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From a Central Laboratory to the Bedside: a Point-of-Care Instrument for Monitoring Wellness and Disease Using Two-Dimensional Immunoaffinity Capillary Electrophoresis Technology

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Abstract

Point-of-care testing (POCT) is a rapidly growing field with potential for improving operational efficiency within healthcare settings. Particularly, the quick delivery of accurate, sensitive, and specific biomarker information at the bedside has shown to facilitate clinical decision-making and may improve clinical outcomes in patient care. While POCT should be one of the most efficient and economical tools for medical management, there are limitations that must be overcome before traditional diagnostic testing can uniformly transition to POCT. In this paper, we introduce a novel portable instrument, manufactured with miniaturized components, based on immunoaffinity capillary electrophoresis (IACE) a two-dimensional technology that can address many of the limitations associated with POCT.

IACE consists of the use of immuno-capture techniques found in some immunoassays, such as enzyme-linked immunosorbent assay (ELISA), and pairs it with a high-resolution analytical separation technique, such as capillary electrophoresis. Distinctively, this promising instrument has the potential to yield no false-positive or false-negative results; this is due to its two-dimensionality format and the use of more than one immobilized affinity ligand to secure the capture of one or more analytes of interest found in biosamples. Additionally, IACE is inexpensive because the immobilized affinity ligands can be re-used multiple times, making it a great asset in both resource-limited and large private healthcare settings. Furthermore, IACE has the potential to be used in outpatient locations such as doctor's offices, remote clinics, or even in patient homes due to its portability. We predict that test results from these locations can be directly sent via secured Internet connections to medical facilities housed by physicians for interpretation, for follow up, and to create an emerging electronic medical record systems known as clinical databases.

In summary, we foresee that this point-of-care technology may have significant implications for healthcare improvement both on an individual and systemic scale. The details, applications, and potential impact of this technology in systems medicine will be described in this manuscript.

Keywords: Immunoaffinity capillary electrophoresis; On-line preconcentration; Affinity capture; Point-of-care device; Miniaturized diagnostic tests; Telemedicine; Reliable and accurate diagnosis; Biosensors; Precision medicine; P4 medicine; Systems medicine

Abbreviations

IACE: Immunoaffinity Capillary Electrophoresis; CE: Capillary Electrophoresis; POCT: Point-of-Care Testing or Point-of-Care Technology; ACM: Analyte Concentrator-Microreactor; SPE: Solid Phase Extraction; ELISA: Enzyme-Linked Immunosorbent Assay; HIV: Human Immunodeficiency Virus

Introduction

Modern diagnostic testing of biomarkers thrives in the presence of sophisticated instruments, a stable electrical supply, availability of various reagents, and highly trained personnel. Although some diagnostic testing can be performed without these resources, most state-of-the-art instruments used for biomarker determination occupy large physical spaces and are predominantly found in modern central laboratories. While urban areas in many countries employ this advanced diagnostic technology efficiently, rural and remote areas across the world struggle to do so due to their limited access to the aforementioned resources [1,2].

In most cases, lack of access to certain laboratory tests can give rise to the absence of, incomplete, or delayed results [3-8]. While clinical management in the setting of limited laboratory results has the potential to be effective in diagnosing a variety of illnesses, doing so for many clinical presentations can result in a lack of or inappropriate treatment [3-8]. This raises the potential for missed diagnoses, worsening antimicrobial resistance and intensifying drug toxicity [7-10]. These potential outcomes, in addition to delaying the initiation of treatment of a correctly diagnosed disease, can contribute to increased morbidity and mortality rates [9-14]. To improve health outcomes in areas with limited access to modern laboratory testing, easy-to-use point-of-care testing (POCT) devices have emerged as tools for physicians globally to diagnose diseases rapidly and accurately.

While POCT is important for diagnostic testing to be carried out in resource-limited areas [13-19], it has also been shown to facilitate clinical decision-making and improve clinical outcomes in resource-abundant areas [18,19]. For example, healthcare facilities have shifted towards using glucose biosensor strips for rapid determination of glucose levels and polymerase chain reaction (PCR) assay for the rapid determination of infectious diseases, such as detection of Clostridium *difficile* [20]. In general, a major benefit of POCT is to facilitate a healthcare provider's ability to deliver appropriate and timely treatment to patients. Additionally, POCT can address limitations that exist with current modern laboratory techniques.

During the last two decades, enzyme-linked immunosorbent assay (ELISA), a convenient and cost effective plate well-based assay technique, has played a central role in diagnosis and clinical research applications [21,22]. ELISA is commonly used in urban laboratories for the quantification of a wide range of substances [21-23]. ELISA utilizes a mono-dimensional format, in which all reagents are mixed in a single plate well from sample incubation to signal generation [21-23]. Each plate is coated with antibodies or antigens, which in theory are specific for a biomarker of interest. The technique is used once and then the plate is discarded. If the target analyte of interest is detected, a colorimetric, fluorimetric, chemiluminometric, or bioluminometric signal is produced. With appropriate known concentrations of standards, a calibration curve is made to calculate the concentration of the analyte under study. Unfortunately, many antibodies are polyreactive [24], which may result in recognition and binding of structurally unrelated targets in addition to their target antigens. Consequently, a substance other than the one of interest can compete with the antigen-binding

site, bind to it, and yield a false-positive test result [25-27]. Imprecise biomarker identification accordingly leads to inaccurate diagnosis and treatment.

A setting in which inaccurate diagnoses can be seen, following the use of mono-dimensional techniques such as ELISA, is in the identification of infectious diseases. For example, infectious mononucleosis caused by the Epstein-Barr virus and mononucleosis-like illnesses caused by other viruses, particularly cytomegalovirus and human immunodeficiency virus (HIV), are often clinically indistinguishable, requiring definitive diagnosis to be made by serology. Current mono-dimensional techniques, such as the monospot test or the sandwich ELISA test for HIV for example, may yield a false positive result and lead to the incorrect preliminary diagnosis of acute retroviral syndrome, thereby requiring a Western blot analysis or other two-dimensional techniques for confirmation [28]. Similarly, when testing for schistosomiasis, it is recommended to use two or more assays in parallel to obtain a confirmatory diagnosis [29]. Examples of contradictory results using ELISA for the diagnosis of non-communicable diseases have also been demonstrated for the determination of the miokine irisin [30-32], the pancreatic ductal adenocarcinoma biomarker CUZD1 [33], and the appearance of a cross-reacting human chorionic gonadotropin (hCG)-like antigen in septic shock [34]. Other examples are described in references [23,30].

To further expound upon the degree of diagnostic limitations that ELISA invokes on modern testing, newer research studies using techniques capable of characterizing and discerning differing forms of the same protein [35-37] have suggested that specific isoforms of biomarkers can improve insights into disease diagnosis. Prostate cancer, for example, is the second most frequently diagnosed cancer in men worldwide. However, recommendations for prostate-specific antigen (PSA) testing are equivocal because the risk of pursuing pathologic diagnosis and treatment of prostate cancer based on PSA levels can be more harmful than the pathology itself. Further investigation into the use of PSA as a biomarker, has suggested that changes of the composition of the sugar moieties of PSA, a glycoprotein, occur in pathological conditions such as malignant prostate cancer [35,36]. If a technology more advanced than ELISA could distinguish isoforms of PSA, based on changes of the composition of the sugar moieties of this glycoprotein, there would be significantly fewer false-positive results for malignant prostate cancer [35,36]. Subsequently, recommendations on diagnosing and treating prostate cancer could be more conclusive.

Similar to the determination of isoforms of PSA, certain isoforms of metallothionein have been suggested as biomarkers in patients with chronic hepatic disease to determine the extent of liver disease and subsequent risk of developing hepatocellular carcinoma, a complication of chronic liver disease [37]. Detecting not only the presence of metallothionein, but distinguishing the concentration of each isoform through advanced separation techniques would allow more cost-efficient and appropriate surveillance of patients with chronic hepatic disease. In fact, there is some evidence that the concentrations of metallothionein isomers change depending on the progress of the tumor and further information about these isomers is very useful in determining the stage of the chronic hepatic disease [37].

In summary, reliable and accurate diagnosis of disease biomarkers has the potential to be medically valuable at all stages of the disease process from diagnosis to subtype identification, management and prognosis [38]. Unfortunately, diagnostic errors, such as inaccurate or delayed diagnoses can persist given current diagnostic methods, and therefore, may result in inappropriate management and possible harm for an unacceptable number of patients [39]. While ELISA has been considered the gold standard in bioanalysis for the quantification of a wide range of substances, its methods suffer drawbacks in sample size, equipment cost, analysis time, and, in some cases, yielding false-positive and false-negative results, as explained above [23,30]. Although POCT has been of use as a resource in access-limited areas, POCT supplements current laboratory testing in resource-rich areas [40]. Newer applications of POCT have the potential to replace and surpass even the most commonly used current technology, such as ELISA and other mono-dimensional techniques with the aim to improve diagnostic testing quality and to prevent medical errors [40-43]. However, in order to generate consistent and reproducible data, POCT should be implemented within a quality framework that is tailored to recognize the resources of non-laboratory environments, while at the same time producing accurate results equivalent to those produced by a centralized clinical laboratory where state-of-the art technology is available to enhance clinical care [17]. Hybrid techniques, which are best represented by merging immunoassays and analytical separation instruments to form a single two-dimensional capture and separation system, can therefore be used to achieve the goals of POCT while overcoming limitations of mono-dimensional testing.

Miniaturized Diagnostic Tests

In the last two decades, significant advancement has been reported in microfabrication techniques, stimulated primarily by the growth in the semiconductor and microprocessor industries [44]. Such miniaturized devices and components have impacted the manufacturing of small-sized analytical instrumentation and methods [45]. Smaller-scale analysis often reduces requirements for samples, reagents, electrical power, and allows the manufacture of portable instruments that can be used bedside and in remote locations [23,46,47]. The assets of microfabrication techniques include facilitation and rapid analysis of small molecules, biomolecules, cells, subcellular structures, nanoparticles, and the contents of detergent-treated cells, subcellular structures, and nanoparticles present in a sample with high sensitivity, high-throughput, multidimensional/multi-task capabilities, cost effectiveness, ease of operation and ease of results interpretation [23,46-48]. If coupled with such advanced microfabrication techniques, POCT can become a standard tool for providing affordable worldwide healthcare through rapid testing at, or near the site of patient care [49-52].

Although there are many miniaturized diagnostic tests available commercially, it is crucial to perform a careful validation of each new test to determine its usefulness for the intended goal sought in the target animal and, more importantly, the human population (e.g., clinical diagnosis, screening, confirmation) to prevent medical errors [42,43]. It is also important to remember that the quality of laboratory medicine is still an advancing field [53], with the goal of developing rapid, sensitive and accurate detection technology that offers improved diagnostics for patient management and early detection of disease outbreaks [54].

Herein, we report the development of a portable, miniaturized, POCT instrument, which combines the use of antibodies and/or other affinity ligands as highly selective capture agents with the superior resolving power of capillary electrophoresis. This miniaturized instrument can meet many challenges with the potential of being used as a point-of-care instrument and holding promise for precision medicine, P4 medicine and systems medicine, known as the application of systems biology, to the challenge of human disease [55]. With advancements in telemedicine, this technology can reach its full potential in low-resource environments.

Capillary Electrophoresis Technology

Capillary electrophoresis (CE) is a powerful tool for the analysis of a wide range of small molecules, biomolecules, cellular entities, subcellular structures, nanoparticles, and the contents of detergent-treated cells, subcellular structures and nanoparticles, and has been applied to a variety of clinical fields and human samples [23,46,56-68]. CE is an analytical technique that separates ions based on their electrophoretic mobility with the use of an applied voltage [23,46,56-68]. Charge and size, as well as buffer constitution, buffer viscosity, temperature and other parameters, influence the movement of the

separated substances. However, neutral compounds can also be separated by CE when the separation buffer contains micelles. CE is defined as a family of electrokinetic separation methods performed in submillimiter diameter capillaries (conventional capillary electrophoresis) and in micro- and nanofluidic channels (microchip capillary electrophoresis) [23,46,56-71]. There are many advantages of CE when compared with traditional analytical separation technologies, including rapid method development and optimization; faster analysis time; higher number of theoretical plates in a liquid-phase separation; a high peak capacity; the use of a variety of background electrolytes, such as totally aqueous buffers, partially aqueous buffers, and non-aqueous buffers; a choice of separation modes; the separation of ions, small molecules, biomolecules, cellular and subcellular structures, and nanoparticles; the use of low volumes of samples and small amounts of expensive reagents; a reduction in the generation of toxic organic waste; and relatively lower operating cost [23,46,56-71]. However, a major disadvantage of the CE technology is the low concentration limits of detection (CLOD). The main reasons for this problem are the limited sample-volume capacity of the capillary and the short optical pathlength of the capillary, which limit the sensitivity of the detector. This limitation, which has been voiced since the inception of CE, has delayed the onset of new applications in several fields of science. In attempts to overcome the poor CLOD, investigators have developed a number of methods to improve sensitivity levels of detections of analytes [23,56,67-89]. One method is the use of derivatizing agents, which is often the method of choice of many laboratories. A derivatizing procedure usually requires one step for conjugation, small volumes of reagents, and a choice of chromophores commercially available [70-74,85,86]. The process is used to create a conjugated end product, in this case the analyte of interest, enhancing sensitivity and changing selectivity. Unfortunately, numerous analytes cannot be derivatized. Another method for enhancing sensitivity is increasing the concentration of the sample prior analysis using a series of preconcentration techniques [23,29,60,62,63,76-81,87-89], of which selective extraction, isolation, and preconcentration are commonly used [23,29,56-60,62,63,76,79,87]. Some of these methods have been carried out using off-line procedures (when reactions are performed prior to introduction into the separation capillary or channel), or on-line procedures (when reactions are carried out within or in direct connection with the separation capillary or channel) [23,35,56-58,62,63,68,72,75,78,79,83,87]. The captured analytes can be further separated by various modes of CE followed by detection, quantification, and characterization approaches. Currently, a variety of detectors are available, including ultraviolet, fluorescence, laser-induced fluorescence, electrochemistry, mass spectrometry, circular dichroism, and nuclear magnetic resonance, among others [66,84-92].

A typical diagram depicting the main selector formats for on-line extraction and preconcentration to perform immunoaffinity capillary electrophoresis is shown in Figure 1. The standard protocol since its inception, reported in the early 1990s [56-58], is the one known as the "linear IACE" or "unidirectional IACE" format separation system (Figure 1-A). This protocol has been utilized in both conventional capillary electrophoresis and in microchip formats. In the linear IACE format, a small area or linear configuration design of the capillary or channel, near the inlet portion, is used to immobilize an affinity ligand, usually an antibody, an antibody fragment, a lectin, an aptamer, or any substance or material that has an affinity for a chemical, biochemical, cellular or subcellular entity [23,56-58,63,76,81]. Also, a combination of affinity ligands can be immobilized to capture more than one analyte of interest or to secure the capture of an analyte with weak binding properties. The linear configuration design or area where an affinity ligand is immobilized to a matrix or to the inner surface of the capillary or channel is known as the solid-phase extraction (SPE) preconcentrator area, the analyte concentrator-microreactor (ACM) area, or just simply the ACM device if a device is positioned in that area and is further connected to two capillaries. Similarly, an ACM area can be created within a microchip channel. The term ACM device, however, is most appropriate. Because of the dual functionality of the device, it functions as both an on-line preconcentrator and an on-line microreactor, where chemical, biochemical, and cellular reactions can be performed using one or more affinity selectors [23,65].

Figure 1-B depicts a different design known as the "orthogonal IACE" format separation system, where samples and cleaning buffers or solutions can be introduced in an orthogonal manner into the ACM orthogonal configuration design, without contaminating the separation capillary or channel. Furthermore, the geometrical configuration of the ACM device permits the repetitive introduction of an accurate amount of liquid into the inner channel of the ACM device, where a

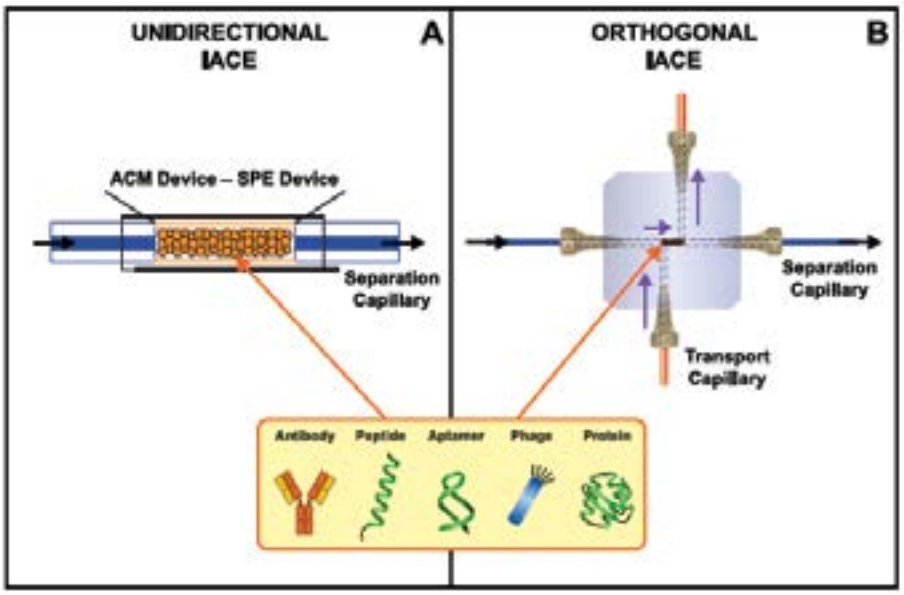

Figure 1: Schematic diagram of an on-line immunocapture device containing one or more affinity selectors in immunoaffinity capillary electrophoresis technology (IACE), prior to CE separation and detection of the captured and eluted analytes. (**A**) A selector device format, known as linear or unidirectional analyte concentrator-microreactor (ACM) device or area. (**B**) A selector device format, known as orthogonal ACM device or area. The area where an affinity ligand is immobilized has been reported in the literature as the solid-phase extraction (SPE) analyte preconcentrator area, or the analyte concentrator microreactor (ACM) device or area. Figure modified from references 23,56,65,95,100.

matrix containing immobilized affinity ligands is contained within a uniform confined space and surface area. The immobilized affinity ligands can be re-used multiple times. This unique feature allows for a better reproducibility of the quantification of the target biomarkers using appropriate internal standards, and avoids sample carry-over and contamination of the separation capillary when exposed to excess amount of matrix constituents.

The affinity ligands can be immobilized to the surface of micro- or nano-beads and this beaded matrix can be held in place within the ACM device by porous frits to avoid leakage of the beads outside the confine space of the concentrator. Alternatively, the matrix can be made of monolithics, or so-called "continuous block of porous structures" [93,94], without the need for frits. Other means of retaining the beaded structures within the inner channel of a microchip or in the channel of an ACM device in capillary electrophoresis is to use magnetic particles coated with the desired affinity ligands [23,95-97]. The magnetic particles can be held in place by positioning one or more magnets on the external sidewalls of the ACM device.

Other approaches include restriction points, which employ different diameters of the tubing forming the ACM device and the diameter of the separation capillary. This configuration prevents the leakage of the contained beaded structures outside the ACM device and into the separation capillary. An additional method of retaining the coated beads within the ACM device consists of interconnecting the beaded microstructures to each other and to the surface of the wall of the inner cavity or channel of the ACM device [23,65].

Another method for coupling one or more affinity ligands to a solid support involves attaching the affinity ligands directly to the surface of the inner cavity wall or channel of the ACM device, or to another protein or chemical entity already immobilized at the inner surface of the ACM device [23,65]. The open cavity facilitates the free flow of complex biosamples such as blood and other biological fluids. It should be taken into consideration that immobilization methods to surfaces vary largely with the type of surface, protein properties, and the goal of the immunocapture assay or enzymatic reaction [23,97,98]. An ideal immobilization surface should have a large surface-area-to-volume ratio, a protein friendly environment, minimal nonspecific protein adsorption, mechanical and chemical stability, and a reactive moiety for protein coupling [23,99].

Affinity Selectors

Highly selective affinity-based separations, for enrichment and isolation of substances and cellular entities, have greatly evolved during the last two decades to improve characteristics related to target specificity, dynamic adsorptive capacity, and chemical robustness of the affinity matrix. As mentioned before, some of the most commonly used affinity ligands to be immobilized, directly or indirectly, onto surfaces are antibodies, antibody fragments, lectins, aptamers, and enzymes. Nevertheless, small molecules, phages, ions, numerous biomolecules, and synthetic materials are also immobilized to surfaces and used as affinity ligands to capture and concentrate substances of interest found in simple or complex mixtures [23,100].

Immunoaffinity capture techniques for the selective determination of biomolecules in complex matrices are gaining much interest in the scientific community. From a point of view of target therapies for the treatment of many chronic diseases, we are in the era of monoclonal antibodies, which are transforming healthcare. The development of therapeutic antibodies has evolved over the past decade into a mainstay of curative options for patients with autoimmune and inflammatory diseases [101]. Similarly, a major advancement has been achieved in the development of antibody drug conjugates, where cytotoxic drugs are conjugated via a linker to a monoclonal antibody. These molecules are designed to selectively bind to target-expressing cells, thus delivering therapeutic agents directly to a targeted tumor [102]. Therefore, there is a need for advanced analytical technologies to characterize these biomolecules for human use, prior to administration to a patient and after being internalized in the body. Experimental forced degradation studies used to evaluate the purity and integrity of a recombinant monoclonal antibody have become integral to the development of these therapeutic molecules. These types of fragmentation studies serve a variety of objectives from early stage manufacturing evaluation to supporting comparability assessment both pre- and post-marketing approval [103,104].

Fragmentation is a degradation pathway ubiquitously observed in proteins despite the remarkable stability of peptide bonds that allows for study of the fine structure of antibodies; proteins differ only by how much and where cleavage occurs [104]. Hybrid technologies, such as capillary electrophoresis or high-performance liquid chromatography coupled to mass spectrometry have become a practical and robust tool to study protein heterogeneity and it is used as a quality control to study the purity and to decipher the complexity of proteins [105-107]. Quality control of purified proteins is the final and critical checkpoint of any protein production process. In most of these quality control studies, there is no need for preconcentration since there is significant amount of material to perform many studies. However, in the case of bioanalysis where the antibody or other therapeutic proteins and degradation products are required to be quantified in biological fluids, there is a need for extraction, isolation and concentration of the protein molecules before characterization studies. The complexity of biological fluid's proteome, in particular the plasma/serum proteome, exceeds the analytical capacity of conventional approaches to isolate lower abundance proteins that may prove to be informative biomarkers. Microscale immunoaffinity liquid chromatography, or immunoaffinity capillary electrophoresis, coupled to powerful detectors, such as laser-induced fluorescence or mass spectrometry, are currently popular technologies being used to capture, separate and characterize a wide range of biomolecules present as low-abundance substance in complex human samples [23,65].

IACE Applications

While significant progress continues to be made in the fields of genomics and proteomics, additional evidence of biological end points of human diseases is highly desired for disease diagnosis, prognosis and therapeutic development. Thus, detection and quantification of protein isoforms, protein degradation products, and peptide metabolites can reveal crucial information for early diagnosis of a disease, prediction of pathology trajectory and treatment efficacy. Immunoaffinity capillary electrophoresis has demonstrated to be a valuable tool in analyzing and decoding the enormous structural diversity of molecular entities [23,57,57,60,62,63,65-68,76,79,81,87-89]. Figure 2 shows representative applications of IACE for the identification and quantification of structurally related peptides, which cannot be identified by conventional immunoassays, such as ELISA. Comparative studies between IACE and ELISA were carried out for the immunosuppressant cyclosporin cyclic peptide (Figure 2-A) and for the beta casein A1 milk-derived beta-casomorphin peptides (Figure 2-B). ELISA was unable to identify structural related peptides, but rather generated single data points that provided information only about the total amount of the sum of the related peptides (e.g., peptides corresponding to the intact molecule having no modifications, plus those modified peptides). Conversely, IACE is a two-dimension technology capable of capturing, releasing, and separating each related peptide, modified or non-modified. Furthermore, the separated peptides can be individually quantified and characterized when coupled to detectors capable of yielding structural information [23,65].

Although many chronic diseases have modifiable risk factors that contribute to disease, there is a need to better understand other causes of diseases and to improve disease-modifying therapies. This can potentially be addressed through the study of effects of certain

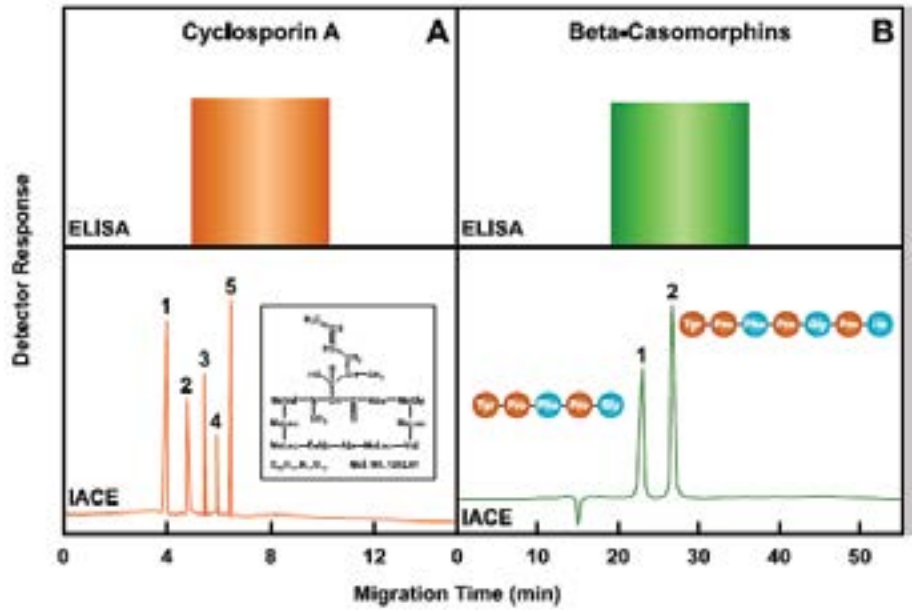

Figure 2: A comparative profile of immunoassays performed by the traditional sandwich ELISA method and by the IACE method. Determination of serum cyclosporin A, a cyclic undecapeptide used as immunosuppressant medication, was quantified by ELISA (upper panel A) and by IACE (lower panel A). Determination of urinary beta-casomorphins, 5 and 7, was quantified by the ELISA method (upper panel B) and by the IACE method (lower panel B). Figure modify from references 23,46,58,62,88,89.

peptides in causing diseases. For example, it has been demonstrated that the immunosuppressant cyclosporin, a cyclic undecapeptide, is metabolized in the body to multiple metabolites [108]. Additional information has reported that cyclosporin metabolites may contribute to organ toxicity [108,109], and therefore, it is crucial to quantify each metabolite in the blood. Similarly, some food-derived peptides, such as the opioid peptides casomorphin and gliadin have been associated to certain non-communicable diseases such as autism, schizophrenia, type I diabetes, heart disease, celiac disease, sudden infant death syndrome, mild allergies, and other disorders [23,110,111]. It is important to mention again that one-dimensional immunoassays are not capable of identifying these individual molecular entities.

Several useful applications of IACE have been recently reported in clinical and pharmaceutical areas. For example, protein engineering is at all-time high in biopharmaceuticals. As a result, absorption, distribution, metabolism and excretion (ADME) of proteins has become more important to understand in the context of engineering strategies to optimize therapeutic properties of potential lead constructs [112]. Han el al. [112] has reported the importance of IACE coupled to MS for the determination of intact and truncated forms of certain protein constructs. IACE-MS provided a qualitative and quantitative analysis of the pharmacokinetic profiles of the two fusion proteins. This platform to assess the in vivo stability of two fusion proteins and for the characterization of the ADME properties of proteins will be impossible to achieve if ELISA techniques were to be used. Similarly, experiments carried out by the group of Wang et al. [113] using immunoaffinity-capture capillary electrophoresis coupled to high-resolution mass spectrometry (IACE-HRMS), were able to differentiate minor structural differences in highly similar probody drug variants. Probodies are proteolitically-activated antibodies engineered to remain inert until activated locally in disease tissue. These investigators concluded that IACE-HRMS strategy could be the only feasible strategy to differentiate probody drug variants in a single assay. Shimura and Nagai [79] reported the coupling of IACE with isoelectric focusing (IEF) and fluorescence detection in a single capillary. After sample introduction and capture of the target protein, the whole capillary was filled with a carrier ampholyte solution, prior to elution. Multiple benefits were obtained by using IACE-IEF-LIF, in particular the quantification of proteins found at small concentrations in complex mixtures and the simultaneous determination of the isoelectric point of the isolated and concentrated target protein.

In the field of clinical diagnosis, several applications have been reported [23,67,68]. One interesting application was reported by Mai el al. [114] in search of biomarkers of Alzheimer's disease in cerebrospinal fluid. A sensitivity determination of three amyloid-beta peptides was achieved by magneto-immunocapture of the peptides followed by on-bead fluorescent labeling of the peptides, thermal elution, and finally separation and detection by CE coupled to laser-induced fluorescent detection. The developed strategy was adapted into a miniaturized fluidized bed configuration that has the potential for coupling with a microchip separation system. Another application is the use of on-line IACE-MS for the determination of serum transthyretin [81] a protein involved in different types of amyloidosis, which most often involves the kidneys and the heart.

Another interesting application is the determination of immunoglobulin E in people suffering of allergy. In most industrialized countries, allergies have increased in frequency quite dramatically during the past 50 years. Approximately 30% of the populations are affected [115]. Allergies have thereby become one of the major medical challenges of the twenty-first century. Allergic reactions can range in severity from mild to severe and life threatening [116]. For many physicians, quantification of serum drug-specific IgE (sIgE) antibodies constitutes the first measure in the diagnostic approach of immediate drug hypersensitivity reactions (IDHR) [117]. Decuyper et al. [117] carried out a careful review on the accuracy and limitations of the main drug-sIgE tests, especially those that are commercially available. These investigators concluded that although drug-sIgE assay can provide valuable information they should not be performed in isolation to establish correct diagnosis, as their predictive value is not per se absolute. As a consequence, larger comprehensive studies are urgently required to determine the accuracy of drug-sIgE assays.

Several investigators have carried out determination of IgE by IACE and IACE-MS. Our group reported for the first time in 1995 the use of IACE for the quantification of IgE in serum using immuno-capture and UV detection [60]. Later in 2008, Chen et al. [118] using magnetic beads, transient isotachophoresis, and laser-induced fluorescence detection, quantified IgE in serum at the low picomolar range sensitivity. In 2014 and 2015 Gasilova and Giraud [63,119] quantified IgE in serum

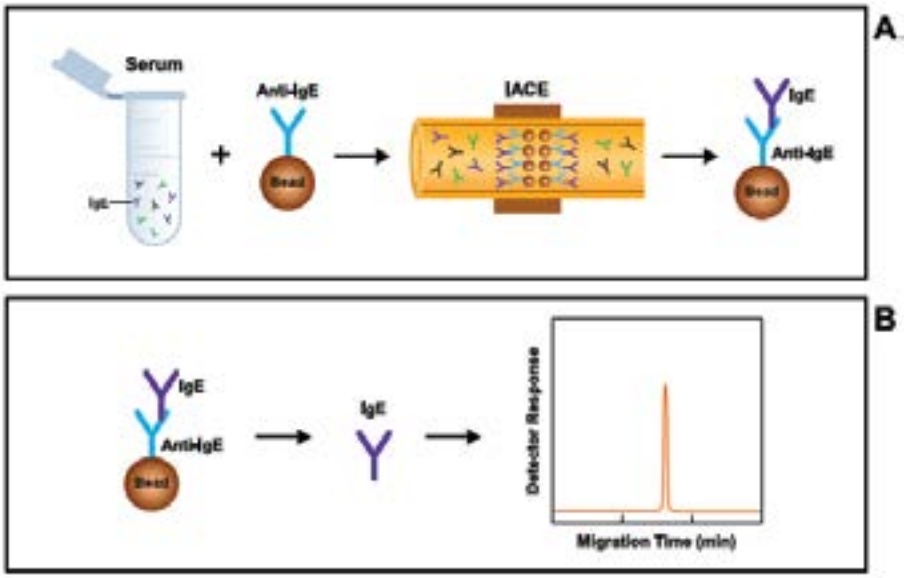

Figure 3: A schematic representation of the experimental workflow used for the determination of serum IgE by IACE. (**A**) Anti-human IgE antibodies are covalently bound to the surface of microbead particles made of glass, plastic or magnetic material. An analyte concentrator-microreactor (ACM) device or area, localized commonly near the inlet side of the capillary, is filled with these beads containing anti-IgE. Micro beads are usually retained within the ACM device or area by employing porous frit structures or capillary constrictions to hold the non-magnetic beads within the device or area. Alternatively, one or more magnets located externally are normally used to retain magnetic beads. Once the ACM device or area is filled with beads covalently bound to anti-human IgE, a serum sample containing the target IgE is introduced into the capillary and a complex IgE-anti-IgE-bead is formed. The excess amount of unwanted materials, and non-specifically adsorbed compounds to the surface of the capillary and/or ACM device, are removed by washing buffers. (**B**) The separation capillary is filled with fresh separation buffer and the bound IgE to the anti-IgE-bead is released by a small amount or plug of an elution buffer or solution. Separation of the eluted IgE is performed by free-solution capillary zone electrophoresis or any other mode of CE. The process of detection and total quantification of the separated IgE molecules is carried out by UV detection. Other detectors, such as laser-induced fluorescence, mass spectrometry or a combination of detectors can be used as well. Figure adapted from references 60,63,118,119.

from a milk allergic patient by IACE-immuno-capture magnetic beads coupled to UV detection. Additional experiments were performed to isolate, quantify and identify the corresponding allergen that bound to IgE isolated from the patient's serum. Using the immunocomplex of antihuman IgE antibodies bound to the patient IgE antibodies, they were required to be crosslinked to chemically stabilize the noncovalent immunocomplex formed. The IgE-anti-IgE stable complex bound to magnetic beads support was used to further capture, quantify, and identify the milk allergen to which the patient was allergic to by UV detection coupled to MALDI-MS. Figures 3 and 4 depict in details the experiments carried out for the determination of IgE in serum and the corresponding allergen(s) present in a sample.

POCT Instrument

There are many challenges related to manufacturing a miniaturized portable POCT instrument that contains micro components (e.g., mechanical, optical, temperature regulators, and electronic) providing optimal conditions for functioning. A fundamental aspect of POCT versus clinical laboratory tests is the portability of the instrument and the capability to provide accurate and cost effective results in a shorter time frame. Therefore, the goal is to fabricate an easy to operate POCT instrument capable to work with standard electrical power, or with a small rechargeable battery. Basic training should be sufficient for a user to operate the computer-controlled automatic instrument. Additionally, the instrument should be able to use biological samples obtained by non-invasive procedures, such as exhaled breath fluid, saliva, tears, sweat or urine. This can be achieved through the installation of a small dispenser cup or vessel containing the collected saliva, tears, sweat, or urine sample that should be an integral part of the POCT instrument [23,45,64], or with the use of a tubing and adaptor bridging and positioning the ACM device to the mouth of a breath provider [120]. Once a sample is loaded, the mixing of reagents with the sample is performed prior to introduction into an AMC device, followed by a series of automated steps controlled via computer commands. Appropriate buffers and solutions will facilitate the capture of the target analyte(s), wash-off unwanted non-specifically bound compounds to the surface of the transport capillary or even in the space of the ACM device, and further elute bound biomarkers from the ACM device prior to separation of the released substances within a separation capillary or channel. One or more detectors, positioned at the outlet end of the separation capillary or channel, will identify and quantify the separated analytes [23,65].

Figure 5 depicts the design of a POCT instrument showing a detailed view of its external and internal layout. Several configurations of the instrument can be manufactured, as for example, a model having an interchangeable platform, or removable cartridge, containing one or more orthogonal ACM devices. Each device can be color-coded to indicate the presence of one or more affinity selectors for capture and isolation of a corresponding single target biomarker or panel of biomarkers. Alternatively, the entire removable cartridge containing two or more ACM devices can also be color-coded to indicate the analysis of specific biomarkers. Color-coded devices or reagents may help to prevent errors derived from human and/or technical factors, since each color is intended for the identification of one disease only [23,65].

Another important feature of the POCT instrument is that it includes several miniaturized valves. Each ACM device is surrounded by four fluid-control mini- or micro-valves, with the purpose of creating an isolated microenvironment, to perform either a concentration procedure, or to perform chemical, biochemical, cellular, or subcellular reactions. Although all ACM devices are connected in tandem for sample and buffer introduction, the process of elution and separation is carried out separately and sequentially. Once the entire process is finished, the system can be regenerated with appropriate cleaning and conditioning buffers [23,65]. A crucial feature of the IACE device is

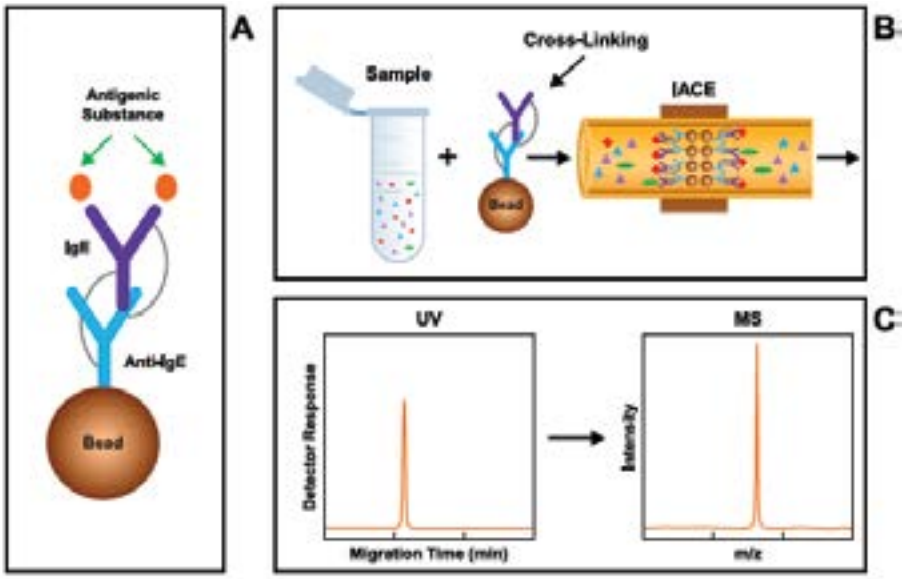

Figure 4: A schematic representation of the experimental workflow used for the determination of an allergen present in a sample by IACE. For quantification and identification of an allergen present in a sample, it is necessary to form first an IgE anti-human IgE complex, followed by a cross-linking reaction in order to chemically stabilize the noncovalent immunocomplex formed (**A**). Once the capillary with the ACM device or area, containing the stabilized IgE-anti-IgE-bead immunocomplex is filled with an optimization buffer, a sample containing a target allergen is introduced into the capillary (**B**). The capillary is washed with a cleaning buffer to remove the excess amount of sample matrix and non-specifically bound materials. The capillary is equilibrated with an optimal separation buffer and the target allergen bound to the IgE of the patient's serum is eluted from the complex allergen-IgE-anti-IgE-bead by a plug of an elution buffer or solution. The released allergen is then quantified and identified by UV detection and mass spectrometry respectively (**C**). Other detectors can be used as well. Figure adapted from references 63,118,119.

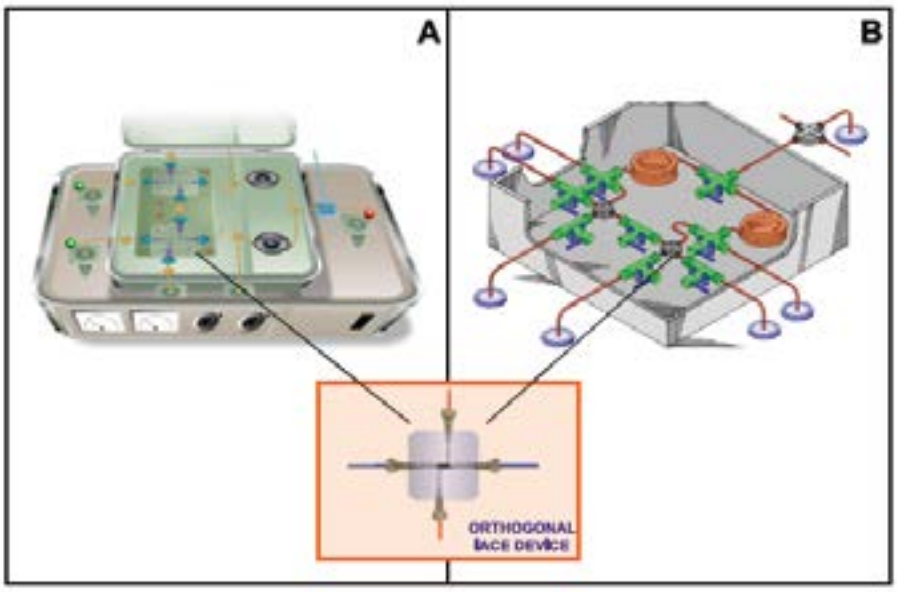

Figure 5: Schematic representation of a multi-dimensional, multi-task, immunoaffinity capillary electrophoresis instrument, equipped with two ACM devices using an orthogonal design. Diagram A shows an external view of the instrument. Diagram B shows an internal view of the instrument. Samples and buffers are introduced through a transport passage orthogonally positioned from the inlet end of the transport capillary or channel to a waste container passing through orthogonally the ACM device (shown in the inset). After removing with a cleaning buffer the excess amount of sample and unwanted non-specifically bound materials, the separation capillary is equilibrated with an optimal separation buffer. Captured analytes of interest are released from their respective ACM devices by an elution buffer or solution and further separated at a corresponding separation capillary or channel, independently and sequentially. One or more detectors positioned at the outlet end of the separation capillary, or along the capillary with a charge-coupled device (CCD) camera, can monitor each separated analyte. For further details of the design and functioning of the instrument see references 23, 62, and 65. Figure modified from references 23,46,62,65.

its orthogonal configuration. Samples are introduced orthogonally through a plastic tubing or channel, known as the transport capillary or passage, with the purpose of avoiding sample-to-sample carry-over, which is an inherent risk of contamination that may cause erroneous results. Another important characteristic of the orthogonal ACM device is that a single transport passage can cross-over multiple ACM devices, allowing a certain volume of sample (microliters-milliliters) to pass-by each individual ACM device containing one or more affinity selectors immobilized to a matrix localized within the internal channel of the ACM device, or directly to the inner surface of the ACM

device [23,65]. Large volumes of samples are needed in cases where analytes of interests are found at very low concentrations, and when the sample is too diluted, as it is in the case of microdialyzates. An additional feature of the instrument depicted in Figure 5 is the presence of an auxiliary ACM device or auxiliary capillary with the purpose of introducing a different buffer to the separation capillary. A two-buffer capillary system can have some benefits. For instance, one buffer can be aimed to maintain the functional integrity of the immobilized affinity ligand localized within the primary ACM device, while the second buffer can be aimed to provide an optimized separation condition for the one or more analytes bound to and released from the primary ACM device. Furthermore, the second buffer can be made compatible for a mass spectrometer when necessary to be used. Therefore, advanced instrument designs are key requirements for delivering meaningful decision support that meets high-quality diagnostic tests.

Many affinity ligands have been commercially available for several decades, in particular antibodies, which have become dispensable research reagents. Antibodies currently represent the gold standard of affinity reagents used for regulated bioanalysis of therapeutic proteins. Over time, traditional antibodies have been refined to the point where they can be specific, sensitive, and reasonable reliable. Unfortunately, the widespread use of antibodies and natural ligands has their limitations and can present challenges with supply, stability and batch-to-batch variation on assay performance. Consequently, the use of antibodies has generated enormous controversy: the inability of investigators to replicate published data due to the use of antibodies that have not been properly validated [121-123]. Validation of antibodies by commercial suppliers is usually inadequate and frequently unreliable [121-123]. Consequently, alternatives to the use of antibodies as affinity selectors are actively being investigated. The most commonly used are lectins and aptamers [124,125]. The goal of shotgun proteomics, also known as bottom-up proteomics, would be to identify and sequence the entire cellular proteome. Therefore, other methods are being explored for the enrichment of low abundance proteins found in biological fluids, including the use of combinatorial peptide libraries [126,127]. As shown in Figure 2-B, we have tested the orthogonal IACE device format for the isolation and characterization of casomorphin peptides, using antibodies directed against the casein-derived casomorphin 7, as the affinity-binding selector. However, due to the lack of reproducibility of results encountered when using different batches of commercial antibodies, we are exploring new affinity-capture selectors to yield highly reproducible data (manuscript in preparation). Validation of an affinity selector is very important to demonstrate that the performance characteristic of an analytical method is suitable for its intended analytical use [128]. Specificity, selectivity and reproducibility are crucial parameters to rely on the routine use of affinity selectors. Significant work is currently in progress in several laboratories in the development of high selectivity affinity selectors, and of new and novel immobilization technologies for the effective quantification and characterization of biomarkers.

Future and Perspective

The demand for the manufacturing of POCT instruments capable to be linked to global telemedicine systems is on the rise. Figure 6 depicts a diagram of an IACE biomarker analyzer connected via Internet to a central laboratory equipped with state-of-the art technology, including one or more supercomputers containing a large molecular database of defined protein-peptide sequences and other molecules. The goal is to identify sequences of one or more unknown molecules and match them with the sequence of well-known biomarkers, modified biomarkers, or new discovered biomarkers. The aim is to complement this information with clinical information obtained by a healthcare provider to define the health status of an individual. The desired outcome is for individuals, even in remote global areas, to routinely measure their own health status with the help of healthcare providers. Information obtained by the biomarker analyzer and supercomputer database can be sent to the individual's family practice physician and further stored in the supercomputer to continue enriching its database.

Although clinical use of telemedicine in the world is still limited, rapid changes in technology trends suggest that within the next decade, health care providers will be able to see patients more often at remote sites by using a desktop workstation or laptop computer in a mobile, wireless configuration. Telemedicine will become even more prevalent, with some experts predicting that virtual visits will soon outpace in-person visits. However, as telemedicine plays an even greater role in global health delivery, it will be increasingly important to develop a strong evidence base of successful, innovative telehealth solutions that can lead to scalable and sustainable telehealth programs [129].

Conclusion

Many diseases are caused by the dysfunction of low abundance proteins, deemed biomarkers, and their post-translational modifications. To speed the discovery of these biomarkers involved in disease, high

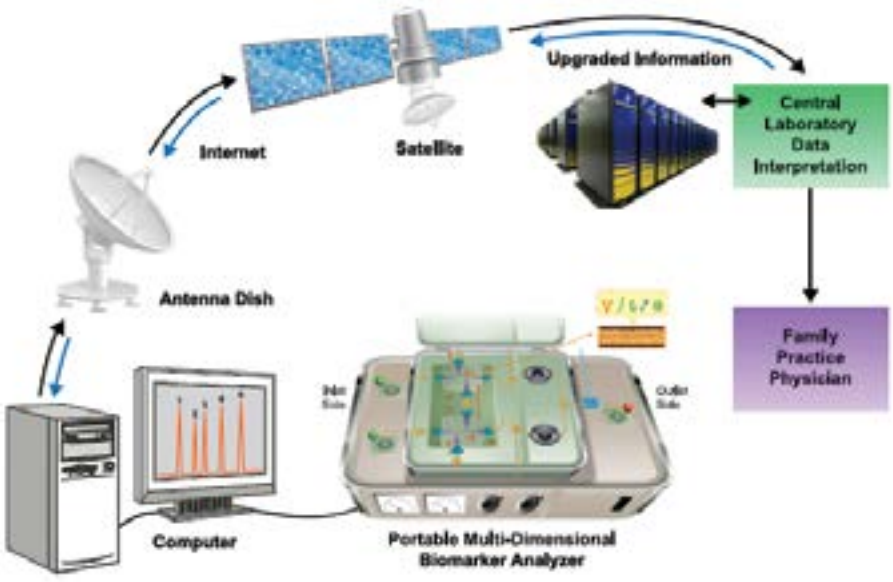

Figure 6: Depicts a diagram of an IACE biomarker analyzer connected to a central laboratory, equipped with super computer, for data interpretation. Figure modified from references 23,65.

sensitivity, comprehensive proteomic diagnostic methods are essential. Furthermore, a deeper analysis of the proteins and peptides present in a biological fluid provides more complete understanding of the biological system under investigation. Proteomic technologies have emerged as an important addition to genomic, metabolomics and other technologies for the accurate diagnosis of many diseases at the early stage of formation. The field of disease proteomics is evolving rapidly with the goal to discern how protein expression, structure and function cause illness. Proteomics has already identified proteins that offer promise as diagnostic and prognostic markers, and it has the potential to allow patient-tailored therapy.

In this paper we described a portable POCT biomarker analyzer that utilizes this advanced proteomic technology coupled with IACE and has many advantages over existing diagnostic technologies. In particular, this device supersedes the accuracy of existing diagnostic assays and addresses many of the limitations that exist in both urban and rural health centers. The major challenge with this technology is to continue improving the capabilities of detecting low-abundance proteins and correlating changes in proteins, such as co- and post-translational modifications. The use of the orthogonal ACM device design, however, permits the introduction of samples and buffers without contaminating the separation capillary. This novel zigzag configuration was designed and manufactured to contain a uniform surface area, with the goal to reproducibly capture the same number of analytes of interest, present in a known dilution of the sample, from assay to assay. Furthermore, this orthogonal design configuration avoids sample-to-sample carry-over during the passage of sample through the transport capillary, exposing the separation capillary or channel only to the released analytes from the ACM device and not to the bulk of components present in the sample to be analyzed. Sample introduction through the transport capillary is followed by washing-off the unwanted materials bound non-specifically to the inner surface of the transport capillary using special cleaning buffers. The release of the bound analyte(s) to the affinity selectors immobilized to the ACM device, by one or more types of elution solutions, result in purified and enriched substances free of contaminating materials. Detection, quantification, and identification are achieved after separation of the target analytes. The color-coded ACM devices, or color-coded interchangeable cartridges containing two or more ACM devices, to identify the one or more affinity selectors to capture specific biomarkers, should facilitate the identification of disease-specific biomarkers accurately avoiding possible errors.

Utilizing such a POCT that supports communication between primary care providers and specialists can overcome some of the traditional problems of patient's access to up-to-date specialist care while still enabling patients to be treated in their communities. Furthermore, utilizing our device to address rural healthcare barriers through wireless health and telemedicine will facilitate routine health care worldwide.

This portable POCT biomarker analyzer described here is not a futuristic vision, but is within our grasp. It would cut costs by utilizing multiple times the immobilized affinity selectors and the use of small amounts of reagents, in addition to reducing professional responsibility for routine tasks and record keeping. This will make it possible for patients to obtain accurate, sensitive, specific, reliable and rapid biomarker information in order to receive higher quality healthcare and be more satisfied with the end-result. The orthogonal configuration of the ACM device permits consistent sample introduction, without contamination of the separation capillary. This feature may be a competitive advantage with existing clinical laboratory procedures to obtain highly accurate results. A pivotal role of this POCT biomarker analyzer in systems medicine is foreseen.

Competing Interest

Dr. Norberto Guzman. Ph.D., is the inventor of several patents issued and pending related to the IACE technology.

Ethics Approval and Consent to Participate

Informed consent was obtained from volunteer individuals before entering the various studies (this apply to papers previously published of which some Figures are used in this review).

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Author's Contributions

Both authors contributed to data analysis and manuscript writing.

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