

Mini-reviews

Protein profiling of blood is going to lead to huge changes in the development of biomarkers and perhaps also in producing targets for the medical treatment of cancer. Experts in this field from Los Angeles have written an outstanding mini-review about this topic.

Renal cancer has been an area of interest to all of those involved in urological oncology, particularly from the point of view of adjuvant medical treatment either to prevent metastatic disease or to treat it. Authors from Los Angeles have produced a review of great interest on this subject.

There are three other reviews on important issues. Authors from Paris have written about microsatellite instability and TCC of the upper urinary tract, and authors from Bristol have written a mini-review about the options available when considering orthotopic bladder reconstruction. Finally, authors from Dublin have written about a significant problem in renal transplantation, delayed graft function.

A perspective on protein profiling of blood

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INTRODUCTION

The first decade of the 21st century will be remembered as the era in which biological sample profiling came of age (again), where the promise of taking a minimally invasive quantity of blood, urine, saliva or other easily obtainable specimen, and performing a rapid analysis to guide patient management decisions started to become a reality. Despite the current focus, the future of personalized healthcare goes beyond early diagnostics. Patient management decisions from advanced cancer treatment to choice of diet and vitamin supplements are only the surface of how healthcare will be improved.

In this review we examine the common working methods for profiling biological samples, concluding with a description of our current work and a description of the problems that we are currently attempting to address.

MASS SPECTROMETRY, PROTEINS AND PROFILING

Many diseases, including cancer, can be described as diseases of the regulation of protein function, and as such should be amenable to study by proteomic techniques. To date, these techniques have yielded

promising, yet limited, results. While molecular profiling based on mRNA levels has met with some success [1], it has been criticized because of the frequent disparity between mRNA and protein expression levels [2–4]. Two dimensional (2D) SDS-PAGE has been useful for analysing proteomic differences between, e.g. cancerous and healthy tissue, even before the use of mRNA profiling [5–7]. However, protein gels have been criticized for issues of reproducibility, sensitivity and protein resolution [8]. While issues of sensitivity and reproducibility have been addressed to some degree by sample pre-fractionation techniques [9,10] and mixing experiments with fluorescently labelled proteins [11], these advances come with an additional cost in money and processing time. Also, while the identification and quantification of proteins of interest from SDS-PAGE gels is possible, it is clearly a low-throughput method with only moderate sensitivity.

Mass spectral techniques, although criticized for sensitivity issues (especially compared to ELISA and RT-PCR) [12] have the strong advantage that they are useful in the analysis of components of a mixture without prior identification. We think that the rapid generation of these 'blind' profiles from serum, despite criticism, has great promise for diagnosis and biomarker development.

WHAT IS MASS SPECTROMETRY?

Mass spectrometry comprises a variety of techniques for determining the mass to

charge ratio (m/z) of gas-phase ions. The methods of producing gas-phase ions from analytes and their subsequent detection vary considerably, but only a few techniques are commonly used for protein/peptide profiling. The two common forms of ionization are: electrospray ionization (ESI), in which charged analytes within a liquid sample are converted to gas-phase ions through high voltage, heat and drying gases [13]; and matrix-assisted laser desorption ionization (MALDI), in which analytes are mixed with photoreactive small molecules (which form the matrix), dried on a metal surface and subsequently exposed to laser irradiation [14].

After the ions are formed, they are analysed to determine their m/z ; typically, ions formed by MALDI contain only a single charge, whereas ESI frequently produces multiply charged species, +2 to +4 for smaller peptides and often double-digit charges on whole proteins. Common detection techniques are slightly more varied: time-of-flight (TOF) accurately measures the time it takes for the ion to travel from the ion source to the detector plate (more massive objects take more time); quadrupole ion traps trap all generated ions and then selectively eject and measure ions of a particular m/z [15]; and, of increasing interest, Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometers introduce the ions into a strong magnetic field and measure the induced frequency of resonance, yielding very high resolution measurements of m/z [16]. Some of these methods are illustrated in Fig. 1.

WHY BLOOD?

There is a two-fold advantage in profiling blood: it is readily obtainable and, as it perfuses all tissues, it hypothetically represents a sampling of the state of the entire body. The organs of the body contribute to the proteomic profile in the blood through regulated secretion, unregulated 'leakage' and necrosis (very unregulated leakage). For profiling purposes, the cellular components are generally removed. This can be done immediately, in the presence of an anticoagulant, yielding plasma, or, in the absence of an anticoagulant, allowing the blood to clot, yielding serum. Much proteomic work to date has been with serum; presumably because of the historical use of serum in clinical diagnostics and because serum, by definition, is less complex than

plasma. However, recent studies make compelling arguments for the use of plasma. While many components of blood show remarkable stability over the period required to prepare serum, there are nevertheless some notably unstable compounds (e.g. adrenocorticotrophic hormone) [17–19]. Also, the peptide/protein complement of plasma tends to be more stable because of EDTA-mediated inhibition of proteases [18]. Not all differences between 'true' levels and levels after blood processing are due to degradation effects. In the preparation of serum, cells present in the blood have been reported, for example, to increase the vascular endothelial growth factor level by as much as 10-fold through an active secretion mechanism, compared to what is measured in plasma [20,21].

While it is common to remove the cellular components from blood, the cells themselves have been used as the targeting of profiling. For example, prognostic predictions have been made based on circulating tumour cells in breast and lung cancer [22,23].

WHAT IS THE PROTEOMIC CHALLENGE WITH BLOOD?

While there are arguably many problems associated with proteomic analysis of blood, the greatest problem is the huge span of protein concentrations. The abundance of identified species spans more than 10 orders of magnitude [24]. Fractionation methods are frequently applied to reduce this complexity before analysis; arguably, mass spectrometry is a separation method, separating total analyte abundance into discrete m/z abundance signals.

A commonly used separation is simple chromatography, either liquid or gel. In liquid chromatography (LC), the proteins are usually enzymatically proteolysed (trypsin being the most common choice) and then separated via reverse-phase HPLC. This process allows for much higher resolution mass spectrometry with a concomitant ability to resolve more species (for a recent review of these methods, see [25]). The gel-based methods typically involve separating whole proteins first by isoelectric focusing and then by PAGE. Typically, the gels are subjected to densitometry and proteins of interest cut from the gel and identified. Further progress has been made in sensitivity, reproducibility,

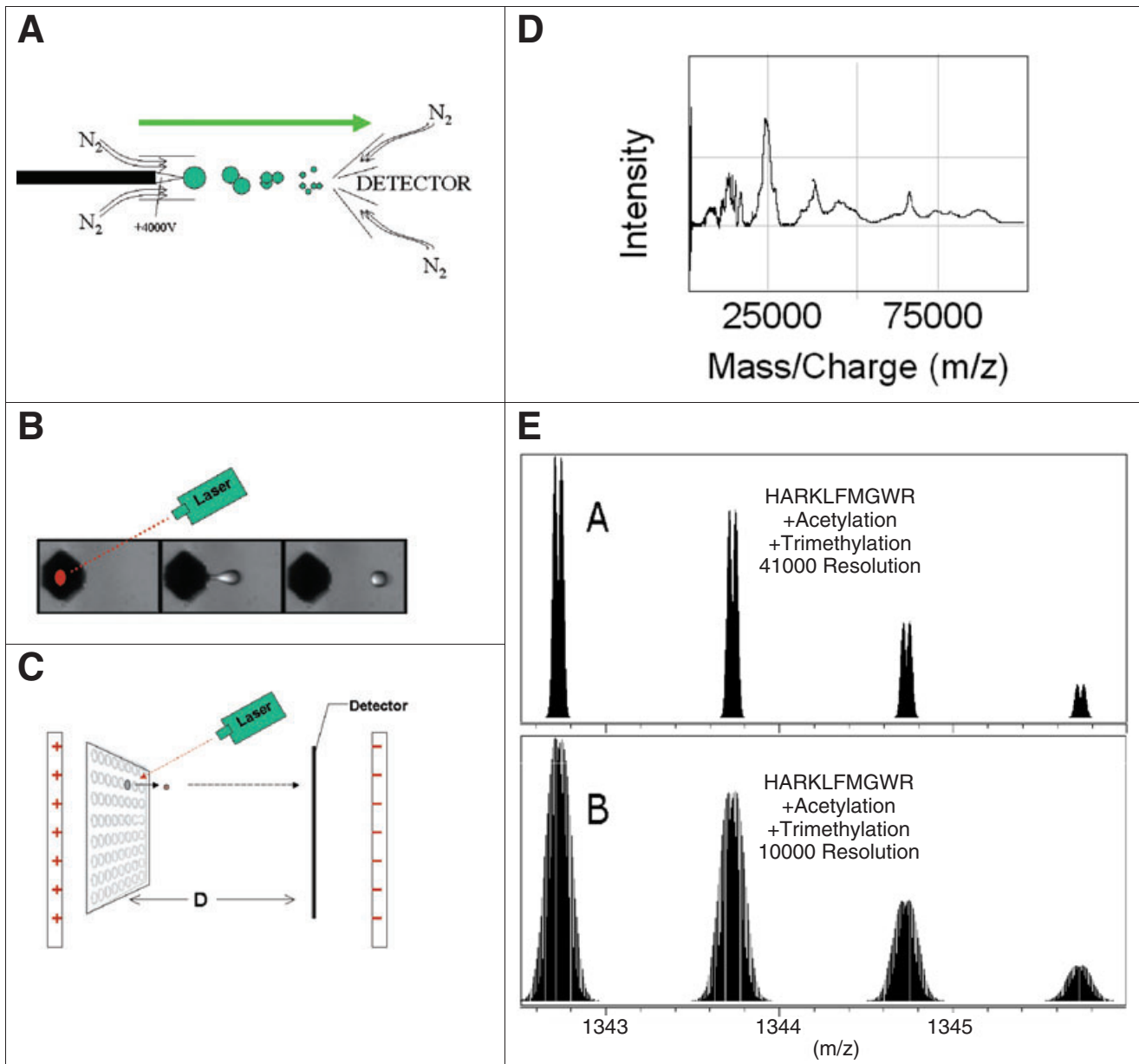
linearity and expense, with the introduction of the fluorescence based 2D-DIGE process from Amersham Int. [26,27].

Despite fundamental differences between these methods they share a common confounding factor, in that the dynamic range of the blood analytes is vastly larger than the dynamic range of the detection method. One approach to this problem is to remove large-abundance proteins. In a recent review, five commonly used depletion kits were evaluated [28]. All of these target the few most abundant proteins, such as albumin, transferrin and immunoglobulin, which can account for as much as 80% of the total protein content of serum [24]. The problem of the dynamic range of analytes can also be approached with selection, rather than filtration, methods. For example, phosphorylated species can be isolated based on their differential affinity to immobilized metal species and antiphospho antibodies [29,30]. Similar work was recently developed for isolating and analysing glycosylated species, common in secreted proteins [31,32].

APPROACHES TO PROFILING

The surface-enhanced laser desorption ionization (SELDI) approach has strong face validity; simplification followed by automated mass spectrometry profiling enables rapid sample preparation/analysis, making it very desirable to develop SELDI for analysing clinical samples. SELDI represents a modification to standard MALDI. With MALDI profiling, samples are mixed with a laser-reactive matrix solution and deposited on a metal MALDI plate. With SELDI, analytes are placed on one of a variety of SELDI chips which are coated with different active moieties. These moieties bind differentially to the analytes within a sample allowing unbound (or weakly bound) analytes to readily be washed from the chip before analysis. The use of these reactive surfaces is thought to obviate more time-consuming separation methods. Surfaces exist that mimic ion-exchange resins (both cationic and anionic), hydrocarbon chains (C18, C4), immobilized metal, and others. In addition to chemical interactions, protocols exist to bind antibodies to SELDI chips to perform rapid immunoprecipitation followed by MALDI analysis. In all cases, samples are incubated on the chip, washed with appropriate solutions

FIG. 1. Common mass spectrometry ionization and detection techniques. These are pictorial representations of the two common forms of ionization, one of the common forms of detection used in mass spectrometry for protein and peptide analysis, and sample data. Panel A depicts ESI; liquid flowing from the black tube becomes highly ionized by the applied voltage. The nitrogen sheath gas, combined with the repulsive-like charges within the large droplet, cause the droplet to both lose moisture and to break apart into smaller droplets. This process continues until (hopefully) only droplets containing a single analyte remain. The process is further facilitated by the countercurrent gas coming from the detector side of the ionization source. Only ions will be attracted into the lower voltage potential 'sample cone' which precedes the detector. Panel B depicts MALDI; laser irradiation causes co-ablation of the reactive matrix and the analyte with a concomitant ionization. Panel C depicts a MALDI TOF configuration. Produced ions are repelled toward a detector plate at some distance 'D'. The travel time is a linear function based on the m/z . Panel D shows an example of a SELDI profile. Panel E shows the effect of resolution on species discrimination; shown are two simulated measurements from the HARKLFMGWR peptide derived from a trypsinization of human histone H3. It is common to find a mixed population of this peptide containing both acetylations (+ 42.01056 Da) and trimethylations (+ 42.04695 Da). Panel E-A shows the theoretical spectrum of this mixed population at a resolution of 41 000 (as would be measured on a 7 Tesla FT-ICR mass spectrometry). Panel E-B is the spectrum at 10 000, as expected on a high-end MALDI-TOF instrument. A theoretical resolution of 35 000 is required to resolve these two species.



and the remaining bound analytes are analysed by MALDI-TOF.

The general SELDI analysis is as follows: a biological fluid, e.g. plasma, is replicate-spotted on one or more different types of affinity chips. The chips are washed with the appropriate solution to remove unbound analytes within the samples, overlaid with the laser-reactive matrix and subjected to mass spectrometry measurements. The data are then analysed for mass spectral features that differentiate the samples.

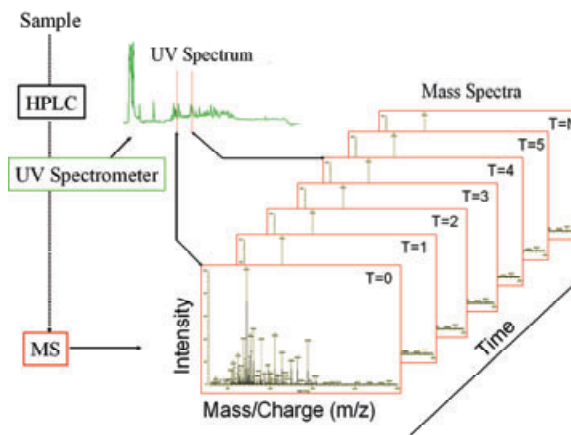
There have been many reported initial successes with this technology, including diagnostics for ovarian and prostate cancer [33,34]. Unfortunately, early promising approaches have met with subsequent strong criticism (reviewed in [35]). There is currently a multilaboratory study design in process to address these criticisms and demonstrate proof-of-concept for this technology [36]. While the study is far from complete, the current findings show that within- and between-laboratory variability is similar with properly operated instrumentation [37].

The comparison of SELDI separation to LC-based separation is similar to the comparison of dialysis to SDS-PAGE. SELDI separation provides a single, discrete, reading; everything that was retained by the chip is seen and that which was not retained is not.

Because of the complexity of blood and the limited resolution of MALDI, the selection tends to be very stringent, to reduce the complexity of the sample enough to yield informative spectra. LC, coupled in-line to a mass spectrometer (LC/MS), adds an additional dimension to profile data; a series of m/z vs intensity spectra over time (Fig. 2). LC adds a highly controllable level of separation to the profiling experiment. While not as practical for high-throughput profiling, some laboratories have used either single- or multiple-column chromatographic separations over tens of hours in an attempt to individually characterize all the species within a sample. Because of the nature of the chromatography, the elution time correlates with a physical property of the analytes (e.g. how hydrophobic a peptide is).

The common LC/MS method is as follows: a biological sample is proteolysed (typically with trypsin, yielding ≈ 10 -amino-acid

peptides) and injected into an inline LC/MS configuration. The peptides are eluted from the LC column into the mass spectrometer over typically 1–2 h. The mass spectrometer makes a series of measurements at 1–10/s. As with SELDI profiling, identifying the peptides/proteins is secondary to looking at the overall 'snapshot' of the original sample. The output is not a 'shopping list' of observed proteins, rather it is a three-dimensional topology of m/z , time and intensity values.



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Because of the time expense of multidimensional chromatography, another approach to sample separation is to use mass spectrometry with higher mass-resolving power; the higher the resolving power, the more similar two analytes can be and still be distinguished.

Figure 1E shows the advantage of high resolving power (FT-ICR vs MALDI) when comparing subtly different peptides. What is sufficient resolving power? Consider the following 'mass collisions': RVMRGMR and RSHRGHR differ by 0.00045 Da (an electron's mass is 0.00055) yet they have been resolved using special FT-ICR techniques [38]; however, LASADLAK will never be mass-resolved from IASADLAK as they have identical masses. The hope is that these nearly ' m/z identical' species will have different elution times as the species, if resolved in time, do not need to be

resolved in m/z . The higher the resolving power, the less dependence there is on two species of similar mass being time-resolved. The lower the mass-resolving power, the more the need for longer or higher dimensional LC runs.

THE NEXT STEPS

Despite advances in screening and awareness of prostate cancer, a significant proportion of patients will be first diagnosed with an advanced stage of the disease when there are fewer treatment options available and, because of disease progression, the number of different treatment regimens that can be tried is also limited. Even with early diagnosis, the balance of quality of life with unknown clinical outcome makes the choice between expectant management and early aggressive therapy unclear.

Patient variability greatly complicates patient management. Which patient will have an aggressive form of the disease rather than one with a long latency? Why do some patients respond well to a given therapy while others do not? These questions make the physician's, and the patient's, choices incredibly difficult. Simply put, better therapies are needed, and better methods of selecting patients for those therapies.

Existing outcome indicators for patients with prostate cancer, e.g. PSA levels, performance status, serum haemoglobin, body weight and other markers of either general health or cancer load, have been used to predict outcome [39–41], although such studies have not always agreed on the significant factors [42]. These studies, while of some use for patient counselling, do not provide information as to the underlying biological mechanisms of outcome and are not as useful for longer term (>1 year) predictions of outcome or therapeutic choice for advanced disease.

We have begun a programme to identify biomarkers of outcome by the computational analysis of LC/MS profiles. These techniques are not specific for prostate cancer and, indeed, the power of our techniques increases by including profiles from many different biological states from many disease models. The LC/MS profiles include proteins not only from the cancer, but also from the cancer stroma, host metabolic pathways and other host factors, yielding a more comprehensive picture of the disease studied.

In our system, proteolytic digests of serum are analysed by one-dimensional reverse-phase chromatography on-line with a Fourier-transform mass spectrometer to generate high-quality, informative protein profiles. As the electrospray signal intensities from the mass spectrometry depend on the analyte concentration [43], the resultant profiles are highly enhanced compared to the one-dimensional, low-resolution profiles obtained by SELDI and MALDI methods.

Our programme is currently in the phase of data analysis. In recent studies assessing SELDI/MALDI mass spectra and using feature recognition, researchers have found the use of neural networks [44] and decision trees [45] to be effective in addition to traditional multivariate analyses [46,47]. The three-dimensional nature of our profiles complicates direct application of these techniques to our datasets, as they first require additional techniques to normalize LC retention times over many different analyses.

We think that the higher resolution mass measurement in the LC/MS systems will reduce the need for sample simplification, thereby increasing sample throughput and ultimately reducing 'discovery' time. Fortunately, after discovery, specific

diagnostics can be used with targeted ELISAs or other similar less-expensive, high-throughput methods other than LC/MS. However, even without methods to reduce the costs, the savings produced by outcome studies such as these, even as a first-line diagnostic, are still dramatic, e.g. when used to determine therapeutic regimens and to determine inclusion criteria for clinical trials.

In the future, we see high-resolution profiling of biological samples transforming our approach to healthcare. The sequencing of the human genome, the dramatic development of computational and analytical equipment, and the rapid advances in bioinformatics, are achievements that are coming together in ways that will revolutionise our ability to diagnose and manage diseases, and general healthcare. Effective, personalized healthcare is within the grasp of this generation. Technological advances have allowed for the development of new and exciting methods to be applied to existing clinical trial datasets and hopefully to yield important information on patient clinical outcome. Coordination and standardization of the methods involved in these outcome studies will be critical to ensure the timely success and application of these technologies to managing patients with prostate cancer.

CONFLICT OF INTEREST

None declared.

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Abbreviations: 2DE, two-dimensional electrophoresis; SELDI, surface-enhanced laser desorption ionization; MALDI, matrix-assisted laser desorption ionization; LC, liquid chromatography; LC/MS, LC coupled in-line to a mass spectrometer; ESI, electrospray ionization; TOF, time-of-flight; m/z, mass to charge ratio; FT-ICR, Fourier transform-ion cyclotron resonance.