiplex array technologies are increasing in popularity, likely because of their ability to analyze large numbers of analytes with low sample volume compared to ELISA. When evaluating both approaches to measuring cytokines, the results indicated that serum cytokine concentrations were not compatible between these two systems [133].

The limitations of traditional approaches to identifying and quantifying proteins have led to the evolution of new technologies for protein biomarker discovery that are highly sensitive and capable of high-throughput analyses. The advent of modernized and more sophisticated tools has granted opportunities for the exploration of biological indicators. A contemporary and prominent example of a high-throughput proteomics technology is an aptamer-based proteomics platform called SOMAscan [134]. The SOMAscan platform is a highly sensitive platform that uses slow off-rate modified DNA aptamers (SOMAmers) as high-affinity protein-capture reagents to simultaneously quantify more than 1300 human proteins in all types of protein extracts, including bodily fluids, tissue, and cells. Despite its potential, this aptamer-based proteomics technology has only been used in pilot studies and has yet to be applied to samples from population-based cohort studies [134]. As artificial single-strand oligonucleotides, aptamers possess unique advantages compared to conventional antibodies. They can be flexible in design, have low immunogenicity, relative chemical/thermos stability, easy synthesis, as well as convenient modification [135,136]. In contrast to using aptamers, antibody-based methods excel in both analytical sensitivity
and specificity and are therefore extensively employed to detect and quantify specific proteins [135].

An alternate multiplatform for protein research is the highly sensitive targeted immunoassay known as the Olink proximity extension assay (Olink PEA) technology, and when combined with other platforms, offers in-depth proteome analysis [137–139]. Also, once PEAs are coupled with next-generation sequencing (NGS) for high-throughput proteome-wide analysis, they enable parallel measurement of about 1500 proteins with 96 samples, resulting in close to 150,000 data points per run [125]. The basic principle behind PEAs is signal generation; when two antibodies linked to corresponding DNA oligos (with the complementary sequence) find the same target, they form a double-stranded DNA (dsDNA). The presence in the reaction mix of a polymerase and other compounds allows for elongation and subsequent amplification, and each antigen will lead to a unique DNA code. The sequence then uniquely identifies a target antigen, and its abundance correlates with the protein antigen concentration [137–141].

These multi-dimensional platform technologies will continue to play an important role in deciphering essential biomarkers and improving the substantiation of intricate diseases. For example, long-haul COVID is broadly defined as a non-monolithic, multisystemic disease that impacts multiple organ systems; it is like a disease with many subtypes that can have many different risk factors and distinct biologic mechanisms that may respond differently to treatments [142]. Because long COVID is a heterogeneous entity, it is unlikely that a single or a few biomarkers will explain its complexity. A creative rethinking of the approach to biomarker development is needed, such as harnessing the power of artificial intelligence to analyze large numbers of variables and identify multidimensional biomarkers to classify distinct long COVID subtypes and predict prognosis and treatment responses [142].

There has been a surge in recent years in the implementation of proteomics in the study of biological fluids, revealing disease mechanisms that are linked to chronic and infectious diseases. The human proteome contains thousands of protein mixtures, and the consensus is that most remain unexplored in their associations with multiple diseases. Even with the first-rate attributes of immunoaffinity-based technologies, weaknesses such as insufficient specificity and antibody cross-reactivity persist [41,125–128,143]. These limitations are lessened via the Olink system, using complementary DNA oligonucleotides that attach to antibody pairs to decrease this undetailed cross-reactivity. Still, evaluations juxtaposing the Olink and SomaScan platforms found that the matching range of coefficients varied greatly among all proteins compared. It is believed that the cross-reactivity of aptamers and antibodies is a factor in the low correlation investigations for proteomic analysis, and their cross-reactive likelihood increases as amino acid homology elevates [143,144].

Even with assays that have varying benefits and drawbacks, more analysis is needed to establish the potential for integrating data across proteomic platforms fully. Investigators should be careful to treat discovery platforms appropriately, confirming key results with additional methods, using comparisons to reference standards to obtain exact protein quantification [144], and specifically acquiring valid clinical biomarkers. Reliable clinical biomarkers are still limited, restricted by the suboptimal methods of biomarker discovery [135,136]. When using modern technologies in the diagnostic process, careful care must be taken to guarantee safe, efficient, and trusted care, and to avoid diagnostic errors that may lead to devastating consequences for the patient.

How can we take advantage of these emerging technologies to create an error-free, point-of-care instrument that yields accurate diagnostic tests based on a selection of biomarkers taken from a wide range of concentrations in biological fluids that are also affordable and accessible to everyone? Section 7 describes the added value of the two-dimensional capillary electrophoresis technique, which allows for the targeting of more than one selective biomarker in a biological fluid or tissue extract to be accurately, promptly detected and quantified at a low cost. These excellent characteristics are ideal for the future design of a portable, point-of-care instrument. Figure 5 highlights two examples
validating the advantages of this two-dimensional affinity-capture–separation technology. Figure 5A shows the separation and identification of multiple biomarkers using a single microchip electrophoresis system simultaneously in the same preconcentrator, while a dozen immobilized antibodies target 12 distinct antigen biomarkers. During a 9-minute cycle, 12 biomarkers were analyzed, during which all analytes were reliably affinity-captured, labeled with a fluorescent chromophore, eluted, separated, and detected with a laser-induced fluorescence detector [145]. The covalently attached antibodies served both as selective capture agents and as a pre-analysis clean-up system. With the aid of a semi-automated miniaturized immuno-separation system, it was possible to repeat an evaluation cycle of six samples/hour to identify and quantify 12 biomarkers taken from detergent-extracted micro-dissected skin material related to mild and chronic skin lesions [145]. Figure 5B presents a microextraction-preconcentration area containing two immobilized affinity-capture ligands that can effectively be used 45 times and exhibit excellent repeatability and stability. In this case, the immobilized affinity-capture ligands used were aptamers, instead of antibodies, capable of trapping two distinct analytes. The process of capture, elution, separation, and detection was performed using a conventional capillary electrophoresis method coupled to a mass spectrometer [146]. The limits of detection of the analytes were 1 pg/mL.

Figure 5. Diagrammatic representation of a multi-biomarker analysis based on affinity-capture–separation analytical technology performed with microchip electrophoresis (panel (A)). The peaks in the electropherogram resolved in the following order: 1, TGF-β (transforming growth factor beta); 2, IL-6 (interleukin-6); 3, IL-1β (interleukin-1 beta); 4, IFNγ (interferon gamma); 5, MIP-1α
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dyspepsia were chemokines that vary from different types of dyspepsia, whereas the latter was the same chemokine regardless of dyspepsia type. The chemokine levels were further categorized into high and low expression levels. The expression levels were based on the median chemokine level found in healthy controls. The chemokine expression levels were compared using the Mann-Whitney U test.

A small amount of free dye was always present. All antibodies directed to the targeted biomarkers were co-immobilized to disposable glass fiber disks and inserted within the extraction port of the microchip. The captured analytes were labeled with a 635 nm light-emitting laser dye and electroeluted into the separation channel as described in ref. [145]. On (panel (B)), single-stranded aptamers were used as affinity-capture ligands for the selective recognition of two molecular-weight compounds (1, aflatoxin B1 and 2, ochratoxin A). These two analytes were co-immobilized via covalent bonds on the surface of the inlet end of the capillary. The separation, detection, and quantification were performed by capillary coupled to a mass spectrometer. The figures were adapted and modified from ref. [145,146].

Although Figure 5 does not show an example of the separation and identification of sputum specimen constituents, it illustrates the advantages of using at least two immobilized affinity-capture ligands in the same ACM device. This allowed the use of one of them to capture and separate a known substance as an internal standard to improve accuracy and can additionally serve to monitor the life cycle of an ACM device after multiple uses. A second immobilized affinity-capture ligand is applied as a bait to selectively attract a specific target biomarker. It is promising to think that the two-dimensional affinity-capture–separation techniques, carried out in microchip electrophoresis or capillary zone electrophoresis, can be used as a miniaturized point-of-care instrument. Such a portable instrument will enable the quantification of two or more biomarkers containing immobilized affinity-capture ligands immobilized to the pre-concentrators that can be used multiple times. Results can be obtained in short analysis times, with significant improvements in analytical sensitivity and specificity. It is foreseeable that such multi-dimensional miniaturized instruments can be cost-effective and ideal for point-of-care use in countries with limited healthcare systems.

9. Discussion and Future Perspectives

There is growing evidence regarding the utility and merits of two-dimensional affinity-capture–separation analytical systems to address biomedical challenges [147–166]. For example, coupling microchip electrophoresis or conventional capillary instruments to specialized detectors, such as radiometric, electrochemical, laser-induced fluorescence, and/or mass spectrometry, offers enhanced analytical sensitivity capable of reaching limits of detection in the lower picogram or at the nanogram scale. Such features enable the analysis of biomarkers found at low concentrations in biological fluids. The affinity-capture agents can be a variety of substances or a combination of them; the most popular are antibodies, antibody fragments, nanobodies, lectins, aptamers, protein A/G, and multimodal peptides, including many others that have high-affinity binding to one or more moieties of another molecule. These agents can act as selective capture molecules or preanalytical concentrators and can effectively serve as a clean-up process in removing excess amounts of non-targeted analytes. Preselection of the affinity-capture ligands and optimization of immobilization also guarantee that equal capture and release will occur for all analytes during a set window of time [145,146]. The immobilization of the affinity-capture ligands can be performed on glass or plastic beads, monolith structures, magnetic particles, or directly on the surface of the inlet side of the channel or capillary. The analyte concentrator-microreactor (ACM) device is so named because of its dual functions of concentrating and cleaning; it is also used as a microreactor that permits the performance of biochemical reactions (e.g., enzymes immobilized to cleave proteins with the ACM device or area) [167,168].

Regarding applications, the analytical technology of affinity-capture–separation can be used beyond solving biomedical problems; for example, it can be extended for the purpose of identifying and characterizing analytes in complex matrices related to food products,
pharmaceutical products, environmental monitoring, space, and other research areas. These qualities of miniaturized analytical technologies offer unique benefits that cannot be matched by current traditional larger-scale equipment. Miniaturization of analytical instruments makes it possible to decrease demands on working space, reagents, and power [169,170].

In the last decade, there has been a marked improvement in the availability of laboratory and point-of-care testing for the diagnosis of microbial infections [171–174]. Unfortunately, balancing the requirements for affordable, easy-to-use, sensitive POC testing is difficult. Raising the expectations for POC testing by requiring POCTs to match or exceed the accuracy and performance of centralized laboratory testing should be an immediate goal [175]. POC testing is generally supported by two types of technologies: handheld single-use devices and small bench-top/portable analyzers. In addition to manufacturing an ideal POC testing instrument, reagents must be considered. A key component for successful, high-accuracy testing is the use of affinity-capture ligands, and today there are many options beyond commercially available compounds, such as antibodies, lectins, and aptamers. Recently, a new technique involving biopanning with phage-displayed peptides has been developed, offering the ability to identify small peptides that can be used in a similar manner to antibodies for on-target capture of biomarkers [175]. When detecting the presence of microorganisms in samples, using capture ligands to selectively extract one protein of interest from a complex mixture offers the advantage of revealing a single biomarker to indicate the presence of bacteria or viruses. Biopanning for phage-display peptides allows for the development of affinity ligands from virtually any organism, permits ease in utilizing these peptides with affinity-capture mass spectrometry, and holds great potential for diagnosing emerging pathogens [175]. This in vitro, antigen-driven affinity selection, employing phage display biopanning, is now a commonly used technique to isolate monoclonal antibodies [176].

POC testing can deliver rapid diagnostics in environments where results can be used to direct clinical management during patient visits and where centralized laboratories are limited [171]. The two-dimensional affinity-capture–separation POC testing instrument that we discuss in this review can be designed not only to work in normal environments but can also function in extreme environments, including harsh temperatures, pressure, and radiation, as required in space and deep marine conditions. Evolving technology in healthcare continues to shape the way we provide care to patients. Advances in artificial intelligence, data generated in proteomics research, and the more mainstream use of telemedicine allow patients, families, and their caregivers to improve diagnostic quality anywhere in the world [103,126,163,164,177]. Sputum tests offer hope in determining protein and nucleic acid biomarkers for the diagnosis of respiratory infectious diseases, pulmonary diseases, and lung cancer far in advance of clinical symptoms [33,178]. Sputum biomarkers have detected other diseases; for example, autoantibodies in subjects with rheumatoid arthritis [179]. Another publication reported that immunoglobulin A could be detected in sputum at an early stage in COVID-19 patients [180]. In addition, another study demonstrated the detection of antibodies against Pseudomonas aeruginosa in the sputum of cystic fibrosis patients and that the sensitivity of the assay was greater in sputum than in serum [181]. In immunodeficient patients with COVID-19, regardless of treatment, persistent viral positivity was obtained in sputum samples where contemporaneous nasopharyngeal swab samples were negative [182]. Persistent viral positivity in sputum, detected by PCR or antigen test, presents infection control challenges, especially in groups with frequent hospital attendances in clinical spaces with other vulnerable patients. Monitoring of sputum samples is therefore recommended in situations where patients can provide them [41,182].

The two-dimensional POC testing (2D-POCT) instrument described in this review has the potential for high-throughput testing. Based on the handheld instrument illustrated in Figure 3, it is possible to separate at least three proteins in less than 7 min. If the instrument described in Figure 4 was commercialized, approximately 3–4 extra minutes would be
required for the entire testing process—sample introduction, cleaning, and removal of the non-specific bound analytes, plus the elution process. Using only one ACM device to test three bound and released biomarkers would take 12 min. To highlight the cost and time-saving benefits, if two ACM devices were utilized, it would be possible to analyze at least six protein biomarkers from a single biological specimen in 25–30 min, or 12 biomarkers per hour. As the process is sequential, both ACM devices are in contact with the same biological fluid but only one ACM device would be in operation for complete analysis at one time. Therefore, one low-cost 2D-POCT instrument can analyze 120 biomarkers in 10 h. If two or more instruments are used simultaneously, then it is possible to capture, release, separate, detect, and quantify a few hundred biomarkers per day.

A further advantage of using one or two internal standards per ACM device is that it yields accurate diagnostic information with virtually zero chance of false results, thus improving the accuracy of the test. This is especially true if the levels of pro-inflammatory cytokines and specific organ biomarker signature(s) are quantified simultaneously in the same biological sample. It is important to state that the quantification of cytokines has significant value in both clinical medicine and biology as the levels provide insights into physiological and pathological processes and can be used to aid diagnosis and treatment [183]. In order to secure the binding of the immobilized affinity-capture ligand with the corresponding target biomarker, it is helpful to use two affinity-capture ligands, for example, with one immobilized antibody and one immobilized aptamer. Not only does this process allow for an increase in the binding capture, but the binding would happen in only a few minutes instead of several hours. Finally, the ACM device has been demonstrated to be re-useable at least 45 times with significant reproducibility of results [138], as illustrated in Figure 5B, dramatically reducing the cost per assay.

In conclusion, we emphasize the importance of protein biomarkers as crucial molecules to discern the complexity of diseases. Proteomics continues to be at the forefront of scientific discovery, providing invaluable insights into the intricacy of living organisms at a molecular level. Proteins are not only the product of the transcription and translation of genetic information; the process of protein biosynthesis underlies a variety of processes at the genome and transcriptome levels, such as splicing, recombination, gene shuffling, and more. Also, a plethora of different co- and post-translational modifications can occur during the lifetime of a protein molecule. These processes lead to the formation of numerous protein species out of a single gene, for which the term “proteoform” has been coined, encapsulating the array of molecular expressions now employed in place of previously used names such as “protein forms”, “protein isoforms”, “protein species”, and “protein variants” [69–71,184–189]. A major advantage of a two-dimensional affinity-capture–separation technique is that polyclonal antibodies are ideally suited for proteoform retrieval. The high-affinity selection of target protein biomarkers coupled with the high-resolution characteristic of capillary electrophoresis and/or high-resolution mass spectrometry can provide information to monitor minor differences in molecular changes in structurally related proteins [70,184,188]. Mass spectrometry is expected to be used for the discovery and characterization of new protein biomarkers but should not be a routine method when biomarkers are known and validated with affinity-capture–separation capillary electrophoresis. The utilization of one or two internal standards to control the performance of separation of the captured and released known targeted biomarkers in microchip electrophoresis or conventional capillary electrophoresis should be sufficient to obtain an accurate diagnosis for those biomarkers known to be affected in a specific disease. We are confident that the two-dimensional, portable, miniaturized point-of-care testing instrument described in this article, designed for use with biological samples obtained by non-invasive methods such as sputum, saliva, urine, and sweat, will become mainstream and be scaled up when its advantages and definite savings are realized.

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