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New technologies in affinity assays to explore biological communication

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Abstract

The language cells use to communicate consist of the small molecules, peptides, and proteins that are released into the extracellular environment. To decipher this language, analytical assays are needed that have high selectivity, high sensitivity, and fast temporal resolution. Affinity assays are a group of analytical methodologies that are adept at studying this communication. In this review, we highlight several examples from the literature on various types of affinity assays used in different platforms to monitor biological communication of peptides and proteins.

Keywords

Bioanalytical methods; immunoassays/ELISA; PCR; microfluidics/microfabrication; capillary electrophoresis; affinity

Introduction

One method by which cells communicate is through the secretion of small molecules, peptides, and proteins. Secretion is governed by signal transduction pathways that are made more complex by the interaction with other pathways depending on temporal and spatial variations in the local protein concentrations, post-translational modifications, and extra-cellular signals. A combination of these variables ultimately control secretion and there are many analytical challenges associated with unraveling and deciphering these pathways. Common examples of communication by cellular secretion are the exocytotic release of neurotransmitters and endocrine hormones.

Biomarkers are not thought of as traditional cellular communication molecules as most are not known to bind extracellular receptors and induce some type of response. However, in a broad sense, several of these proteins do communicate to physicians or clinicians the state of a disease. For example, the American Cancer Society suggests to clinicians the measurement of prostate specific antigen (PSA) in men who are at risk of prostate cancer.[1] We and others are developing affinity-based methods to monitor cellular release and biomarkers which may aid in understanding, and potentially controlling, biological communication.

Affinity assays take advantage of the binding specificity inherent in certain biomolecules to their target. By far, the most common biomolecule used in affinity assays are antibodies; however, in the last 20 years, other agents have been utilized in affinity assays such as cell-surface receptors and oligonucleotides. Regardless of the binding molecule, the specificity of

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the binding interaction allows the quantitation of target in the presence of a myriad of other, potentially interfering agents. We refer the reader to other reviews for a more general discussion on affinity assays [2,3].

The goal of this Trends article is to provide an overview of affinity assays utilized in quantifying the analytes involved in biological communication, either secretory products from cells or biomarkers. One common aspect of the assays discussed in this review is the ability to measure multiple analytes simultaneously. We believe that the ability for simultaneous measurement is necessary in this field as biological communication is often composed of multiple analytes secreted or used as biomarkers for a disease. Even with these qualifications, the number of publications relating to this topic is extensive and this article is not meant to be a comprehensive review. Rather, we have focused on selected reports from recent years highlighting emerging technologies.

Overview of field

Electrophoretic immunoassays

Capillary electrophoresis (CE) immunoassays have been used to measure expression and/or secretion of a variety of proteins and peptides from cells. In these assays, bound and free labeled-antigens or labeled-antibodies are separated by CE and the ratio of these peaks provides a quantitative value on the amount of antigen in sample. This separation mechanism is ideal for monitoring applications as high voltages can be used with rapid dissipation of Joule heating resulting in fast separations. Faster separations allow higher temporal monitoring enabling the observation of acute changes in analyte concentration.

In our laboratory, we are interested in monitoring the peptides secreted from tissues involved in glucose regulation, for example, from pancreatic islets of Langerhans. Much work has been done on developing affinity assays to monitor insulin release from single islets with high temporal resolution [4], although relatively little has been done to multiplex the assay to simultaneously quantify the other peptides secreted in concert with insulin from this tissue. One of the difficulties when attempting to multiplex CE immunoassays is the similar mobilities of the bound antigen peaks, resulting in overlapped peaks that hinder quantitation [5]. Another difficulty associated with multiplexed CE immunoassays lies in attempting to resolve the various components that may be at much different concentrations. Large differences in concentrations can result in some peaks outside the dynamic range of the detector or again losing electrophoretic resolution as the higher concentration compound tails into the other, lower abundant peaks.

To circumvent these difficulties, we have developed a multiplexed competitive CE-IA for insulin and glucagon using a two-color detection scheme and have applied this assay to determine the levels of these peptides in islets of Langerhans [6]. In this assay, separate fluorescent dyes were used for detection of each antigen: fluorescein isothiocyanate (FITC) for insulin and Cy5 for glucagon. An Ar^+ laser at 488 nm was used for excitation of FITC-insulin and a diode laser at 635 nm for excitation of Cy5-glucagon. The fluorescence emission was split and passed through a 520 ± 20 nm bandpass filter and a 665 nm longpass filter before being made incident on separate photomultiplier tubes for detection of FITC-insulin and Cy5-glucagon, respectively.

This experimental configuration enabled simultaneous quantitation of both analytes even though electrophoretic resolution was low due to the difficulties mentioned above (similar mobilities and large differences in concentrations). Examples of electropherograms and calibration curves are shown in Figure 1. The intracellular amounts of insulin and glucagon measured in islets were 56.6 ± 3.2 and 1.0 ± 0.5 ng/islet, respectively, which agreed with

literature values. We have since applied this technology to simultaneous monitoring of secretion from islets on microfluidic devices.

To increase throughput, the Kennedy lab has utilized microfluidic devices as platforms for electrophoretic immunoassays. Building on earlier work using an online competitive immunoassay to monitor insulin release, secretion from four islets was monitored in parallel on a single planar device [7]. This type of methodology represents one of the goals of single cell (or single organism) research – high throughput measurements to discern heterogeneities in biological samples. A total of 1450 analyses were performed in less than 40 min, monitoring secretion every 6.25 s.

In another effort to combine the selectivity of affinity assays with the high resolving power of electrophoresis, a cleaved-tag immunoassay has been developed for detection of myoglobin, the muscle isoform of creatine kinase, cardiac troponin T and I, all biomarkers of acute myocardial infarction [8]. In this type of sandwich immunoassay, analyte is captured from solution by antibody-coated beads. A secondary antibody tagged with a unique fluorophore is then added to sandwich the analyte. The fluorescent tags are cleaved from the secondary antibody and separated by micellar electrokinetic chromatography. One benefit of this assay is the ease of multiplexing for different antigens by combining the high resolving power of the separation step and the spectroscopic resolution of the tags.

Micromosaic immunoassays

Micromosaic immunoassays are a powerful method for multi-analyte determination. These types of immunoassays involve the immobilization of a series of antibodies or antigens on a solid surface at varying concentrations and the corresponding antigens or antibodies delivered to these reagents. With these devices, a calibration curve or cross-reactivity can be performed in parallel allowing a large decrease in assay time.

In one example of this type of assay, secretion of tumor necrosis factor α (TNF-α) from dendritic cells was measured. Using a non-competitive format, a 1 pM limit of detection was achieved with a 600 nL sample volume [9]. To perform this assay, the surface of a poly (dimethylsiloxane) (PDMS) device was coated with capture anti-TNF-α, and subsequently placed on an array of capillary systems containing a flow of cell culture supernatant (Figure 2). This exposed PDMS device was then placed on a second array of capillary systems containing a flow of fluorescently-labeled detection antibody solution. To gain further insight into micromosaic immunoassays, a theoretical modeling of these surface immunoassays has been published [10]. In this report, a combination of experimental data and theory were applied to better understand and improve the transport of analytes in microchannels, as well as the binding kinetics between analytes in a flow of solution and immobilized antibodies.

These micromosaic immunoassays have been performed for a variety of analytes and in a multitude of formats. Some of the biomarkers that have been detected include C-reactive protein [11, 12, 13], cardiac troponin [11], myoglobin [11], prostate-specific antigen [12], ferritin [12], vascular endothelial growth factor [12], and thyroxine [13]. As these assays are dependent on flowing reagents over the immobilized antigens or antibodies, the use of microfluidics to facilitate fluid handling has been intimately linked to several of these devices [11, 12, 13]. With highly automated control of reagents, the potential to use these devices in clinical settings is much increased.

Bead-based multiplexed immunoassays

Bead-based multiplex immunoassays are powerful methods for simultaneous detection of cellular communication networks. In these types of assays, fluorescent beads with unique

intensities and wavelengths are coated with specific capture antibodies. By using a variety of beads each coated with different antibodies, multiple analytes can be detected in a single sample. Some of the advantages of these bead-based affinity assays over enzyme linked immunosorbent assays (ELISA) include the multiplex nature of the technique, smaller sample volumes, lower dilutions, and higher-throughput evaluation of multiple analytes in a single platform.

In a recent manuscript, this technology was used to simultaneously quantify 27 proteins to estimate age of cerebral wounds [14]. The use of this multiplexed assay was successful and the authors were able to categorize the 27 measured analytes into 5 subgroups corresponding to those that were expressed early, middle, late, varying, and with no response to wound age. In addition, the ability to measure this large number of proteins simultaneously allowed for mapping protein interactions.

Ultra-sensitive detection schemes for immunoassays

For competitive immunoassays, limits of detection are dependent on the dissociation constant of the binding molecule, typically in the nM range for antibody-based competitive immunoassays. For ultra-sensitive detection of proteins and biomarkers, several new methods have been developed. Several of these assays have recently been reviewed [15, 16]. Other non-traditional detection systems, such as surface plasmon resonance, quartz crystal microbalance, and cantilever-based immunosensors have also been recently reviewed [17, 18].

Bio-barcode assays have been utilized for several different antigens, for example, prostate specific antigen [19]. In one form of this assay, a sandwich format was utilized with antibody-functionalized magnetic particles to capture antigen. Nanoparticles coated with both DNA and secondary antibodies were then added to the mixture. Release of the DNA followed by downstream capture and subsequent detection was used as an indirect marker of antigen. This type of bio-barcode assay allowed for detection of 300 copies of prostate specific antigen in serum [19].

To multiplex these types of assays, microparticles have been encoded with micrometer-sized diffractive bar codes [20]. The encoded microparticles were fabricated from a commercial functional polymer (SU-8), and functionalized with molecular probes exhibiting high affinity for the analytes of interest. In this system, each bar code is associated with specific recognition probes, allowing for simultaneous detection of a wide variety of molecular events in parallel. The method was tested by reacting immunoglobulins G (IgGs) with anti-IgGs from multiple animal species in small sample volumes (~ 1 μL), and detection of low amounts of analyte was achieved (~ 20 fmol).

A hybrid method termed immuno-PCR combines the selectivity of immunoassays with the amplification power of the polymerase chain reaction (PCR) [21]. In immuno-PCR, DNA strands are directly linked to antibodies through covalent attachment, biotin-streptavidin linkage, or other chimeric proteins [22]. These assays are typically performed in the same manner as an ELISA. A conventional sandwich assay is performed but a DNA-linked detection antibody is used in place of the conventional enzyme-linked detection antibody. Quantitation of this DNA can be accomplished via real-time amplification of the DNA, which is proportional to the amount of antigen. These assays have been used to detect various biomarkers of diseases, such as prostate specific antigen [23]. The major advantage of immuno-PCR is signal amplification; in theory, single copies of secreted proteins can be detected due to the exponential amplification of DNA by the PCR.

Immuno-PCR has been multiplexed for simultaneous detection of human thyroid stimulating hormone, human chorionic gonadotropin, and β-galactosidase [24]. These three analytes were

simultaneously detected by amplifying different sized DNA for each of the three analytes. This result demonstrates that the immuno-PCR method could be used for the simultaneous detection of multiple biomarkers or cell secretion molecules.

Using a similar technique, termed immuno-rolling circle amplification (immuno-RCA), multiplexed detection of cell communication molecules has also been performed. With this technique, circular primers are used and the amplified DNA continuously grows out from the DNA-Ab complex (Figure 3). While only linear amplification is achieved (in contrast to exponential amplification with PCR), the amplified DNA remains attached to the antibody, which aids detection in certain applications such as microarrays. This method has been multiplexed in an array format on a glass slide and utilized for the simultaneous detection of 51 cytokines secreted from human dendritic cells induced by lipopolysaccharide and tumor necrosis factor-α [25]. The authors emphasize the importance of measuring multiple cytokines simultaneously to obtain global expression patterns instead of single protein secretion values.

Mass spectrometry immunoassays

Mass spectrometry (MS) has also been utilized with immuno recognition to provide information on various analytes, including cell communication markers. MS-immunostaining is becoming more common where antibodies tagged with specific isotopic elements are added to a sample and allowed to bind. MS can then be performed to gauge the absence or presence of the antigen [26]. This technique has been reviewed elsewhere [27].

An inductively-coupled plasma mass spectrometer (ICP-MS) has been used to quantify multiple antigens in an immunoassay. In these types of assays, a conventional sandwich immunoassay is performed for the analyte(s) of interest using a secondary antibody tagged with a particular element. The plate is washed and acid hydrolysis releases the element, which is then detected by the ICP-MS. Due to the high resolving power of MS, there is a large potential for utilizing these types of assays for a variety of multiplexed analyses.

These element-tagged immunoassays have been utilized to determine cellular secretion and measure biomarkers. In one example of a multiplexed ICP-MS immunoassay, antibodies to alpha fetoprotein and beta-human chorionic gonadotropin were labeled with Eu^{3+} and Sm^{3+}, respectively [28]. A typical non-competitive sandwich assay was used where the labeled-antibodies were incubated in the presence of the immune complexes. After washing to remove non-specific antibodies, Eu^{3+} and Sm^{3+} were dissociated and quantified by ICP-MS. The amounts of these antigens determined by the element-tagged immunoassay were not significantly different from the values quantified by conventional immunoradiometric analysis.

Outlook

Measurement of the secreted molecules and biomarkers used in biological communication is an analytical challenge. The concentrations of these molecules may fluctuate rapidly in blood or in the extracellular space, the spatial release locations may be tightly regulated, and the large number of analytes that are released demand the use of analytical methods that are selective, sensitive, and have rapid response times. Thus, immunoassays and affinity assays are ideal methodologies to measure various aspects of cellular communication.

To further advance this field, future developments in immunoassay technology ought to include better automation of the procedure to remove manual input as much as possible. This automation should provide more reproducible analyses, higher throughput, and allow researchers or clinicians outside the analytical chemistry field to benefit from these assays. Additionally, improvement in detection sensitivity should be a goal of new method development as a majority of biological events occur at very low analyte levels. Finally,

multiplexed assays for simultaneous quantitation of different analytes will be valuable to the scientific community. The biological language that cells use involves multiple peptides and proteins and only their simultaneous detection and observation of their behavior in the context of the other secreted analytes will allow us to understand and potentially control the processes that go awry in disease states or lead to the disease state itself.

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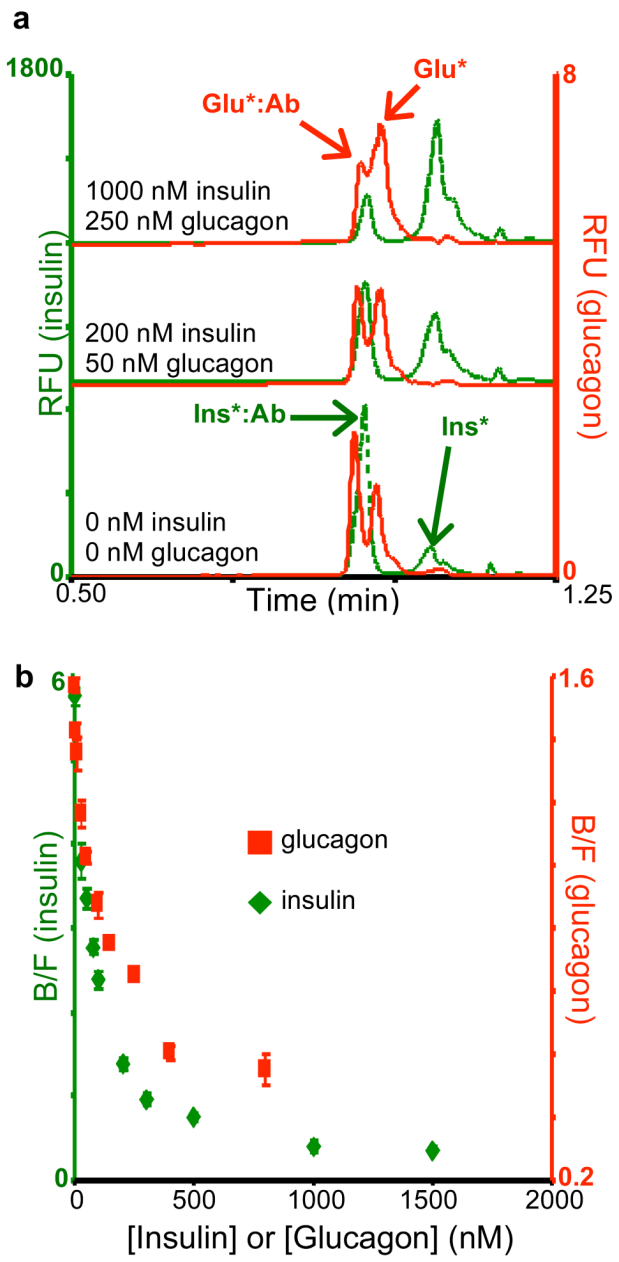

Figure 1. Two-color capillary electrophoresis immunoassay

a. Example electropherograms are shown for the simultaneous separation of free and bound FITC-labeled insulin (Ins* and Ins*:Ab, respectively), and free and bound Cy5-labeled glucagon (Glu* and Glu*:Ab, respectively) at varying unlabeled insulin and glucagon concentrations. Due to similar mobilities, the components in these separations were not spatially resolved; however, spectral resolution enabled simultaneous quantitation. **b.** A series of separations were performed as shown in **a** and the ratio of Glu*:Ab and Ins*:Ab to Glu* and Ins* were plotted as a function of unlabeled antigen. Limits of detection were 7 nM insulin and 3 nM glucagon. Reprinted with permission from reference 5 (Copyright 2008, Wiley-VCH).

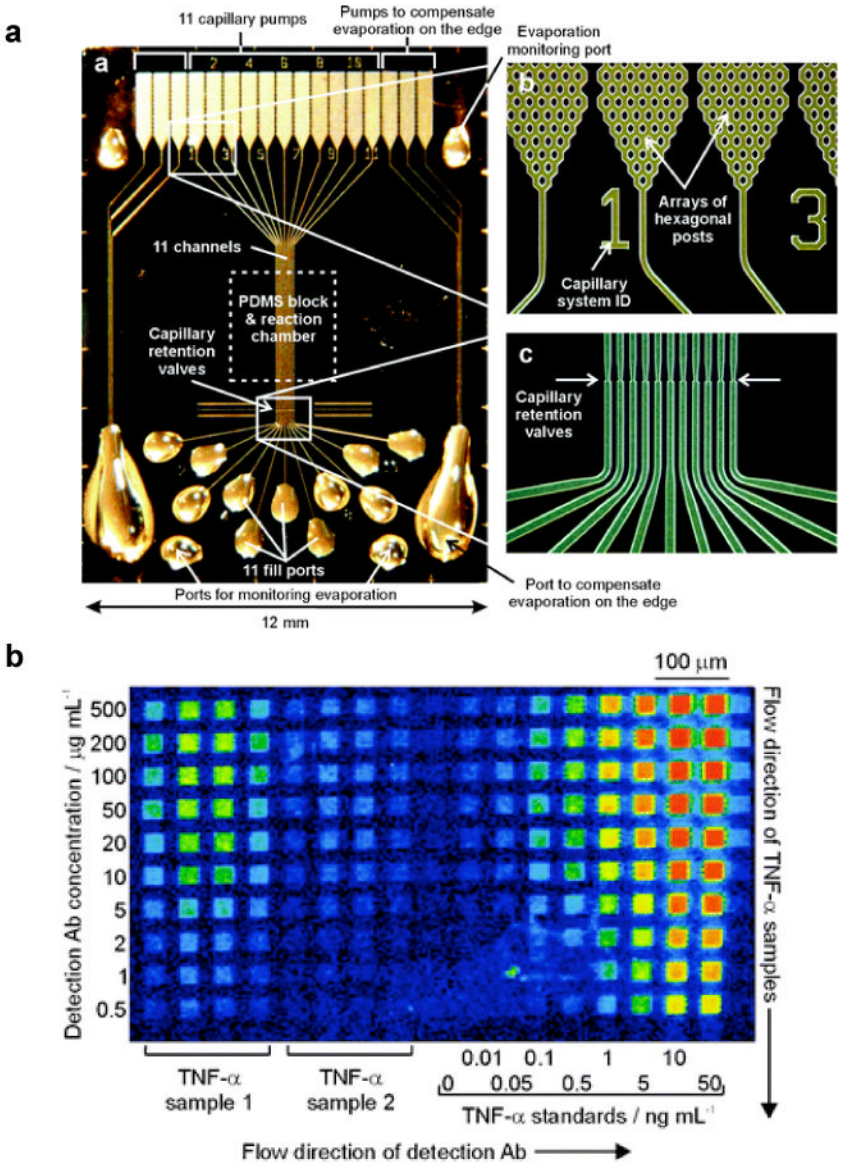

Figure 2. Micromosaic immunoassay

a. Microfluidic device for movement of liquids through the reaction chamber where the antigens are presented to the immobilized antibodies. This device used capillary pumping for control of fluid flow. **b.** Results from a micromosaic immunoassay for TNF-α. On the left, two samples of TNF-α were run and compared to a series of standard solutions for quantitation. The colors on the images correspond to the fluorescence intensity of the detection antibodies where the warmer colors indicate higher intensity. Reprinted with permission from reference [8] (Copyright 2004, The Royal Society of Chemistry).

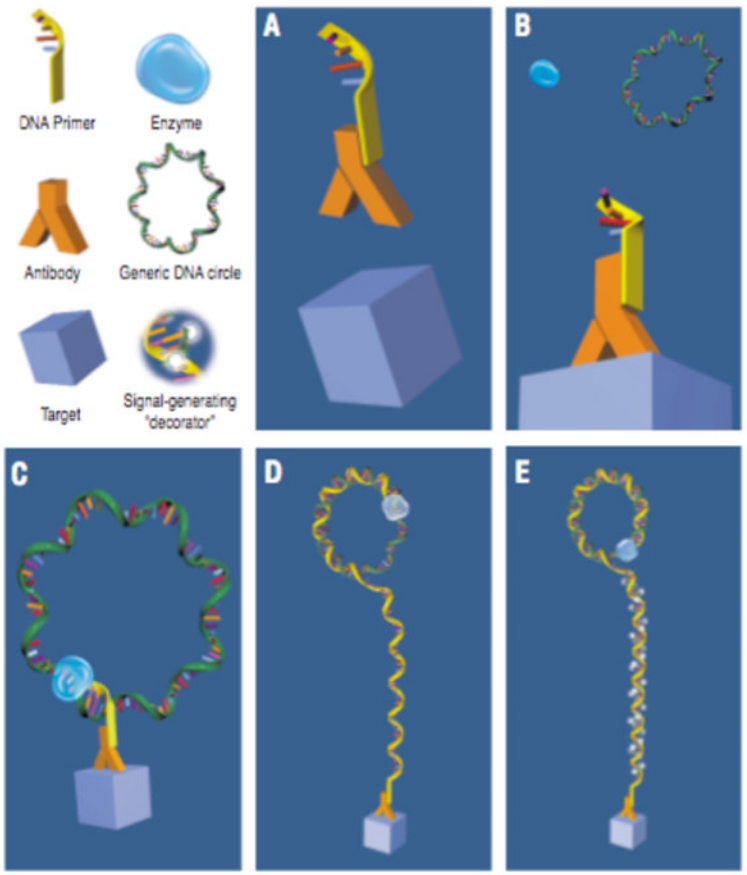

Figure 3. Method of immuno-rolling circle amplification (immuno-RCA)

a. DNA-labeled antibody is added to antigen and allowed to equilibrate. **b.** Circular DNA template is added with DNA polymerase. **c.** The template binds to the DNA on the antibody and is extended via the polymerase. **d.** Extension continues as the replicated strand grows longer while remaining attached to the antibody. **e.** Detection occurs by addition of complimentary strands of DNA labeled with a fluorophore. Reprinted with permission from reference 24 (Copyright 2002, Nature Publishing Group).