

Published in final edited form as:

*Analyst*. 2010 October ; 135(10): 2496–2511. doi:10.1039/c0an00204f.

## Measurement of biomarker proteins for point-of-care early detection and monitoring of cancer

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### Abstract

This critical review evaluates progress toward viable point-of-care protein biomarker measurements for cancer detection and diagnostics. The ability to measure panels of specific, selective cancer biomarker proteins in physicians' surgeries and clinics has the potential to revolutionize cancer detection, monitoring, and therapy. The dream envisions reliable, cheap, automated, technically undemanding devices that can analyze a patient's serum or saliva in a clinical setting, allowing on-the-spot diagnosis. Existing commercial products for protein assays are reliable in laboratory settings, but have limitations for point-of-care applications. A number of ultrasensitive immunosensors and some arrays have been developed, many based on nanotechnology. Multilabel detection coupled with high capture molecule density in immunosensors and arrays seems to be capable of detecting a wide range of protein concentrations with sensitivity ranging into the sub pg mL<sup>-1</sup> level. Multilabel arrays can be designed to detect both high and ultralow abundance proteins in the same sample. However, only a few of the newer ultrasensitive methods have been evaluated with real patient samples, which is key to establishing clinical sensitivity and selectivity.

### 1. Introduction

Broadly defined, biomarkers for cancer consist of any measurable or observable factors in a patient that indicate normal or disease-related biological processes or responses to therapy.<sup>1,2</sup> Biomarkers for disease thus encompass physical symptoms, mutated DNAs and RNAs, secreted proteins, processes such as cell death or proliferation, and serum concentrations of small molecules such as glucose or cholesterol. In this review, we focus on serum levels of *proteins* as biomarkers that can be used as indicators of the onset, existence or progression of cancer.<sup>3</sup> In addition to early detection of cancer, measurement of panels of protein biomarkers holds enormous potential for directing personalized cancer therapy and treatment monitoring.<sup>4</sup> However, these applications have yet to be broadly realized in a form that can be readily adapted to point-of-care.<sup>5,6</sup> This critical review is aimed mainly at assessing progress toward these goals.

A realizable hope to decrease deaths from cancer and improve therapeutic outcome for patients may be offered by earliest possible detection coupled with new targeted drug delivery therapies featuring personalized biomarker-based monitoring.<sup>7,8</sup> Protein biomarkers can be measured in serum and tissue for early cancer detection,<sup>9–14</sup> although reliable methodologies have been established clinically for only a handful of biomarkers.<sup>1</sup> The poster child of protein cancer biomarkers is prostate specific antigen (PSA),<sup>15</sup> which began its career as a clinical biomarker for prostate cancer several decades ago. PSA in serum is the only protein biomarker currently recommended by the American Cancer Society as an early cancer screening tool.<sup>16</sup> The danger zone for PSA serum concentration is 4 to 10 ng mL<sup>-1</sup>, a level indicating the possibility of early stage prostate cancer, while normal levels are typically<sup>1</sup> 0.5 to 2 ng mL<sup>-1</sup>. Late stage prostate cancer is characterized by values<sup>1</sup> of 10 to 1000 ng mL<sup>-1</sup>.

It is now apparent that *collections or panels* of protein cancer biomarkers, as opposed to single biomarkers, will be necessary for reliable cancer detection and monitoring.<sup>1,3,10–14</sup> For example, detecting 5 or more biomarkers for a given cancer by liquid chromatography-mass spectrometry (LC-MS) has provided >99% reliable diagnostics.<sup>10,12,17,18</sup> On the other hand, single biomarkers often have inadequate predictive value, *e.g.* about 70% for PSA, which is one of the better single biomarkers.

Increasing predictive power is both a statistical and a biochemical issue. Measurement with equivalent accuracy of elevated levels of 4 to 10 biomarkers for a given cancer should provide a much better statistical basis for successful prediction than measurement of a single biomarker. On the other hand, there are many types of cancers, and even different types of cancers specific to a given organ. A single biomarker expected for a given cancer may for some biochemical reason be poorly expressed in a particular patient, but it would be unlikely that an entire panel of protein biomarkers indicative of that cancer would fail to be expressed. In addition, false positives and negatives may appear quite frequently with a single biomarker, but would be minimized by using a biomarker panel.

Another issue to be considered is the fact that many protein biomarkers are indicative of more than one disease, *e.g.* serum PSA is elevated in some benign prostate diseases as well as prostate cancer.<sup>15</sup> Another biomarker protein, interleukin 6 (IL-6), is overexpressed in oral, prostate, lung, multiple myeloma and renal cell cancers.<sup>19</sup> Thus, single cancer biomarkers are often not unique to a specific cancer, but it should be possible to define collections of biomarkers whose elevated individual levels taken together would be reliable predictors for specific cancers.

Detection of panels of biomarkers is further complicated by the fact that ideally both normal and elevated serum levels of biomarkers need to be accurately measured. In addition, concentrations may vary widely for different analyte proteins in the serum. As mentioned for PSA, normal levels are typically 0.5 to 2 ng mL<sup>-1</sup> and patients with early stage prostate cancer have levels of 4 to 10 ng mL<sup>-1</sup>. However, serum IL-6 levels of patients with oral cancer range from  $\geq 20$  up to thousands of pg mL<sup>-1</sup> compared to  $< 6$  pg mL<sup>-1</sup> in healthy individuals.<sup>19</sup> Thus, the serum concentrations for IL-6 that need to be measured in a given sample may be 1000-fold smaller than those of PSA. In general, collections of cancer biomarkers in serum will have some members at very low concentrations, and other members at much higher concentrations. Also, there are thousands of proteins at significant concentrations in serum, some at much higher than ng mL<sup>-1</sup> levels.<sup>3</sup> Thus, strategies need to be developed to measure both high and low concentration members of the biomarker panel in the same sample without interference from thousands of other serum proteins, many at higher concentrations than the target analytes.

Owing to low inherent predictive ability of some biomarkers and their overexpression by multiple cancer types, highly reliable prediction for a specific cancer will ultimately require measuring a number of relevant biomarker proteins *for each cancer*. However, panels are likely to provide accurate prediction and monitoring with a small number of carefully selected members,<sup>20</sup> *e.g.* 4–10. Ideally, measurements need to be done at high accuracy and cheaply at point-of-care, *e.g.* in a physician's office or clinic, to reduce costs, minimize sample decomposition, facilitate on-the-spot diagnosis, and alleviate patient stress. Such point-of-care biomarker measurements can also help to guide therapy,<sup>4</sup> especially when timely adjustments in treatment become critical. Last but not least, point-of-care biomarker measurements for cancer detection and monitoring will need to be accepted and embraced by the clinical community. Given these realities, development of point-of-care bioanalytical devices to measure multiple cancer biomarkers presents a daunting challenge, but one that nevertheless should be realizable.

Enzyme-linked immunosorbent assays (ELISAs) have served as important methods for clinical protein determinations. Detection limits (DLs) approach  $1 \text{ pg mL}^{-1}$  for some protein biomarkers,<sup>15,19–22</sup> but ELISA is difficult to develop for point-of-care use and requires significant technical expertise. Classical ELISA methods suffer limitations in analysis time, sample size, equipment cost, and measuring collections of proteins. On the other hand, ELISA-type approaches have been quite useful in assays utilizing immunoarrays, as discussed below.

While modern LC-MS proteomics can achieve multiple biomarker measurements approaching acceptable sensitivity, the current analysis technology appears to be too expensive, often semi-quantitative, time consuming, and technically complex for routine diagnostic point-of-care applications.<sup>1–3,20</sup> At present LC-MS is more suited for biomarker discovery research. While vigorous efforts are underway to make LC-MS methods more suitable for point-of-care,<sup>1–3,23</sup> arrays for proteins employing optical or electrical detection are also being developed that may have more immediate promise to achieve relatively simple yet highly accurate and sensitive point-of-care measurements at low cost. Unlike gene arrays that are widely available commercially, accurate, simultaneous, sensitive multiple protein arrays are still under development.<sup>14,20,24–26</sup> Nevertheless, protein microarrays hold the promise of high selectivity and sensitivity, ease of use, reasonable cost per assay, and a good possibility for future automation. These arrays typically feature a series of analytical spots on a chip with each spot having a selective protein capture agent for a specific biomarker. Following ELISA strategies, the spots in the array may contain primary antibodies or aptamers to capture the desired analyte proteins, and after washing with a cocktail designed to block non-specific binding, a labeled secondary antibody dispersion is added to bind to the analyte proteins (Fig. 1). In the measurement step, the label provides an optical or electrical signal, which can also monitor a reaction catalyzed by an enzyme label. This approach is well suited to early cancer detection and monitoring since it has the potential for relatively rapid high sensitivity determinations of limited panels of biomarkers in serum with good precision and accuracy.<sup>20,27</sup>

This critical review focuses on methodologies for sensitive, specific protein determinations in biomedical samples that we believe have the potential for development for multiplexed point-of-care applications. The paper considers only this limited application, and is not intended as a comprehensive review on protein detection. The next section of this review provides a summary of therapeutic potential of cancer biomarker protein measurements. In subsequent sections we summarize progress in measuring cancer biomarker proteins by optically based, electrochemical, electro-optical and other methods. Our criteria for selection of methodology include the current or future potential for use in multiple protein measurements for cancer detection and monitoring at point-of-care, with all the desired

properties of that application as described above. The final section provides future predictions for this field. We have not included LC-MS in this review for the reasons outlined above, although future LC-MS methodology may well reach point-of-care applications. The reader is referred to excellent review articles describing efforts to approach this goal.<sup>1-4,23,24</sup>

## 2. Therapeutic value of cancer biomarker proteins

The US National Institutes of Health defines biomarkers more narrowly than above, as “molecules that can be objectively measured and evaluated as indicators of normal or disease processes and pharmacologic responses to therapeutic intervention”.<sup>28</sup> By this token, biomarker panels hold value by their remarkable ability to distinguish between two or more biological states. Consequently, the excellent clinical and preclinical applications that these molecules afford have resulted in an area of intense investigation. Significant contributions have recently been made in biomarker identification with the use of powerful discovery and screening technologies such as DNA microarrays and proteomic approaches including mass spectrometry, to essentially accelerate the number of potential biomarker candidates.<sup>29-32</sup> Analogous to how automated gel-filled capillaries allowed the early completion of the Human Genome Project, a trend in proteomics that has had a large impact is using capillary electrophoresis separation, other new methods, and sample handling protocols for biomarker protein discovery.<sup>33</sup>

Most biomarker studies generate data that have the potential of improving our understanding of the underlying disease.<sup>34</sup> In the case of human cancers, we now know that progressive changes in cellular behavior from slightly deregulated proliferation to full malignancy are a result of the accumulation of mutations and epigenetic alterations in a sub-set of genes. Identifying their aberrant forms including expressed products such as mRNA or proteins, which may be causal for conversion of normal to malignant tissue, can yield vital information on novel biomarkers for tumor development and progression. Such studies can also suggest candidate drug targets for pharmacological intervention.<sup>35</sup> However, a caveat to consider before embarking on detection of cancer biomarkers is that solid tumors generally develop from one or several defective cells, which then proliferate to a self-sustained mass which leads to further growth to a point when patient care is compromised.<sup>21</sup> Thus, the single most important goal of a cancer biomarker is the reliable detection of the presence of the smallest number of tumor cells before further growth and when clinical outcome and prognosis are still favorable for the patient.<sup>16</sup>

To realize the full potential of clinical biomarkers in diagnosis, new bioanalytical technologies are being developed as outlined later in this review. Accompanying the explosive interest in cancer biomarkers is the real potential to improve the accuracy and sensitivity of detection of cancer sub-types as well as markers of prognostic value and predictive of treatment outcome.<sup>36</sup> Nevertheless, a limiting factor likely to be encountered is the reliable and accurate detection of cancer biomarkers which is primarily due to insufficient sensitivity of the assays used. Many current methods lack the needed dynamic range to detect proteins of interest that are often expressed as discussed above at levels in the low pg mL<sup>-1</sup> range in serum, and in some cases below the detection limit. In contrast, prostate-specific antigen (PSA) levels are normally at a few ng mL<sup>-1</sup> or less in healthy subjects, which can be detected by current methodologies including ELISA. The Food and Drug Administration has approved a blood test to measure elevated PSA levels to detect prostate cancer at early or asymptomatic stages of development. However, this test is non-cancer-specific as PSA is also elevated in benign prostate conditions. Thus, at present, higher levels of PSA are not always indicative of disease, but accepted as a biomarker of disease when combined with clinical examination. Unfortunately prostate cancer can still

only be definitively diagnosed with a positive biopsy.<sup>37</sup> In this regard, the use of new technologies is essential to simultaneously and accurately measure biomarkers that are inherently elevated in cancer and as members of panels of multiple biomarkers. This approach can then result in early cancer detection, as well as in improvement in patient prognosis and care.

Validated biomarkers of cancer development and progression thus need to have excellent *specificity*, which can be defined as the ability of the assay to rule out a condition when it is absent and, *sensitivity*, defined as the ability of the assay to identify a condition when it is present. These clinical specificity and sensitivity parameters are closely linked to method used for measurement, and need to be high (*e.g.* >90%) to avoid false positives and false negatives, which is crucial to avoid misdiagnosis.<sup>38</sup> Notably, it is during the validation step where low specificity of selected biomarkers often fails to move them to the next stage.

However, with well designed experimental systems using large collections of clinical samples, the chances of validating potential biomarkers increase, together with our knowledge of the biology of the underlying disease condition.<sup>39</sup> In this regard, immunohistochemistry remains an important technique in biomarker validation in cancer tissues. Combined with the availability of using multiple antibodies against potential cancer biomarker proteins using tissue microarrays (TMAs) consisting of hundreds of normal and tumor tissues on a single slide, the expression and activity status of relevant molecules can be explored and validated simultaneously in large sample collections from a single immunostaining procedure. This provides valuable information about suitable biomarkers and drug targets, for example for the development and testing of novel therapies.<sup>40</sup>

Currently, fully validated biomarkers hold great promise for use as diagnostic, prognostic, and predictive markers for neoplastic diseases, especially when used in panels. Diagnostic markers are used to detect early stage disease where intervention can provide a favorable outcome under the current standard of care. The success of improving patient prognosis is dependent on the existence of evidence establishing the advantage of *early* intervention, as is clearly the case in cancer.<sup>41</sup> Similarly, diagnosis can be applied to histopathological tumor classification, primarily to understand the biological complexity underlining the disease. When metastasis is likely, an aggressive adjuvant therapy can be often prescribed. For example, a sub-set of breast cancer patients correctly diagnosed with early stage and high-risk tumors can benefit from appropriate personalized treatment. Similarly, if biomarker detection suggests that metastasis is likely in a patient, then an aggressive adjuvant therapy can be prescribed.<sup>42</sup> On the other hand, biomarker levels indicative of disease progression can be used to identify those patients who are likely to benefit from a specific targeted treatment, and may also provide molecular endpoints that can be used to predict and monitor treatment efficacy.<sup>43,44</sup> Appropriate biomarkers may constitute components of dysregulated signaling pathways and can be effectively screened with increased sensitivity and specificity by using available antibodies and TMAs. Ultimately, this knowledge may provide tremendous opportunities not only for the diagnosis of disease, but also for the development of novel molecular-targeted strategies. Realization of these opportunities depends on the development of accurate, sensitive, cheap, automated devices for the detection of multiple protein biomarkers in patient samples, preferably at point-of-care.

### 3. Optical methods and arrays

#### 3.1. Introduction

Array-based optical methods, fluorescence spectroscopy in particular, are important approaches for the multiplexed analysis of important cancer biomarkers with good sensitivity and low detection limits.<sup>25</sup> There are two distinct types of goals that need to be

addressed by these methods. One is the determination of a small panel of specific protein biomarkers in multiple samples that are obtained at different stages of cancer diagnosis, prognosis, treatment and monitoring. Success with this goal is critical for personalized medicine where each individual patient is cared-for in an appropriate manner. The other major goal is the determination of specific biomarker panels in samples obtained from a group of patients for the early detection of cancer or other diseases. These studies are directed toward validation or discovery of biomarkers. Progress in this second area is expected to accelerate early screening of high risk populations at point-of-care.<sup>45</sup>

To achieve these two distinct goals, arrays of antibodies,<sup>46</sup> nucleic acid aptamers,<sup>47</sup> peptide-like molecules called peptoids,<sup>48</sup> peptide aptamers,<sup>49</sup> and small molecules,<sup>50</sup> are being used to determine the concentrations of proteins in a variety of biological samples that include serum, saliva, sputum, tears, tissue lysates, and urine.<sup>25</sup> The following sections of the article focus mainly on frequently used antibody arrays for detection of single or multiple analytes by optical methods. The reader may consult excellent articles, suggested above, for progress on other types of arrays and detection methods. Next, we focus on fluorescence-based detection by using the sandwich immunoassay approach.

### 3.2. Fluorescent arrays

Strategies for high sensitivity fluorescence detection of biomarkers by sandwich immunoassay on an antibody array involve the following steps: (1) capturing the analyte on spots of the array using high affinity antibodies, called the capture anti-bodies ( $Ab_1$ ), which are immobilized on a solid support without diminishing its bioactivity;<sup>51</sup> (2) blocking or minimizing the non-specific binding (NSB) of the analyte to the solid support, usually with a protein such as bovine serum albumin and a detergent; (3) exposure of the array to the sample for capture of the analytes by  $Ab_1$ ; (4) tagging the immobilized analytes with secondary antibodies ( $Ab_2$ ) labeled with a high efficiency fluorophore, while blocking NSB of this species; (5) imaging the array by exciting the fluorophore at particular wavelengths and monitoring the fluorescence such that the signal is highly selective for the fluorophore label while maintaining low background levels; and (6) quantitation of fluorescence from specific spots of the array in terms of the corresponding analyte concentrations.

Detection limits for fluorescence immunoassays, in general, are in the range of mid- to lower picomolar ( $ng\ mL^{-1}$ ) concentrations.<sup>25</sup> However, cancer biomarker detection in patient samples such as serum often requires much lower detection limits, in the range of femtomolar ( $pg\ mL^{-1}$ ) concentrations or lower. The need for low detection limits is essential for early detection of cancer, since a large number of important cancer biomarkers exist at very low levels at early stages of cancer and at normal levels. Ultralow detection levels are also essential for the prompt detection of relapse after cancer treatment and therapy to ensure positive therapeutic outcomes. An additional challenge is that the real samples consist of complex mixtures of proteins, lipids and carbohydrates where achieving high specificity and low levels of NSB are major challenges. NSB to un-intended targets, where the analyte of interest is in very low concentrations and many other components are in high concentrations, can severely interfere with the assay. To achieve desired detection limits for biomedical applications, it is almost always essential to integrate specific signal amplification strategies by biochemical<sup>52,53</sup> electrochemical (see below), chemiluminescent,<sup>54</sup> or nanoparticle-based amplification strategies.<sup>55,56</sup> These amplification strategies need to be compatible with biomarker arrays, while providing high sensitivity and low background signals. One major challenge for signal amplification is that the amplification agent that results in the enhanced signal should not migrate from spot to spot within the array such that spatial resolution of the signal is maintained.

Fluorescence-based multiplexed detection methods are still in developmental stages, and a representative set of examples is given in Table 1 to illustrate the type of antigens that have been successfully detected with corresponding detection limits. These methods illustrate the detection of cytokines, toxins, antigen/antibody pairs and other proteins of clinical importance at levels that are biologically significant. These examples demonstrate the feasibility of the antibody array approach for the detection of biomarkers at physiologically relevant levels in serum, tissue and other biological media. While these examples of biomarker detection are impressive, significantly improved sensitivity and detection limits are still desirable for practical applications. While many studies focused on single analyte measurements in buffer or serum, simultaneous analysis of multiple biomarkers in relevant biological fluids, with little or no pre-treatment, will be of great significance in cancer diagnosis, treatment and management. Such an approach is particularly relevant for point-of-care (POC) applications. Currently, there are no commercial multiplexed biomarker detection systems specifically designed for POC applications. Such devices are highly desirable for improved treatment and lowering health care costs, and have the potential to provide diagnostic cancer testing for millions of patients worldwide.

The xMAP technology marketed by Luminex employs 5.6  $\mu\text{m}$  beads labeled with two fluorescent dyes at different levels for identification of multiple beads addressing different analytes. The beads in the assay are identified and counted by laser flow cytometry. This approach can be used for multiplexed fluorescent detection of proteins employing commercial kits for up to 100 or more proteins.<sup>64</sup> The sandwich immunoassays employ a bead with a secondary antibody attached to a third fluorescent dye label. The analytical label and two-level identification dyes are read for each bead simultaneously by flow cytometry. While this approach is appropriate for research and hospital laboratory use, it has not yet been developed for point-of-care applications. Flow cytometry is generally expensive and somewhat technically demanding. However, future breakthroughs in this field may overcome current limitations.

### 3.3. Chemiluminescence based arrays

One approach to improve upon the sensitivity of fluorescence-based arrays is to use chemiluminescence to generate the signal while optimizing the method such that the signal is proportional to analyte concentration over a wide dynamic range. ELISA-like arrays have been used to detect cytokines from patient sera using chemiluminescence detection.<sup>65,66</sup> Cytokines were captured on an IgG array supported on polyvinylidene difluoride membrane, and captured ligands were detected by exposure to biotin-conjugated antibodies, which also carried reagents required for chemiluminescence. Binding of the ligand resulted in enhanced chemiluminescence, thereby providing on-chip amplification of the emitted light signal by several orders of magnitude.<sup>67</sup>

### 3.4. Surface plasmon resonance (SPR)

**Introduction**—Surface plasmon resonance (SPR) is a powerful platform<sup>68</sup> to investigate biomolecular interactions with a high degree of accuracy, precision, information content, and sensitivity. Key biomedical applications of SPR include, but are not limited to, antigen–antibody interactions, protein–ligand binding, DNA-hybridization and high sensitivity detection of a variety of biomarkers.<sup>69</sup> SPR is a phenomenon that occurs when light propagates from a high-refractive-index medium (Fig. 2, prism or grating) toward an interface containing a low-refractive-index material (sample solution). When a thin Au or Ag film is placed at the glass–liquid interface, incident photons in the evanescent wave generated at the interface by rear illumination couple with the electrons in the metal (plasmons). This coupling occurs only when the conditions of resonance are established. Experimental variables that control resonance include the polarization of the incident light

with respect to the metal film, the angle of incidence, and the wavelength of incident light, for a given pair of low and high refractive index materials. Resonance coupling between the incident photons and the plasmons of the metal film results in the exchange of momentum between the two entities, and this interaction decreases the intensity of the reflected light from the metal–glass interface. Minimum reflectivity, therefore, occurs at an angle called the SPR angle at a specific wavelength, or at a specific incident angle at the resonant wavelength. Therefore, SPR signals can be monitored as a function of incidence angle or incidence wavelength, and the reflectivity changes monitored with a sensitive optical detector.

When biomolecules bind to the external surface of the metal film, the resulting change in the dielectric properties of the medium adjacent to the metal film results in changes in the resonance conditions, and hence, modulates the intensity of the reflected light. Thus, SPR can serve as a label-free detection technique to monitor the refractive index changes of the medium adjacent to the metal film brought about by a variety of events. The SPR signal is extremely sensitive to the dielectric constant as well as the mass of the material in contact with the Au film.<sup>70</sup> For example, binding of a ligand to a receptor attached to the metal surface will change the intensity of the reflected light, and hence, generate an SPR signal that can be monitored in real time. Kinetics of biomolecular interactions or concentrations of specific analytes such as proteins that bind to molecules attached to the sensor surface can be monitored during the SPR experiment.

Changes in refractive index of the medium up to ~300 nm above the Au film surface influence the SPR signal. Thus, almost any biological binding event can be detected by SPR when one of the binding partners is immobilized on the gold sensor within this distance. SPR can also be used to monitor protein conformational changes, an application that we pioneered to study protein folding thermodynamics on surfaces.<sup>71,72</sup> Angular-dependent SPR is convenient for single analyte samples, optimization and calibration, measuring on and off rates of binding events, and affinity constants. SPR has been extended to array format<sup>25,69</sup> and a number of analytes can be detected simultaneously (Fig. 2). The general strategy has been to spot multiple antibodies, each specific to a particular antigen, and use the array to simultaneously record SPR signals from each spot after the array is treated with a mixture of antigens.

SPR is a high sensitivity, real-time, label-free method for the detection of virtually any biomarker. Recent innovations have resulted in specific signal amplification methods and decreased detection limits (DLs). The DLs can vary depending on the protocol used for the SPR detection, and they are in the range of a few  $\text{ng mL}^{-1}$  when the SPR signal is not amplified.<sup>73</sup> However, DLs are improved to  $\text{pg mL}^{-1}$  levels or better by amplification strategies such as labeling with nanoparticles or DNA-based amplification.<sup>25</sup> While the label-free designation is lost by this improvement, the gain can be exquisite sensitivity. Specifically, the use of secondary antibodies labeled with Au nanoparticles, nanorods or magnetic nanoparticles, as well as enzyme-mediated precipitation reactions, provides excellent methods to amplify SPR signals and improve DLs by as much as 10 000 times with respect to un-amplified signals.<sup>74</sup>

**SPR detection of single biomarkers**—Vascular endothelial growth factor (VEGF), which is a cancer biomarker for breast cancer, lung cancer, and colorectal cancer, as well as rheumatoid arthritis, was detected by SPR at biologically relevant levels of  $45 \text{ pg mL}^{-1}$  (1 pM).<sup>53</sup> In healthy humans, VEGF levels are in the range of 1–2 pM but in cancer patients VEGF levels can be considerably higher.<sup>80</sup> A novel aspect of this approach was that RNA aptamers were used to capture VEGF as an aptamer–protein–Ab<sub>2</sub> sandwich. RNA aptamers are short stretches of RNA sequences that show unusually high affinities and selectivities for



specific antigens, and these are typically obtained by *in vitro*-selection and amplification. A number of aptamers specific for corresponding ligands can be purchased from commercial sources, although there are far less aptamers available than antibodies. In the above study, Ab<sub>2</sub> was labeled with horseradish peroxidase (HRP), which catalyzed a precipitation reaction with tetramethylbenzidine at the sensor surface. Precipitation of the solid amplified the SPR signals by 10 000-fold when compared to the unamplified signals.

SPR was also used for the detection of prostate specific antigen (PSA) in human serum at nanogram levels<sup>75</sup> using a sandwich immunoassay. Capture antibody Ab<sub>1</sub> was first immobilized onto the SPR sensor surface, and binding of PSA to Ab<sub>1</sub> was detected as the Ab<sub>1</sub>-PSA-Ab<sub>2</sub> complex, where Ab<sub>2</sub> is the secondary antibody, which was labeled with biotin. Biotin present on Ab<sub>2</sub>, in turn, was complexed with streptavidin-coated Au nanoparticles (20 nm) to enhance the SPR signals. The signal amplification was on the order of several-hundred fold due to the binding of the streptavidin-coated nanoparticle to Ab<sub>2</sub>, and no binding occurred without PSA. Thus, the Ab<sub>1</sub>-PSA-Ab<sub>2</sub>-biotin-streptavidin-NP configuration facilitated sensitive detection (DLs of 1 ng mL<sup>-1</sup>) of PSA in human serum samples. Note that PSA levels in healthy humans (males) are 0.5–2 ng mL<sup>-1</sup> and the SPR approach was sensitive enough to detect elevated PSA levels of 4–10 ng mL<sup>-1</sup>, which are indicative of the onset of prostate cancer. Preliminary results from our laboratory showed that SPR can be used to monitor PSA in serum at levels into the low fg mL<sup>-1</sup> range. The SPR signals were amplified by using magnetic nanoparticles (MNPs) coated with Ab<sub>2</sub> for off-line capture of PSA. This type of SPR protocol, and other innovative approaches (Table 2), need to be extended for the detection and analysis of multiple biomarkers with improved DLs and reliability.

Protein 53 (p53) is a transcription factor and it regulates the cell cycle and functions as a tumor suppressor. For this reason p53 is considered to be “the guardian of the genome”. Mutation of p53 is one of the most common molecular markers of the squamous cell carcinoma of the head and neck.<sup>81</sup> Recently, SPR was used to monitor the binding of p53 to its target double stranded DNA<sup>82</sup> as well as other biological partners. Interactions between the HPV E6 protein, p53 and ubiquitin ligase E6AP were monitored successfully by SPR.<sup>76</sup>

The analysis required 10 nL of 5–10 μM p53 solution and demonstrated the viability of p53 detection by SPR with high sensitivity, prior to signal amplification, but left room for considerable improvement. The method was neither optimized nor validated but we estimate a detection limit of 2–5 pg in the 10 nL sample, based on data presented in the article. It is likely that the DLs can be further improved with appropriate SPR amplification steps and that the method can be extended for the detection of native p53 as well as mutants.

Recombinant interleukin-6 (IL-6) was detected with good DLs by SPR using the immobilized antibody assay. IL-6 is a multi-functional cytokine and it is implicated in growth regulation and differentiation of cells.<sup>77</sup> IL-6 is a 20 kDa protein with two *N*-glycosylation sites, the human form has been cloned, and the recombinant IL-6 is commercially available. In order to monitor the activities of different batches of recombinant IL-6, SPR methods were developed, calibration graphs constructed and the procedure validated.<sup>77</sup> The binding of IL-6 to its antibody was monitored, which was followed by the binding of soluble IL-6 receptor to the Ab<sub>1</sub>-IL-6 complex (Ab<sub>1</sub>-IL-6-IL-6 receptor). Optimal results were obtained when Protein A was first immobilized on the sensor surface, followed by the binding of anti-mouse antibody and then IL-6 antibody. This approach gave a DL of 4 ng mL<sup>-1</sup> of IL-6, which was highly reproducible from day to day, sample to sample, and did not involve any amplification steps. These data clearly support reliable SPR-based detection of IL-6, but at concentrations above those that are clinically useful. Sensitivity and DL may be improved by introducing specific amplification strategies.

Interleukin-8 (IL-8) is another cytokine which plays an important role in human cancers including breast, prostate, and Hodgkin's disease.<sup>83</sup> Expression levels of IL-8 vary with respect to the cancer type, and there is a significant difference in the salivary levels of IL-8 between healthy individuals (30 pM or 250 pg mL<sup>-1</sup>) and patients with oropharyngeal squamous cell carcinoma (OSCC) (86 pM or 720 pg mL<sup>-1</sup>).<sup>84</sup> Inflammation of the oral cavity increases salivary cytokine levels but the above differences in IL-8 levels are significant when compared to the corresponding IL-6 levels. Elevation of IL-8 levels in the saliva of the cancer patients is elevated over and above levels due to inflammation of the oral cavity. SPR was used to determine the levels of IL-8 in human saliva of oral cancer patients using a sandwich immunoassay. The assay had a detection limit of 184 pM (<2 ng mL<sup>-1</sup>) which is greater than the IL-8 levels in patients or healthy individuals. Therefore, ten-fold pre-concentration of the initial sample was suggested in order to determine IL-8 levels in patients.<sup>78</sup>

Virus-like particles (VLPs) consisting of the human immuno-deficiency virus (HIV) oncoproteins were used in an ELISA format<sup>85</sup> to detect human IgG antibodies against human papillomavirus HPV-16 infection in cancer patients, a known cause of cervical and oral cancer.<sup>86</sup> Subsequent studies showed that ELISA detection of infection by HPV-16 can be improved by the use of full length recombinant biotin labeled E6 and E7 proteins as antigens instead of VLPs.<sup>87,88</sup> Serum samples of patients of head and neck squamous cell carcinoma (HNSCC) (240 HNSCC cases, and 322 matched control subjects without cancer) have been analyzed in this way. These serology studies when coupled with DNA sequencing and histological studies indicated that HPV-16-positive and HPV-16-negative oral cancers in HNSCC patients are distinct and that these should be considered as separate cancers. Such approaches should also be valuable in detection by SPR.

Overall, amplified SPR detection of cancer biomarkers is promising for early diagnosis of cancer, and such technologies may eventually find their way into POC applications. One problem remains the expense of optical detection, and the need for a robust, stable, portable SPR device. Cost might be decreased for POC using simple optics in conjunction with CCD camera detection. Such devices are not currently available for clinical biomarker panel detection.

## 4. Electrochemical immunoassays and arrays

### 4.1. History

Heineman and Halsall pioneered enzyme-linked electrochemical immunoassays for small molecules and proteins several decades ago.<sup>89</sup> They have continued to develop their approach, in which the binding of antigens to capture antibodies is separated in space and time from enzyme-label detection.<sup>90,91</sup> Their enzyme label alkaline phosphatase produces electroactive products that are transported by a chromatographic or fluidic system to an electrode detector. They have obtained excellent detection limits (DLs) in the pg mL<sup>-1</sup> to ng mL<sup>-1</sup> range for small molecules and proteins depending on the design characteristics of the system and the nature of the detector. Interdigitated electrodes have provided the highest sensitivities. For automation and miniaturization, Heinemann's team was among the first to employ microfluidics in electrochemical immunoassays for measurements of proteins.<sup>92</sup>

### 4.2. Single protein electrochemical immunosensors

In the 1990s, significant effort began to be directed toward developing self-contained, single analyte enzyme-linked electrochemical immunosensors.<sup>26,93-97</sup> Most approaches feature antibodies (Ab) attached to the sensor surface, so that antigen capture, enzyme-labeled secondary antibody binding, and detection are all done on the same surface. The most

selective and sensitive electrochemical immunoassays employ the sandwich format (*cf.* Fig. 1).

In addition to alkaline phosphatase and glucose oxidase, horseradish peroxidase (HRP) is a suitable label. It can be activated by hydrogen peroxide, which oxidizes the iron heme HRP enzyme to the  $\text{Fe}^{\text{IV}}=\text{O}$  form that is reduced to produce electro-chemical signals.<sup>98</sup> HRP-labeled  $\text{Ab}_2$  is a good choice for inclusion into arrays since immobilization of the electroactive enzyme label on the electrode minimizes diffusional crosstalk between array elements, which may complicate detecting soluble electroactive products, *e.g.* from alkaline phosphatase.<sup>99</sup> Many other types of labels have been used, including electroactive metal ions and complexes, nanoparticles,<sup>100,101</sup> conductive polymers, and liposomes loaded with electroactive compounds.<sup>97</sup> Savéant and co-workers provided theoretical analyses of amperometric and voltammetric enzyme-labeled immunosensor responses, and identified key factors in sensitivity.<sup>102–104</sup>

A critical issue in an immunoassay is minimization of NSB of the labeled- $\text{Ab}_2$  that arises when this species is bound to non-antigen sites on the sensor. In NSB, labeled- $\text{Ab}_2$  still gives a signal, but it is not proportional to the analyte protein concentration, and can raise the detection limit and degrade sensitivity. NSB can be minimized by washing with casein or bovine serum albumin along with detergents in NSB-blocking steps in the assay, as discussed earlier. The literature on protein adsorption onto solid surfaces suggests that tailoring the sensor surface with appropriate chemical groups may retard protein adsorption, with one of the most effective surfaces featuring polyethylene glycol (PEG) moieties.<sup>97,105,106</sup> However, most studies on which these predictions are based have measured protein adsorption in the absence of a sensitive analytical target, and few have addressed the trace levels of adsorbed proteins relevant to immunoassays. Such functionalized surfaces may still be subject to small amounts of NSB of labeled species that could significantly increase background in  $\text{pg}$  to  $\text{ng mL}^{-1}$  analyte concentration ranges.

More recently, the advantages of labeled *vs.* unlabeled immunoassays have been argued. Unlabeled immunoassays can be achieved with SPR, atomic force cantilevers, and quartz crystal microbalance (QCM), impedance/capacitance, field-effect transistors (FETs) or nanowire transistors.<sup>107,108</sup> While most of these techniques can provide excellent sensitivity, some like SPR (see above) and QCM still require labels to reach sensitivity levels needed for clinical cancer biomarker detection. Label-free methods such as AFM and ultrasensitive nanowire transistors require significant future development and cost reductions before they are able to compete with more accessible, lower cost procedures. In this context, a less-sensitive nanoribbon sensor was recently coupled with a microfluidic sample preparation device for the detection of PSA and a breast cancer biomarker in an approach that shows considerable promise.<sup>109</sup>

Proponents of unlabeled approaches argue that labeling of antibodies is a troublesome and perhaps unnecessary procedure, and adding labeled  $\text{Ab}_2$  requires an additional step in sandwich assays. These are, of course, relevant issues only when high sensitivity can be achieved without labels. In addition, there is a distinct difference in uniformly labeling all the analyte proteins, which is difficult to achieve, and using a labeled  $\text{Ab}_2$  for detection that can be easily prepared in batches and stored prior to the assay. The latter approach shares many of the attributes of true non-labeled methods, but fewer of the problems.<sup>108</sup> Further, NSB of the large number of non-analyte serum proteins in non-specific label-free methods such as SPR, nanowire transistor, and impedance/capacitance can be significant, since any adsorbed molecule will contribute to the NSB background. Proponents of labeled approaches also point out that large numbers of labeled secondary antibodies for sandwich immunoassays are available commercially, and that multi-label strategies using

nanoparticles have already achieved exquisite sensitivities and detection limits for protein biomarkers, as well as good accuracy in real biomedical samples.<sup>100,101,105,106,110,111</sup> In addition, predominantly NSB of labeled Ab<sub>2</sub> will contribute to background signals and presents the main NSB problem to be solved.

### 4.3. Ultrasensitive electrochemical immunosensors

As suggested above, reliable early cancer detection *via* protein biomarker measurements in serum requires methods with detection limits below that of the normal patient concentration of the least abundant protein, and adequate sensitivity for all the protein biomarkers at normal and elevated levels in serum. These considerations suggest that detection limits below the pg mL<sup>-1</sup> level and good sensitivity up to hundreds of ng mL<sup>-1</sup> will be necessary. Considerable effort has been directed toward fabrication of high sensitivity electrochemical immunosensors for proteins with ultralow detection limits (DLs) and excellent sensitivities. An added benefit may be that unknown biomarker proteins with very low serum concentrations could emerge once very high sensitivity can be achieved routinely.

Promising approaches utilizing secondary antibodies (Ab<sub>2</sub>) labeled with nanoparticles in sandwich immunosensors have employed strategies including dissolvable nanoparticles, and nanoparticles with multiple enzyme labels (Fig. 3) or multiple redox probes.<sup>100,101,105–115</sup> These approaches yield high signal amplification by providing many signal generating electrochemical events for each analyte bound to the sensor.

A recent review by Wang<sup>100</sup> summarizes the various approaches using electrochemically detectable metal nano-particles as labels in protein immunosensors. Dequaire *et al.* provided proof of concept for the basic approach,<sup>116</sup> in which the metal labels are dissolved in acid after Ab<sub>2</sub> binding and NSB blocking steps. They used gold nanoparticle–Ab<sub>2</sub> labels, and detected gold ions released after acid dissolution by anodic stripping voltammetry to achieve a 3 pM DL for IgG protein in buffer. Wang developed several strategies to amplify the sensitivity even further,<sup>100</sup> including a cyclic accumulation of gold nanoparticles and the use of these particles to catalyze the subsequent precipitation of Ag. Both approaches provide a large number of metal ions to be detected in the electrochemical stripping measurement step. The Ag-deposition approach was used to achieve a 0.5 ng mL<sup>-1</sup> (22 pM) DL for cardiac troponin I.<sup>117</sup> Multiple gold nanoparticles have also been attached to larger Au spheres and used for Ag-deposition enhancement.<sup>100</sup> CdS quantum dots (Qdots) on magnetic particles have also been used as labels, with magnetic collection and dissolution of the Qdot and electrochemical stripping detection of Cd, which was further enhanced by Cd-deposition.<sup>100</sup> Ag-deposition has also been used in high sensitivity conductivity immunoassays of human IgG in buffer.<sup>118</sup> Other strategies as extensively reviewed<sup>100,101,105,112–114</sup> have included loading Ab<sub>2</sub>-nanoparticles or Ab<sub>2</sub>-polymer beads with electroactive labels such as ferrocene derivatives, and releasing these redox probes for electrochemical detection following the binding steps. Multiplexing strategies using the above approaches are discussed in a later section.

Wang's team also pioneered the use of multilabel enzyme nanoparticles with attached Ab<sub>2</sub>, while first demonstrating this approach for ultrasensitive detection of DNA.<sup>119</sup> Multi-wall carbon nanotubes (MWCNTs) were used to carry thousands of alkaline phosphatase enzyme molecules per CNT and secondary antibodies to achieve a fM detection limit for proteins in buffer. The carbon nanotubes also served to preconcentrate the enzyme reaction product  $\alpha$ -naphthol by adsorptive accumulation. A similar enhancement strategy using layer-by-layer film deposition of alkaline phosphatase with oppositely charged polyions on MWNTs achieved a remarkably low DL of ~70 aM for IgG in buffer.<sup>120</sup>

Our research team has exploited the multi-enzyme label strategy in designing immunosensor protocols for PSA, IL-6, and other prostate cancer biomarkers.<sup>106,112,121–123</sup> Sensitivity and detection limits were further improved by using conductive nanostructured electrode platforms, *e.g.* films of oxidatively shortened upright single wall carbon nanotube (SWCNT) forests<sup>110</sup> or 5 nm glutathione-decorated gold nanoparticles, both of which contain carboxylate groups ready for attachment of large amounts of capture antibodies by amidization.<sup>111</sup> AFM images of these sensor surfaces confirm their large surface areas (Fig. 4).

SWCNT and AuNP nanostructured sensor surfaces were first used to fabricate sandwich immunoassays for prostate cancer biomarker PSA.<sup>106,110,111,121</sup> Two detection protocols were evaluated. As in Fig. 3a, conventional secondary antibodies ( $Ab_2$ ) conjugated with enzyme label HRP were used, as well as a higher sensitivity protocol using oxidized multiwall carbon nanotubes (CNTs) conjugated with HRP and  $Ab_2$  (CNT–HRP– $Ab_2$ ) at high HRP/ $Ab_2$  ratios (Fig. 3c). These CNT–HRP– $Ab_2$  with 170 HRPs per 100 nm length<sup>124</sup> can replace the conventional HRP– $Ab_2$  in the immunoassay procedure to greatly enhance sensitivity.

Fig. 5A shows amperometric SWNT immunosensor responses to PSA in calf serum using CNT–HRP– $Ab_2$ . A PSA detection limit (DL) as a signal 3 times the noise above the zero PSA control was  $4 \text{ pg mL}^{-1}$  (150 fM). Controls (b) and (c) in Fig. 5A show that SWNT forests provided a significant gain in sensitivity over immunosensors without nanotubes, presumably because of an increased density of  $Ab_1$ . Recent unpublished results show that such SWCNT forests can achieve a 10 to 15-fold increase in the number of surface antibodies compared to a flat immunosensor.

Our AuNP electrodes were fabricated by depositing a layer of 5 nm glutathione-decorated AuNPs onto a previously deposited 0.5 nm polycation layer on PG. High sensitivity was obtained by using  $1 \text{ }\mu\text{m}$  magnetic bead– $Ab_2$ –HRP bioconjugates with  $\sim 7500$  HRPs per bead.<sup>111</sup> Using these multiply labeled magnetic beads with AuNP electrodes (Fig. 4C), detection limit at  $0.5 \text{ pg mL}^{-1}$  (20 fM) PSA was eight fold better, and sensitivity was 4-fold better than SWCNT forest immunosensors featuring the multi-label CNT–HRP– $Ab_2$ . Controls (a) and (b) in Fig. 5C show that AuNPs also provided enhanced sensitivity over immunosensors without AuNPs. With both SWNT forest and AuNP immuno-sensors, excellent correlations were found with a standard ELISA for determination of PSA in serum of human cancer patients.<sup>110,111</sup>

A head-to-head comparison was made between SWCNT and AuNP immunosensors for determination of IL-6 in serum.<sup>122</sup> Immunoassays employed HRP labels on streptavidin bound to biotinylated  $Ab_2$ , providing 14–16 labels per analyte (Fig. 3b). The AuNP immunosensor gave DL of  $10 \text{ pg mL}^{-1}$  IL-6 (500 fM) in  $10 \text{ }\mu\text{L}$  calf serum, 3-fold better than  $30 \text{ pg mL}^{-1}$  found for the SWCNT forest immunosensor. The AuNP sensors provided a linear dynamic range of  $20\text{--}4000 \text{ pg mL}^{-1}$ , covering most of the clinically relevant range, and was much larger than the SWNT immunosensors ( $40\text{--}150 \text{ pg mL}^{-1}$ ).<sup>122</sup>

While there has been considerable development of label-free electrochemical impedance immunosensors, a recent review concluded that in general these methods may not be able to provide ultrasensitive protein analyses without some mode of amplification or other improvements in sensitivity.<sup>125</sup> Significant amplification and high sensitivities have been obtained, for example, using metal nanoparticle labels or AuNP labels that catalyze subsequent silver deposition. Furthermore, several carefully designed label-free impedance/conductance methods have been able to achieve notably high sensitivity for proteins. A capacitance method using a ferri/ferrocyanide probe and a potentiostatic step gave a DL of

10 pg mL<sup>-1</sup> (500 fM) for IL-6 in buffer.<sup>126</sup> Optimization of experimental parameters and protocols using flow injection electrochemical impedance spectroscopy led to sensitivity in the low aM range for interferon- $\gamma$  in buffer.<sup>127</sup> Such approaches appear promising for future point-of-care applications provided NSB from the numerous serum proteins can be minimized. A recent review by Tkac and Davis discussed these high sensitivity approaches in detail.<sup>108</sup>

#### 4.4. Multiplexed protein arrays

Two major approaches have been pursued for ultrasensitive multiplexed electrochemical protein measurements. In one, a “barcode” approach is used to label secondary antibodies with distinct nanoparticles having different electrochemical characteristics, *e.g.* different dissolvable metals or quantum dots (Qdots) that can be detected by stripping voltammetry.<sup>103</sup> In the second approach, multi-electrode microelectronic arrays are used in which each electrode has a different antibody attached. Conceivably these approaches could also be combined.<sup>103</sup>

As an example of the first approach, cadmium sulfide, zinc sulfide, copper sulfide and lead sulfide quantum dots (Qdots) were attached to four different secondary antibodies to detect four different proteins by sandwich immunoassay.<sup>128</sup> The four metal ions, each attached to a specific antibody for a different protein, were measured after binding of the antigen by stripping voltammetry and dissolution of the particle. Multiple-metal spheres, striped rods or alloy rods have also been used as labels. The rods can be capped with a gold end for attachment to Ab<sub>2</sub>. Upon dissolution, these materials give a series of stripping peaks whose peak potentials and relative intensities are characteristic of a single analyte protein.<sup>100</sup> In principle, such “barcode” labels can be employed for the determination of thousands of proteins, with computer deconvolution of the identities and concentrations.

Pairs of iridium oxide electrodes to which capture antibodies for two proteins were covalently attached have been used to determine proteins.<sup>129</sup> Separation of the electrodes by 2.5 mm to eliminate cross-talk between the electrodes enabled simultaneous electrochemical immunoassays using alkaline phosphatase-labeled Ab<sub>2</sub> and detection of the electroactive enzyme product hydroquinone. Detection limits were ~1 ng mL<sup>-1</sup> for goat IgG, mouse IgG, and cancer biomarkers carcinoembryonic antigen (CEA) and  $\alpha$ -fetoprotein (AFP). This concept was extended to an 8-electrode array and an electrochemical immunoassay was developed for simultaneous detection<sup>130</sup> of goat IgG, mouse IgG, human IgG, and chicken IgY. DLs of ~3 ng mL<sup>-1</sup> were achieved for these analytes, and the method was validated *vs.* a referee method using synthetic sera containing the 4 proteins. Eight-electrode iridium oxide arrays designed into each well of a 12-well plate were used to simultaneously measure<sup>131</sup> seven cancer biomarkers: AFP, ferritin, CEA, hCG- $\beta$ , CA 15-3, CA 125, and CA 19-9 with DLs of ~2 ng mL<sup>-1</sup>. The method showed good correlation for a standard serum sample against commercial ELISA methods. Unfortunately, none of these systems were useful in the pg mL<sup>-1</sup> range.

CombiMatrix VSLI chips with over 1000 electrodes cm<sup>-2</sup> have been used for sandwich immunoassays of proteins.<sup>132</sup> Oligonucleotides were synthesized on the 100  $\mu$ m diameter electrodes and antibodies were outfitted with complementary oligonucleotide strands so that they could bind to specific electrodes. This method was used to determine human  $\alpha$ 1 acid glycoprotein (APG), ricin, M13 phage, *Bacillus globigii* spores, and fluorescein with a 5 pg mL<sup>-1</sup> DL for the protein APG. A similar approach was used with two or three different enzyme labels on the secondary antibodies for multianalyte detection at several different measuring potentials.<sup>133</sup> While these chips have the potential for massively parallel array detection of thousands of proteins, this has yet to be realized. The approach has been commercialized by CombiMatrix for DNA and RNA arrays. The bottleneck for protein

arrays would seem to be attachment of the oligonucleotide affinity strands to the large number of antibodies that would be necessary for such a chip.

Wong *et al.* reported several approaches to arrays to detect the oral cancer biomarkers IL-8 protein and RNA in the same sample. In 2007, they reported a prototype microfluidic array with detection capabilities of  $\sim 50 \text{ pg mL}^{-1}$ .<sup>134</sup> More recently, they reported a 16-sensor electrochemical chip with each sensor having its own reference and counter electrode.<sup>135</sup> Individual sensors were coated with a DNA dendrimer/conducting polymer film decorated with binding sites for capture antibodies. Two oral cancer protein markers (IL-8 and IL-1b) and IL-8 mRNA were measured in buffer with LD of  $100\text{--}200 \text{ fg mL}^{-1}$  for the proteins and  $10 \text{ aM}$  for IL-8 mRNA in  $4 \text{ }\mu\text{L}$  samples, which was 1000-fold better than without the DNA dendrimer/conducting polymer. This system was applied to measurements in saliva, with considerably higher DL for IL-8 mRNA ( $\sim 4 \text{ fM}$ ) and for IL-8 ( $7.4 \text{ pg mL}^{-1}$ ).<sup>136</sup> Using these arrays to analyze a collection of oral cancer saliva samples and controls gave  $\sim 90\%$  clinical sensitivity and specificity for both IL-8 mRNA and IL-8 protein. These studies illustrate the critical importance of testing arrays in real biomedical samples, as they show considerable degradation of DL for both analytes when moving from buffer to saliva. In addition, the last study is an excellent example of essential method validation and analytical performance evaluation using real patient samples.

Our research team recently used a 4-unit electrochemical immunoarray equipped with single-wall carbon nanotube forest electrodes to simultaneously measure multiple prostate cancer biomarkers in cancer patient serum.<sup>137</sup> The proteins were PSA, prostate specific membrane antigen (PSMA), platelet factor-4 (PF-4) and IL-6. Method sensitivity was tailored to analytical requirements of each protein by combining single and multiply labeled strategies. Biotinylated secondary antibodies ( $\text{Ab}_2$ ) that bind specifically to streptavidin–HRP conjugates to provide 14–16 labels per antibody (see Fig. 2b) were used in a sandwich immunoassay format to give the necessary sensitivity to detect PF-4 ( $\text{DL} \approx 1 \text{ ng mL}^{-1}$ ) and IL-6 ( $\text{DL} \approx 30 \text{ pg mL}^{-1}$ ). Singly labeled  $\text{Ab}_2$ –HRP (Fig. 2a) was used for PSA ( $\text{DL} \approx 1 \text{ ng mL}^{-1}$ ) and PSMA ( $\text{DL} \approx 10 \text{ ng mL}^{-1}$ ) in the  $\text{ng mL}^{-1}$  range. Immunoarray determinations of the four proteins in serum samples of prostate cancer patients and controls gave excellent correlations to standard ELISA assays.<sup>137</sup>

Electrochemiluminescence (ECL) detection is also promising for multiple protein assays. Tris(2,2'-bipyridyl)ruthenium(II),  $[\text{Ru}(\text{bpy})_3]^{2+}$ , or RuBPY, initiates ECL when its oxidized form reacts with a suitable sacrificial reductant, and can be used as a label in immunoassays.<sup>138</sup> The result is light emission with electrical rather than optical excitation, thus simplifying instrumentation. Magnetic bead methods have been most successful with ECL detection, and are available from several companies.<sup>139,140</sup> A typical ECL magnetic bead immunoassay features antibody–streptavidin-coated magnetic beads that capture the protein analyte from the sample. Then, RuBPY-labeled bio-tinylated secondary antibody is added and the beads are washed to remove non-specific binding. The magnetic beads are transported to an electrochemical cell where they are magnetically captured onto an electrode for ECL measurement.<sup>140</sup> Manual and automated systems are available along with kits for up to 10 selected proteins, although most applications measure one or two proteins per sample. Current automated ECL bead technology is relatively expensive and requires a high level of technical expertise and maintenance. While ECL-bead protein detection is making inroads in research and some hospital clinical laboratories, it will require considerable development and cost reduction for widespread point-of-care applications. A recent innovation involved  $\text{Ab}_2$ –ECL–RuBPY beads with a gold-coated fiber-optic array used to demonstrate detection of 3 proteins in sandwich immunoassays.<sup>141</sup> By using 3 different levels of fluorescent labels, single beads representative of 3 different protein analytes were identified and imaged using ECL for quantitation. While promising, this method is in very early development.

## 5. Outlook for the future

Optical and electrochemical approaches have been developed that are capable of detecting proteins at levels at or below a few pg mL<sup>-1</sup>, at concentration at or below the normal levels of many cancer biomarker proteins. Many new approaches using nano-technology have been designed to detect proteins at even higher sensitivity. While only a few of these approaches have been validated against cancer patient samples, they have demonstrated good accuracy and sensitivity for clinically important levels of biomarker proteins. However, cancer detection and diagnostics using protein biomarkers will require accurate detection of panels of an estimated 4–10 biomarkers for each cancer, and much less progress has been made on this front. Extensive testing will be necessary on patient samples such as serum or saliva to establish analytical reliability, and clinical sensitivity and selectivity.

Currently no devices or methodologies for multiplexed cancer biomarker protein detection are in widespread POC use, although some commercial bead methods utilizing ECL and fluorescence are appropriate and reliable for laboratory based sample analyses. POC devices will require simple, low cost, technically undemanding methodology. Electrochemical approaches are inherently inexpensive; optical methods will need technical advances to produce lower cost detection. We can distinguish between methodology where binding events and detection are done simply and directly on a sensor or array surface vs. those that require further processing or dissolving of labels and detection in solution. Clearly the simpler approaches have the current advantage. Finally, the marriage of sensitive reliable, simple detection protocols with microfluidic or other sample handling technology seems to be a necessary future step to reach POC. However, the payoff in future cancer diagnostics and therapy is large, and significant efforts and funding are being committed with the hope of solving these formidable analytical challenges in the relatively near future.

## Acknowledgments

Preparation of this review was supported jointly by PHS grant ES013557 from NIEHS/NIH, by the National Science Foundation (DMR-0604815), by the Intramural Research Program, NIDCR/NIH, and by a Walton Research Fellowship awarded by Science Foundation Ireland to JFR.

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## Biographies



James Rusling obtained his BSc in Chemistry from Drexel University and PhD from Clarkson University. He is Professor of Chemistry at University of Connecticut and Professor of Cell Biology at University of Connecticut Health Center. Current research includes electrochemical arrays for toxicity prediction and early cancer detection, and fundamental bioelectrochemistry. He has authored over 300 research papers, several books, and is also a musician interested in Irish and American folk styles.



Challa Vijaya Kumar obtained his PhD from the Indian Institute of Technology Kanpur, India in 1982 and worked as research associate at the University of Notre Dame and Columbia University. He joined the University of Connecticut in 1988 as assistant professor of Chemistry and he is currently Professor of Biological and Physical Chemistry, and Head,

the Division of Physical Chemistry. His research interests include DNA/RNA binders, Biocatalytic Nanomaterials, and Protein Biomarkers and Photoscissors.



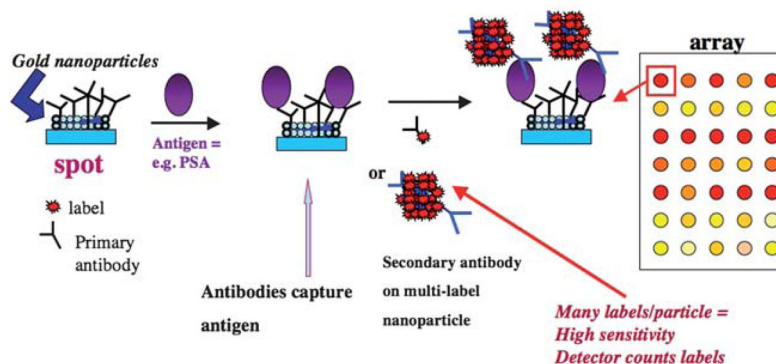
J. Silvio Gutkind received his PhD in Pharmacy and Biochemistry from the University of Buenos Aires, Argentina. He was trained as a post-doc at NIMH and NCI, and joined the NIDCR, NIH, in 1988, where he is currently the Chief of the Oral and Pharyngeal Cancer Branch. His research team studies the molecular basis of cancer, with emphasis on basic mechanisms of signal transduction and cell growth control, and their dysregulation in oral squamous cell carcinogenesis and AIDS-malignancies. Dr Gutkind has published over 320 scientific manuscripts, edited three books, and is currently leading a multi-institutional effort aimed at exploring novel molecular-targeted therapies for oral cancer patients.



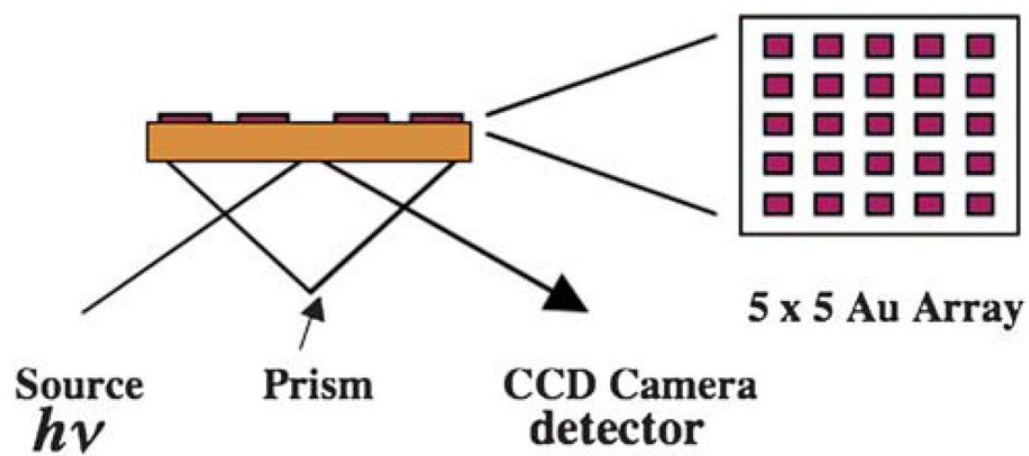
Vyomesh Patel received his PhD from the University of Bristol in 1993. After Post-Doctoral training at the NIH, in Bethesda, USA, he is currently a Staff Scientist. His research interests are in understanding the molecular basis of squamous cancer development and progression, and in the use of technology to drive some of these research areas, to potentially identify biomarkers, that may have use for example at point-of-care, for diagnostic and prognostic application.



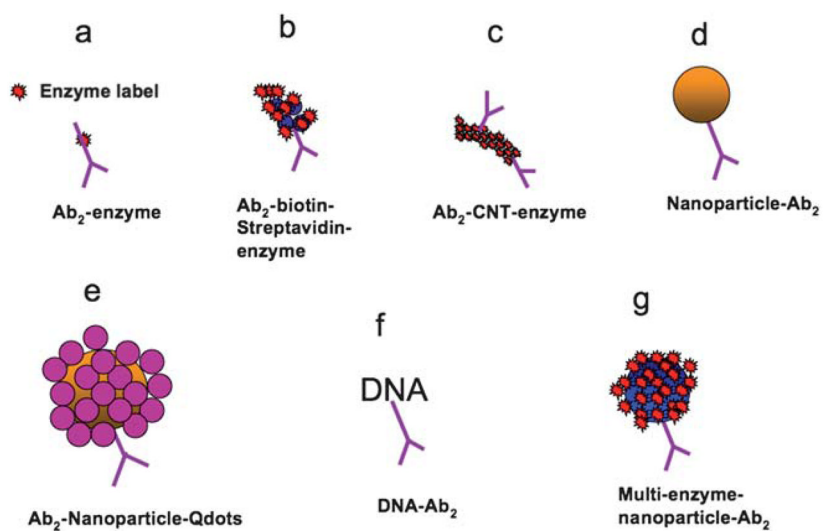
### Sandwich immunoassays using nanoparticles



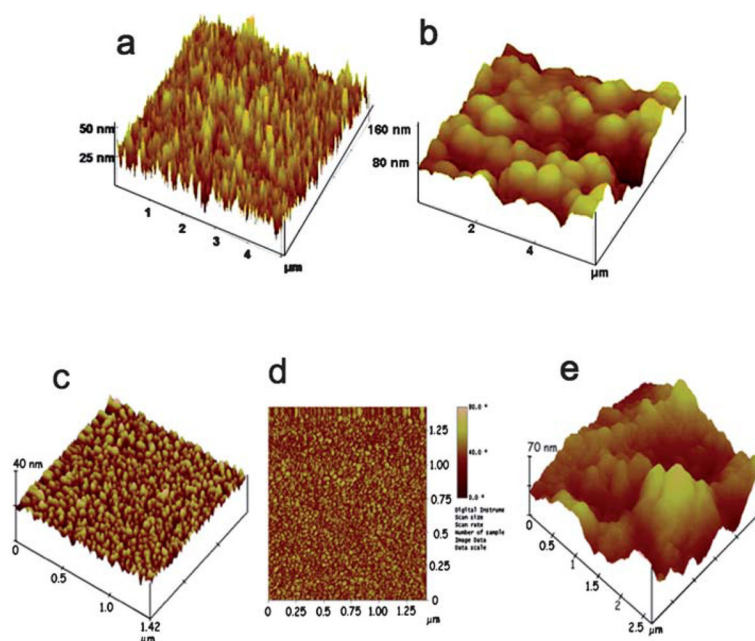
**Fig. 1.** Example of an immunoarray strategy designed to detect cancer biomarker proteins (PSA = prostate specific antigen). Gold nanoparticles providing high spot areas are linked to primary antibodies that capture the protein analytes. After washing, a labeled secondary antibody, or as illustrated here a multi-labeled nanoparticle with attached secondary antibody, is added. This species binds to another site on the captured analyte molecules. After additional washing with blocking agents to remove non-specific binding of the labeled species, electrical or optical detection is used to “count” the number of bound labels that is proportional to protein analyte concentration.



**Fig. 2.** Illustration of SPR arrays on gold films that can be used for measuring multiple protein biomarkers shown as a side view of an array that is coupled to a CCD camera for imaging. On the right is the top view of a  $5 \times 5$  array.

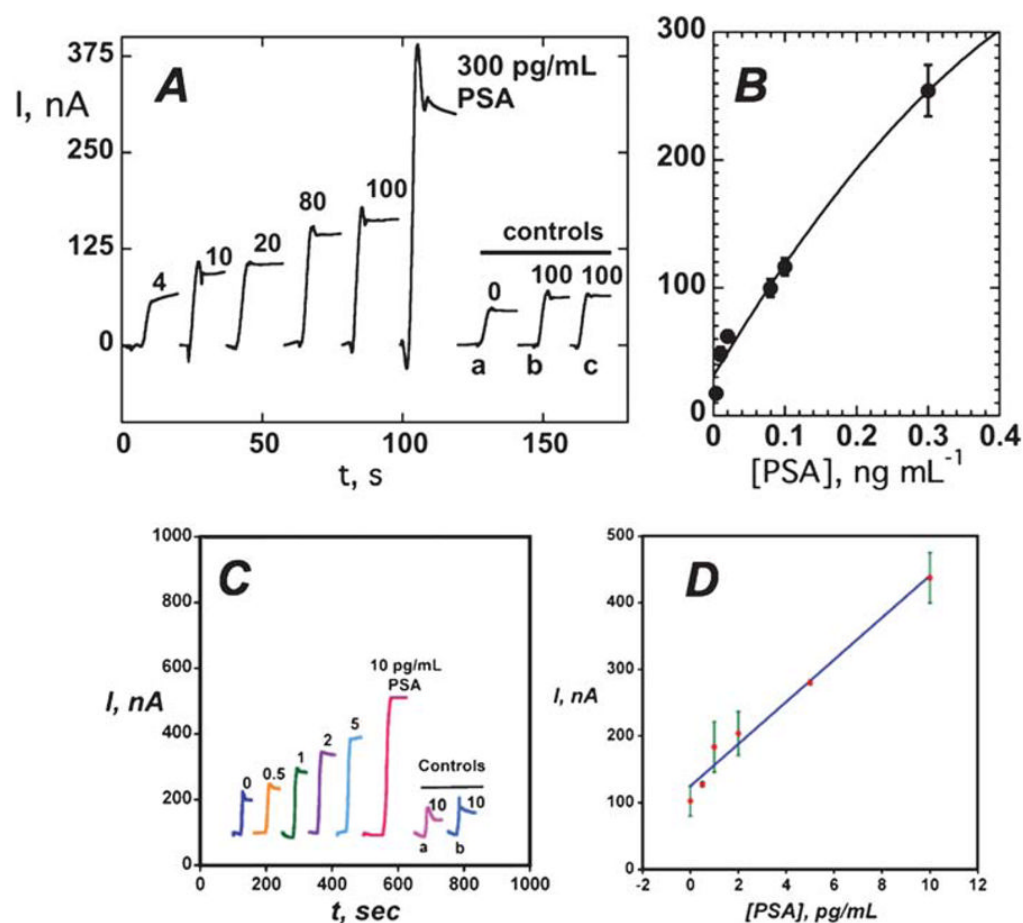


**Fig. 3.** Amplification strategies for electrochemical immunosensors using nanoparticles or other moieties attached to secondary antibody  $Ab_2$ .



**Fig. 4.**

Tapping mode atomic force microscopy (AFM) images of: (a) SWNT forest on smooth silicon and (b) anti-biotin antibody functionalized SWNT on smooth silicon. (c) PDDA/gold (5 nm) nanoparticle bilayer on a smooth mica surface suggesting a densely packed nanoparticle layer; (d) phase contrast image of gold nanoparticle electrode on mica, again suggestive of full coverage with the nanoparticles; and (e) gold nanoparticle electrode as in “c” after covalent linkage of the antibody anti-PSA onto the surface. Images a and b reproduced with permission from X. Yu, S. Kim, F. Papadimitrakopoulos, J. F. Rusling, *Mol. BioSyst.*, 2005, **1**, 70–78. Copyright Royal Society of Chemistry (UK), 2005. Images c, d, and e reproduced with permission from ref. <sup>111</sup>, Copyright American Chemical Society, 2009.



**Fig. 5.**

Amperometric response for nanostructured immunosensors incubated with PSA in 10  $\mu\text{L}$  serum in buffer containing 1 mM hydroquinone after injecting 0.04 mM  $\text{H}_2\text{O}_2$  to develop the signal (A) current for SWNT immunosensors at  $-0.3$  V and 3000 rpm using the  $\text{Ab}_2$ -CNT-HRP bioconjugate with 170 labels per 100 nm at PSA concentrations shown: controls: (a) full SWNT immunosensor omitting addition of PSA, (b) immunosensor built on bare PG surface for 100  $\text{pg mL}^{-1}$  PSA, (c) immunosensor built on Nafion-iron oxide-coated PG electrode for 100  $\text{pg mL}^{-1}$  PSA. (B) Influence of PSA concentration on steady state current for SWNT immunosensor using  $\text{Ab}_2$ -CNT-HRP bioconjugate. (C) Current at  $-0.3$  V and 3000 rpm for AuNP immunosensors using  $\text{Ab}_2$ -magnetic bead-HRP with 7500 labels/bead at PSA concentrations shown. Controls: (a) immunosensors built on bare PG at 10  $\text{pg mL}^{-1}$  PSA; (b) immunosensors built on PDDA coated PG surface at 10  $\text{pg mL}^{-1}$  PSA; (D) influence of PSA concentration on steady state current for AuNP immunosensor using multi-label  $\text{Ab}_2$ -magnetic bead-HRP. A and B reproduced with permission from ref. <sup>110</sup>, copyright American Chemical Society 2006. C and D reproduced with permission from ref. <sup>111</sup>, Copyright American Chemical Society, 2009.

**Table 1**

Biomarker detection by fluorescence methods

Array	Detection	Amplification	Assay type	Detection limits	Ref.
Microsphere	Fluorescence	RCA <sup>a</sup>	Sandwich immunoassay	10 fM IL-6, IL-8	57
DNA barcode	Fluorescence	PCR <sup>b</sup>	Sandwich immunoassay	500 aM PSA	58
Microspheres	Fluorescence	None	Sandwich immunoassay	0.3 pg mL <sup>-1</sup> , toxins	59
Microspheres (Luminex)	Flow cytometry	None	Sandwich immunoassays	Approaching 10 pg mL <sup>-1</sup> for multiplexed proteins	60
None	Fluorescence	None	Sandwich immunoassay	1 ng mL <sup>-1</sup> C-reactive protein	61
Au	Scattering	Au-deposition		1 fM PSA	62
Antibody array	Fluorescence	RCA <sup>a</sup>	Sandwich immunoassay	1–0.5 pg mL <sup>-1</sup> , 71 cytokines	63
Antibody array	Fluorescence	RCA <sup>a</sup>		10 fM various proteins	57
Antibody array	Fluorescence	None	Dye-labeled	100 pg mL <sup>-1</sup> , 115 proteins	46

<sup>a</sup>Rolling cycle amplification.

<sup>b</sup>Polymerase chain reaction.

**Table 2**

Selected examples of SPR-based detection of cancer biomarkers

<b>Biomarker</b>	<b>Amplification</b>	<b>Assay type</b>	<b>DL</b>	<b>Ref.</b>
VEGF	Precipitation	RNA–aptamer microarrays	45 pg mL <sup>-1</sup>	53
PSA	Au-NP	Ab <sub>1</sub> /PSA/Ab <sub>2</sub> –biotin/streptavidin–AuNP	1 ng mL <sup>-1</sup>	75
p53	None	SPR binding assay	0.2–0.5 ng mL <sup>-1</sup>	76
IL-6	None	Protein A/Ab <sub>1</sub> /IL6/IL6 receptor	3.75 ng mL <sup>-1</sup>	77
IL-8	None	Ab <sub>1</sub> /IL8/Ab <sub>2</sub>	<2 ng mL <sup>-1</sup>	78
PSA	DNA barcode	Ab <sub>1</sub> –NP/Ab <sub>2</sub> –magnetic microparticles	30 aM PSA	79