Perspective

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Bioanalysis

Sensors and microarrays in protein biomarker monitoring: an electrochemical perspective spots

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The development of clinically applicable portable sensors and multiplex protein biomarker assays is one of the most important goals of laboratory medicine today. Sensing strategies based on electrochemical devices are discussed in this overview, with special emphasis on detection principles derived from voltammetry, electrogenerated chemiluminescence, bipolar electrochemistry and impedance-based measurements. Up-to-date examples of electrochemical methods in biomedical research and development are highlighted here, including critical evaluation and future directions of the analysis, development and validation of new protein biomarkers.

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The application of chemical sensors, microarray technologies, microfluidic techniques and various flow injection and fully automated analyzers plays an important role not only in the biomedical research (development) of biomarkers, but also in practical diagnostics. With established diagnostic methods, this applies to analytical instrumentation based on the (often discussed) concept of point-of-care testing or to highly robust and centrally controlled multiplex instrumentation [1]. In all the above-mentioned areas of interest, electrochemical detection principles are being successfully applied. The reason for this is the use of screen-printed electrodes or disposable detection surfaces, for example, electrodes embedded into the bottom of the titration plate or microelectrodes or nanoelectrodes forming the microarray surface. Electrochemical approaches can also be easily combined with other complementary analytical methods. In this respect, significant advances can be made in the field of spectroelectrochemical analyzers that have been tested and validated with no apparent interference between partial detection principles. In addition to electrochemical and optical methods, other detection approaches, most often (depending on the application) operating on magnetic, gravimetric or acoustic principles, may be used in miniaturized techniques for protein biomarker analysis.

The principal and common aspect in biosensor or microarray construction is the immobilization of biorecognition elements (b.es) onto the substrate, which for electrochemical methods is a working (metallic or carbon based) electrode (Figure 1). When it comes to protein biomarker analyses, the b.e. is ideally a monoclonal antibody or other molecules capable of molecular recognition, such as DNA/peptide aptamers or affimers [2]. With nonelectrochemical approaches, a slide made of glass, silicone, teflon or conventional synthetic polymers is most commonly used as a solid substrate for immobilizing a b.e. A layer of gold or other metal can be vaporized onto the surface of the substrate, which may be important for immobilizing the b.e. itself, for example via bonding an SH-labeled molecule to the metal. By coating the metal at the same time, an improvement in the mechanical and chemical resistance of the substrate can be achieved or the desired optical or conductivity properties can be obtained. The b.es can be immobilized directly onto the substrate or its modified surface by physical sorption (adsorption) or via covalent bonding; immobilization strategies are described in detail in [3]. In the protein microarray field, an organic polymeric film is often immobilized onto an inorganic (metallic) substrate, which serves primarily to reduce its dehydration

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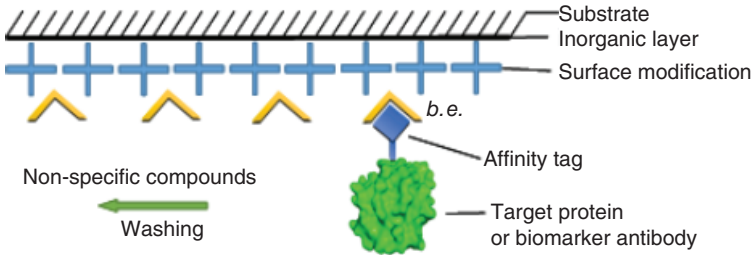

Figure 1. Scheme of protein sensor (array) architecture. The protein is immobilized via an affinity tag onto a detection interface containing the b.e. Other proteins and nonspecific compounds are washed from the surface prior to the detection end point.
b.e.: Biorecognition element.

property. The most commonly used are agarose, dextran and polyacrylamide gels. The process of immobilizing a b.e. or a specifically targeted protein (e.g., when examining post-translational modifications of already known biomarkers) onto the surface of a microarray platform or sensor is a key step in its design. There are countless methods for anchoring biomacromolecules to detection surfaces. In order to immobilize the antibody (or any target protein) to such a surface, it is necessary to modify its structure with a so-called 'affinity tag'. This tag is specifically recognized by the substance (or functional group) that is part of the surface of the substrate (Figure 1).

Proteins can be labeled with biotin, a water-soluble substance with a high affinity for the glycoproteins avidin and streptavidin. The biotin-labeled protein can thus selectively bind to a surface modified with these proteins. Another possibility is modifying the protein with a polyamino acid sequence (e.g., poly [His]) that binds to a surface that has been modified with a metal (e.g., Ni). Recombinant fusion proteins can also be used to immobilize proteins. For example, glutathione-S-transferase fusion protein (a glutathione-S-transferase-labeled protein at the C-terminus) can be bound by an antiglutathione-S-transferase antibody immobilized onto the surface of the sensor or detection platform [4,5].

In addition to the above, it is desirable to modify microarray surfaces with other components that can prevent surface passivation during the analysis or the nonspecific adsorption of other components of the sample. In particular, there are antifouling polymers or supramolecular systems that are capable of forming self-assembled monolayers. Alkanethiols are most commonly used, which can be easily bonded to a metal surface and there are many more; for a review, see [6].

The specific surface area of a microarray that contains an immobilized b.e. is called a 'spot'. A micrometer spot size can be reduced to nanometer size through today's nanotechnologies and screen printing technology [7]. An analysis of a bound biomarker or target biomolecule on the surface of an electrochemical sensor can be done directly by analyzing the electrochemical properties of the surface, or using an electroactive label or molecular probe [8]. Common electrochemical techniques are mostly used for this, in other words, voltammetry and amperometry. For both techniques, the measured quantity is current (I), which is proportional to the concentration of target analyte. Voltammetric techniques involve changes in applied potential (E) during the analysis. In linear sweep and cyclic voltammetry, the potential is swept linearly at a given scan rate. Where low detection limits are required, pulse techniques can be applied, of which the most common are differential pulse, normal pulse and square-wave voltammetries. In all sorts of voltammetry, the measured I is plotted against E, and the resulting analytical signal has the form of a peak positioned at the characteristic potential for a given probe. Amperometric techniques are based on the measurement of I at a fixed potential, sufficient for the probe's redox transition. The solution is usually stirred and the I signal is monitored over time. In this way, the responses to biomarker additions or the kinetics of binding can be monitored. More sophisticated protocols of pulse amperometry allow periodical renewal of the sensing surface by switching between analytical, cleaning and/or regeneration potentials, avoiding passivation phenomena that often make the measurement difficult. For special purposes (monitoring the electrocatalytic H-peak in proteins and DNA [4,5]) a technique of 'chronopotentiometry' is used, featuring the monitoring of E while I is applied to the electrode, in other words, the opposite principle to voltammetry and amperometry.

In addition to the above outlined traditional electrochemical techniques, there are now also interesting applications based on electrochemiluminescence (ECL) [9], bipolar electrochemical detection [10] and the application of electrochemical impedance spectroscopy (EIS) [11]. These detection principles will be described below.

ECL sensors & bipolar electrode arrays

ECL, also called 'electrochemically generated chemiluminescence', is a process in which the substances are excited to higher energy levels via electron transfer reactions. Specifically, radical ions are produced by electrochemical reactions (i.e., a radical anion by reduction and radical cation by oxidation) from a single or two different precursor

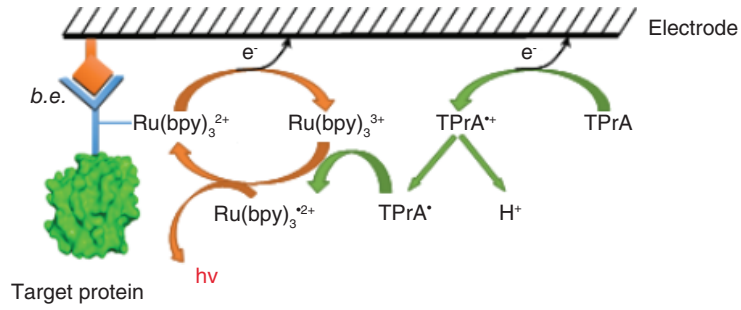

Figure 2. Schematic representation of heterogeneous electrochemiluminescence mechanisms for the couple $Ru(bpy)_3^{2+}$/tripropylamine.

molecules. The electron transfer – 'radical annihilation' between the pair leads to the product in a singlet or triplet excited state. The deexcitation, in other words, the return of the electron from an excited to a ground state, is often accompanied by light emission. The detection of light can be performed over a broad range of intensities, starting at the level of individual photons (photon counting). This allows for ultrasensitive analysis [12]. After several decades of fundamental research, ECL devices have entered commercial use, offering assays for several clinically relevant analytes, including antibodies and protein biomarkers.

In protein biomarker analysis one uses ECL labels which, after the selective binding of the protein to the surface of the sensor (Figure 2), undergo electrochemical conversion, resulting in the generation of luminescence. The original ECL approaches required an electrode potential switching to produce the radical anion and radical cation necessary for the ECL event. Significantly, generating ECLs can be made more efficient (i.e., the generation of an ECL at constant potential) in the presence of a 'coreactant'. The coreactant is a species which is capable of providing the radical ion counterpart at the same potential as that at which the other radical ion in the pair is generated (e.g., the radical anion is spontaneously generated from the primary product of coreactant oxidation). Although many ECL detection systems have been proposed in the last few years, only one has found practical use, namely a redox system with the tris(2,2′-bipyridine)ruthenium(II) complex, $Ru(bpy)_3^{2+}$ as the luminophore and tripropylamine (TPrA), which serves as the coreactant (Figure 2) [13]. To be more specific, $Ru(bpy)_3^{2+}$, available to the electrode surface, is oxidized, and the oxidized $Ru(bpy)_3^{3+}$ is then reduced by the $TPrA^{\bullet}$ coreactant intermediate. This intermediate is formed at the electrode at the same potential as for $Ru(bpy)_3^{2+}$ oxidation. The result of these reactions is the formation of $Ru(bpy)_3^{2+}$ in the excited state. Finally, the $Ru(bpy)_3^{2+}$ is converted to the initial $Ru(bpy)_3^{2+}$, resulting in the luminescence emission [14]. During the generation of ECL, the luminophore $Ru(bpy)_3^{2+/3+}$ undergoes redox cycling and the coreactant is continuously consumed.

In addition to the appropriate selection of a luminophore and coreactant [13], the physicochemical properties of the electrode surface [15] and the properties of the solution in which the reaction takes place play the key roles in generating the ECL. Thanks to their fast electron transfer kinetics, platinum and gold are often used in ECL applications combined with aprotic and aqueous solutions. The disadvantage of using these materials may be their passivation (metal oxide formation) that occurs during ECL generation. This passivation can be avoided by modifying the surface, most often using self-assembled monolayers, see above. In addition to noble metals, carbon-based electrode materials are also frequently used in ECL devices, especially glassy carbon, where water oxidation occurs at high potential, which minimizes bubble formation at the potential required for ECL emission. Carbon nanostructures, such as carbon nanotubes or graphene and transparent electrodes have also found significant application in the development of ECL methods. The most commonly used are glass substrates coated with a conductive layer of metal oxides (indium tin oxide electrodes) or transparent materials modified with carbon nanostructures. In addition to the above, nanomaterials (e.g., quantum dots) can be used as 'ECL emitters' themselves instead of organometallic complexes [16].

An exhaustive overview of ECL bioapplications can be found in [17], or recently ones of the analysis and use of peptides [18,19], proteins and tumor markers [20] as target analytes or b.es. Also, the possibilities of using ECL for point-of-care testing [21], microfluidics and paper-based technologies [22] or the combination of ECL with bipolar electrodes [10] have been evaluated.

The concept of 'bipolar electrochemistry' is based on the application of a bipolar probe (usually an Au plate) that is exposed to an electric field so that a potential gradient is generated from the ends to middle (uncharged) part of the probe. This can be used as a detection principle and for imaging purposes; for more details, see [23]. In the specific and widely-used case of the so-called 'closed bipolar setup', the bipolar probe connects two physically

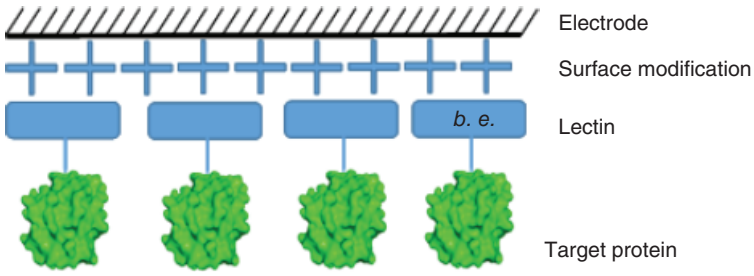

Figure 3. Simplified scheme of lectin impedimetric biosensor constructed for protein glycoprofiling.

separated compartments in the bipolar cell. This way, the analytical and detection steps can be separated, for example, by coupling immunoassay on the cathode end of the bipolar electrode with detection by electrogenerated chemiluminescence in the anode compartment.

Electrochemical impedance & other ultrasensitive approaches

As mentioned in the Introduction, electrochemical sensors are based on voltammetric, amperometric or potentiometric methods. For voltammetry and amperometry, the E (changing with time or constant) is applied to the working electrode (electrochemical sensor) and the I is monitored. The magnitude of the I or E response then corresponds to the concentration of the target protein or redox label. Despite the variety of electrochemical methods, these two detection principles have predominated in the past and are still predominant in modern electroanalysis today [24]. Phase-sensitive methods, such as EIS, can also be used very efficiently for the development of electrochemical sensors [25]. This method is based on resistance monitoring (Z, impedance), often through a charge transfer function, where the charge transfer properties change after the binding of a target protein to the electrode surface. Specifically, an alternating voltage is applied to the electrode where the alternating current component is monitored at different frequencies. The result of such analyses is the Nyquist diagram Z" versus Z', which has the shape of a semicircle. The diameter of the semicircle corresponds to the resistance to the charge transfer. In other words, with increasing resistance, the charge transfer is limited. Impedance, or the charge transfer parameters, can be detected by using redox markers such as $Fe(CN)_6^{3-/4-}$. Generally, the availability of the redox marker to the electrode surface depends strictly on whether or not the target protein covers the surface.

ESI sensors have been applied to detect a wide range of protein biomarkers; recently prostate-specific antigen [11] and selected glycoproteins [26] were mentioned. The bioaffinity principle is based on the immobilization of lectins (as b.es) on the surface of the sensor, which are able to recognize specific sugar modifications of the protein biomarker (Figure 3). After the binding of the protein to the surface of the 'lectin sensor', the impedance characteristics change in the presence of the aforementioned redox marker. By comparing the size of the semicircles in the Nyquist diagrams before and after the binding of the target protein, glycoprofiling can be performed with high selectivity and sensitivity. Similar to ECL, the protein analyses can be performed at the subnanomole level [11,26].

The monitoring of glycosylation and other post-translational modifications, like the study of membrane proteins (receptors), is one of the most complex analytical tasks in biochemical practice today [8]. As for post-translational modifications of proteins, there is now a wide range of bioaffinity methods or MS peptide profiling approaches. The interpretation problem for these highly advanced methodologies is not a low analytical selectivity or sensitivity of determination, but a low knowledge of intra- and interindividual variability in the protein modifications. The fact that electrochemical approaches can play an important role in solving these problems has recently been demonstrated in the electrocatalytic detection of the glycation and carbonylation of serum albumin as the model protein [27]. The results could be used for the further evaluation of the interindividual properties of other proteins or protein biomarkers. Electrochemical methods, sensors and microarrays can thus be considered not only to be an effective analytical (or screening) tool, but also as important instrumentation for fundamental biochemical and biophysical research.

Selected recent applications of protein biomarker assays

Electrochemical sensors and modern electrochemistry-based techniques discussed in this work have been used to analyze a wide range of real and potential protein biomarkers [28] such as tumor antigens, interleukins, alpha-fetoprotein, cardiac troponins, growth factors and their receptors, C-reactive protein, ferritin, prostate-specific

Table 1. Recent applications of electrochemical sensors or arrays in protein biomarker sensing.

Analyte	Real sample, selectivity	Detection surface/electrode	Biorecognition element, principle of detection, conditions	LOD	Ref.
Voltammetry					
IL-6	Human serum	Array (8×) of Au microelectrodes on silicon needle tip	Immobilized cross-linked IL-6 anti-bodies, remaining electrode surface blocked by SAM, detection by DPV of ferri/ferrocyanide, peak rises upon IL-6 binding	N/A, working range 20–100 pg.ml^{-1} of IL-6	[29]
Recombinant spike protein S1 of MERS corona virus	Nasal samples, selective over influenza A, B	Carbon 8-electrode microarray/AuNPs	Competition between free virus in sample and immobilized MERS-CoV protein for fixed concentration of antibody added to sample, detection by SWV of ferri/ferrocyanide, peak decreases upon binding	1.0 pg.ml^{-1}	[30]
Ovarian cancer tumor protein CA 125	Untreated human plasma	GC/nano-ink (containing AuNPs, AgNPs and carbon QDs)	Anti-CA 125 immobilized onto nano-ink through EDC-NHS chemistry, detection by DPV of ferri/ferrocyanide, peak decreases upon binding	0.001 U.ml^{-1}	[31]
Electrogenerated chemiluminescence					
Simultaneous recognition of carcinoembryonic antigen, alpha-fetoprotein and β-human chorionic gonadotropin	Model sample	GC electrode	Ruthenium and iridium complexes with bipyridyl as emitters, loaded onto carboxylate polystyrene beads, modified with specific detection antibodies for AFP, CEA and βHCG on the basis of biotin-streptavidin complexation, sandwich immunoassay with immunomagnetic beads coated with respective capture antibodies, TPrA coreactant	N/A, demonstration with 5 ng.ml^{-1} CEA, 25 ng.ml^{-1} AFP and 5 mIU/ml βHCG	[32]
Early lung cancer biomarker neutrophil activating peptide-2	Human serum, selectivity over interfering proteins including, MMP-14, CD44, PEX-14, chymotrypsin, albumin, myoglobin	GC electrode	$Ru(bpy)_3^{2+}$ anchored to NAP2 binding aptamer immobilized onto poly-4-aminobenzoic acid or thiol SAM-modified GC, ECL triggered by conformational change upon NAP2 binding, TPrA coreactant	0.001 pM for SAM-modified GC	[33]
Systemic inflammation biomarker – C-reactive protein	Model solution	GC electrode	Sandwich immunoassay, magnetic beads with Ab1, Ab2 with substituted iridium 2-phenylpyridine complexes ECL labels, TPrA coreactant	1 ng.ml^{-1}	[34]
Bipolar electrochemistry					
Prostate-specific antigen	Spiked human serum	Closed bipolar setup, gold CDtrode	Sandwich immunoassay – magnetic beads with PSA Ab1/PSA/PSA Ab2 attached to luminol loaded MOF, detection by luminol ECL after immobilization of magnetic beads using a magnet onto anodic pole of bipolar electrode, coated with ruthenium nanoparticles on nitrogen-doped graphene, Cu foam at cathode pole	0.1 pg.ml^{-1}	[35]
Cancer cell surface glycoprotein mucin 1	Spiked human serum; overexpressed MCF-7 cells	Carbon ink-printed bipolar electrode	Sandwich immunoassay performed at anode pole, modified with chitosan-MWCNTs with antimucin1, ferrocene-labeled mucin1 aptamer as Ab2, ferrocene redox at cathode pole, detection at cathodic pole utilizing SPM reduction product fluorescence	10 fM or three MCF cells	[36]
Carcinoembryonic antigen	Model sample	ITO	Sandwich immunoassay, Ab1 immobilized via cross-linking with chitosan-MWCNTs on cathode pole, Ab2 labeled with Pd-Pt bimetallic nanocrystals catalyzing H_2O_2 reduction at cathode pole, detection by ECL of $Ru(bpy)_3^{2+}$ with $C_2O_4^-$ coreactant at anode pole	1 pg.ml^{-1}	[37]

βHCG: β-Human chorionic gonadotropin; Ab: antibody; AFP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; DPV: Differential Pulse Voltammetry; ECL: Electrochemiluminescence; EDC: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; GC: Glassy carbon; ITO: Indium tin oxide; MOF: Metal organic framework; MWCNTs: Multi-Walled Carbon Nanotubes: NAP2: Neutrophil activating peptide-2; NHS: N-hydroxysuccinimide; NPs: Nanoparticles PSA: Prostate-specific antigen; QDs: Quantum dots; SAM: Self-assembled monolayer; SPM: 2-(2-(4-Hydroxystyryl)-6-methyl-4H-pyran-4-ylidene)malononitrile; SWV: Square-Wave voltammetry; TPrA: Tripropylamine.

Table 1. Recent applications of electrochemical sensors or arrays in protein biomarker sensing (cont.).

Analyte	Real sample, selectivity	Detection surface/electrode	Biorecognition element, principle of detection, conditions	LOD	Ref.
Impedance-based sensors					
Cardiac troponin I biomarker	Spiked blood serum	MWCNT-COOH	Paper-based two-electrode device, electrodes from MWCNTs, working electrode with anchored antibody, impedance measured in ferro/ferricyanide solution	0.05 $ng.ml^{-1}$	[38]
Nonstructural protein 1-a dengue virus biomarker	Human serum, selectivity against FBS and lysozyme	Screen printed electrode modified with electrospun nanofibers of polysulfone	Molecularly imprinted polydopamine recognition layer	0.3 $ng.ml^{-1}$	[39]
Prostate specific antigen	Human serum, selectivity against human serum albumin and human glandular kallikrein 2	Gold interdigitated microelectrodes on SiO_2 wafer	Self-assembled monolayer by amino-silanization of SiO_2, carboxyl-terminated antiPSA DNA aptamer anchored through EDC-NHS chemistry	0.38 $ng.ml^{-1}$	[40]

βHCG: β-Human chorionic gonadotropin; Ab: antibody; AFP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; DPV: Differential Pulse Voltammetry; ECL: Electrochemiluminescence; EDC: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; GC: Glassy carbon; ITO: Indium tin oxide; MOF: Metal organic framework; MWCNTs: Multi-Walled Carbon Nanotubes: NAP2: Neutrophil activating peptide-2; NHS: N-hydroxysuccinimide; NPs: Nanoparticles PSA: Prostate-specific antigen; QDs: Quantum dots; SAM: Self-assembled monolayer; SPM: 2-(2-(4-Hydroxystyryl)-6-methyl-4H-pyran-4-ylidene)malononitrile; SWV: Square-Wave voltammetry; TPrA: Tripropylamine.

Table 2. Recommendations for further reading.

Analyte	Ref.
IUPAC terminology on bioanalytical tools, including sensors and portable devices	[42,43]
Surface modification and immobilization approaches used for protein biosensor and microarray construction	[3]
Current opinion and a critical evaluation of electrochemical methods and biosensing strategies for the analysis of proteins (membrane proteins) and peptides	[8,19]
Data on the biosensor validation of analytical reproducibility	[44]
Recent progress in nanotechnologies for biosensors and bioassays	[45]
The current state of the art in the development of other (nonelectrochemical) biosensing principles	[46,47]

antigen, etc. Selected examples of recently (2019–2020) published works on the topic, summarizing the detection surface composition, assay design and the achieved LODs are given in Table 1.

Conclusion & future perspective

The application of electrochemical methods and sensors, microarrays and miniaturized techniques is a key field in the development of new analytical instrumentation suitable for the identification and monitoring of protein biomarkers [41]. For more details on methods used in biomarker research, we refer the reader to Table 2. Despite the very large number of studies targeting the field of interest, we mainly consider ECL-based approaches to be highly promising [13]. The ECL concept is basically similar to ELISA, using luminophore redox cycling instead of enzyme catalysis for signal amplification. Unlike signal amplification by enzymes, signal amplification using the ECL principle is based on an electrochemical reaction in which the experimental conditions are strictly under control. Therefore, variability in the activity of the enzymes cannot contribute to the intrinsic error in the detection step, which results not only in a significant improvement in systemic measurement error, but also in a reduction in interlaboratory differences. The applicability of electrochemical detectors was also demonstrated here with bipolar electrochemistry [10,23] and selected impedimetric sensors [11]. Ultrasensitive approaches based on electrochemical impedance analysis can be used for the research of glycoproteins and monitoring protein modifications. Achieving low detection limits is important for monitoring post-translational modifications where the analyte is a protein molecule, but the real analytical target is a submolecular component, for example a sugar moiety. The submolecular components occupy a tiny fraction of the protein under investigation, and hence there are increased demands on the sensitivity of analytical methods.

In general, microarray technology is now more widely used in DNA research than for protein identification purposes and screening. Microarray devices, however, can offer a highly effective way to detect various covalent and noncovalent modifications of proteins, see interactomics [48]. In this case, the protein is usually not a target

analyte, but directly a b.e. immobilized onto the detection surface of a sensor or microarray platform. The resulting modifications and protein–protein interactions can be analyzed not only by means of electrochemical approaches and optical methods, but also by other advanced techniques, including 'scanning electrochemical microscopy' [49] and mass spectrometric imaging techniques [50].

These multispectral analytical approaches, which we can apply simultaneously with electrochemistry in sensor and microarray technologies, represent additional value for future research and clinical applications. It should be noted that in addition to electrochemical and optical approaches, there has been great progress in the analysis of proteins and other biomolecules using semiconductor and molecular (organic) electronic devices [51] and 'nanopore technologies' [52,53], which are a significant step toward further miniaturization in bioanalysis. Finally, it is important to mention here that the standardization of detection surface preparation procedures, elimination of electrode fouling effects, development of new contactless approaches, improvements to robustness and lowering the demand for user experience could represent critical points in the further development and application of the electrochemical methods described in this contribution in protein biomarker analyses.

Executive summary

- With the growing demands on laboratory medicine and diagnostic procedures, it is desirable to develop new methods for protein research and for the sensitive analysis of protein biomarkers. In this sense, electrochemical methods represent detection-surface-based strategies that are applicable in the development of microarrays and microfluidic devices.
- The application of electrochemiluminescence, the detection of post-translational modifications using the impedimetric principle, and also a completely new concept based on bipolar electrodes and bipolar electrode arrays are highly promising for the future direction of the field.
- Bipolar electrochemistry in combination with electrochemiluminescence was recently proposed. Bipolar electrochemical probes are not physically connected to the control unit, a feature which could contribute to their application in microarray technology, without the need to use multichannel potentiostat instrumentation.
- Transparent or semitransparent electrodes are also a quite effective tool, because they can be utilized for spectroelectrochemical approaches. Complementarities between electrochemistry and spectrometric approaches could be important for the development of robust analytical solutions.
- In addition to the development of new methods, standardization of the development, validation and monitoring of proteins (protein biomarkers) and the pre-analytical phase are equally important tasks. The biological variabilities of the investigated proteins, for example, inter and intra-individual properties, need to be well known. This point is of paramount importance, far more so than technological improvements in laboratory medicine.

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