Molecular BioSystems

Dynamic Article Links

Cite this: *Mol. BioSyst.*, 2011, **7**, 1795–1801

www.rsc.org/molecularbiosystems

REVIEW

Secretome profiling with antibody microarrays

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Received 22nd February 2011, Accepted 30th March 2011
DOI: 10.1039/c1mb05071k

Following the advances in human genome sequencing, attention has shifted in part toward the elucidation of the encoded biological functions. Since proteins are the driving forces behind very many biological activities, large-scale examinations of their expression variations, their functional roles and regulation have moved to the central stage. A significant fraction of the human proteome consists of secreted proteins. Exploring this set of molecules offers unique opportunities for understanding molecular interactions between cells and fosters biomarker discovery that could advance the detection and monitoring of diseases. Antibody microarrays are among the relatively new proteomic methodologies that may advance the field significantly because of their relative simplicity, robust performance and high sensitivity down to single-molecule detection. In addition, several aspects such as variations in amount, structure and activity can be assayed at a time. Antibody microarrays are therefore likely to improve the analytical capabilities in proteomics and consequently permit the production of even more informative and reliable data. This review looks at recent applications of this novel platform technology in secretome analysis and reflects on the future.

In the past few decades, the field of molecular biology has witnessed yet another leap forward, which is associated with the deciphering of the human genome. Genomics established itself as a field of molecular biology concerned with the elucidation and analysis of the information contained in the cellular nucleic acids. Genomic techniques have been developed that permit whole genome sequencing of individuals,[1] the unravelling of epigenetic modifications and gene regulation processes[2] as well as the identification of transcriptional variations,[3] for example. Despite the remarkable progress in our understanding of the complex biological processes

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involved in disease pathogenesis at the level of nucleic acids, our insights into many other molecular levels remain incomplete and blurred. Already the surprisingly small number of (protein-encoding) genes found by genome sequencing indicated and emphasised the fact that regulation and activity occur at many molecular levels.[4] Genomic data are generally inadequate to predict dynamic protein properties, for example. Consequently, the scope of analysis was expanded beyond the merely genomic and transcriptomic approaches to address also events at the proteome level.[5–7]

The term proteome was coined in 1996 by Marc Wilkins and colleagues and is defined as the analysis of the complete set of proteins expressed in a cell or tissue.[8] Proteins are the molecules that execute many biological functions in a cell, and many regulatory processes take place at the protein level. The proportion and importance of protein modification is reflected by the fact that 5% to 10% of mammalian genes encode for proteins that modify other proteins. Proteins are involved in basically all vital biological processes in cells. Consequently, 98% of all therapeutic targets are proteins currently. Their obvious central role in understanding cellular activity at a molecular level promoted proteomics already early on as a second pillar of comprehensive molecular analyses.[9–11]

From proteomics to secretomics

As the proteome as a whole and the very many individual proteins continuously undergo dynamic changes, proteomics faces the challenge of detecting and analysing all these variations. For lack of processes for a really comprehensive investigation, studies are usually aiming at one major type of objective currently, such as functional[12] or structural aspects,[13] which deals with particular protein modifications, for example phosphoproteomics[14] or glycoproteomics,[15] or concentrates on a physiologically or biologically defined sub-proteome. One such sub-population is formed by the proteins that are secreted from cells into the extracellular medium. The term secretome refers to this class of proteins as released under defined conditions at a given time.[16] It is estimated that about 10% of all genes in the human genome encode for proteins of this class;[17] the *Secreted Protein Database* (http://spd.cbi.pku.edu.cn/) lists more than 18 000 entries of secreted proteins along with their sequences.[18] Secreted proteins are considered to be the main group of molecules for intracellular communication. They participate in most physiological processes, such as cell signalling, differentiation, invasion, metastasis, cell adhesion and binding, angiogenesis, and apoptosis. Common proteins in any secretome include cytokines, chemokines, hormones, immunoglobulins, neuroproteins, lipoproteins, growth factors and extracellular matrix degrading proteinases.[19–21] However, most secreted proteins are expressed during specific growth stages, by particular cell types or during specific cellular responses. Therefore, they could represent a reliable source of biomarkers in body fluids.[22,23]

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Current techniques used in secretome analysis

Currently, mass spectrometry (MS), either coupled to other preparative and analytical methods or on its own, is the main method in proteomic research, even achieving in few cases a data quality and reproducibility that is sufficient for use in a clinical setting. The classical approach is a combination of two-dimensional gel electrophoresis and MS. More recently, a modified version called differential in-gel electrophoresis (DIGE) has improved performance at the gel-based part.[24–26] Overall, however, analysis is moving away from gel-based systems,[27] with chromatography and MS taking over. Multidimensional protein identification technology (MudPIT) and isotope-coded affinity tags (ICATs) are two more recently developed methods in this field. There are also numerous MS-techniques and adaptations; matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS), surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF MS), and electrospray ionisation mass spectrometry (ESI–MS) are the most prominent forms.

Antibody microarrays

Antibody microarray analyses represent a methodology that is complementary to the MS techniques, adding quite a few features toward an overall comprehensive analysis.[23,28–33] As depicted in Fig. 1, the procedure is equivalent to the chip-based transcriptional profiling analyses. Antibodies (or other appropriate binder molecules) are arrayed on a solid support. The relevant protein mixture of interest is isolated, labelled with a fluorescence dye and applied to the array. As with transcript analyses, two samples labelled with two dyes can be applied at a time. Signal intensities obtained at the various binder molecules provide the basic information.

Antibody microarray analyses have the big advantage that different kinds of data can be obtained in a single assay. Aspects that can be studied include variations in the abundance of proteins,[23] the occurrence of structural differences in the form of protein isoforms[34] or protein modifications[35] and the definition of biochemical activities and regulative

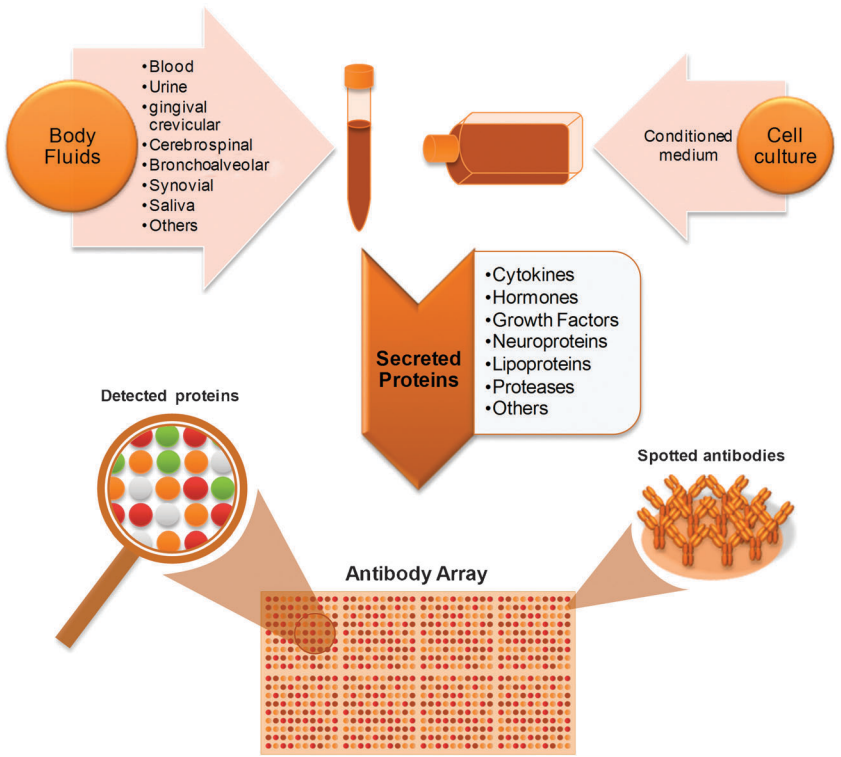

Fig. 1 Scheme of the protein profiling process with antibody microarrays. Proteins are isolated from the respective samples, mostly labelled with a fluorophor and applied to the array. If two different samples are applied that are labelled with different dyes, not only signal intensity but also the colour at the spots provides information about variations in protein abundance.

processes by virtue of detecting interaction partners, for example. Technically, assay processes have been established which permit analyses with a degree of robustness and reproducibility that meets the requirements of clinical applications.[23] Although in complex analyses a sandwich assay format is made impossible by the number of antibodies displayed on the array platform, specificities better than those achieved with ELISA assays are possible. Mass transport and kinetics were identified as factors that limited severely the speed and the sensitivity of analysis. To overcome this, appropriate processes were established that on standard detection devices permit sensitivities in the attomolar range without signal amplification[36,37] and allow to reach binding equilibrium and thus reproducible and quantifiable measurements within reasonable time periods. More recently, using appropriate hardware, sensitivities down to single-molecule detection were achieved.[38] Also, various auxiliary facets such as appropriate protocols for protein extraction have been established,[39] although this is a relatively minor aspect with regard to secretome analysis.

The main obstacle for thorough proteome studies is the availability of antibodies or other binders.[40] There are many different types of affinity reagents available today. However, currently still missing is a comprehensive set of binders of appropriate specificity and affinity that covers all human proteins. Efforts are ongoing, however, toward the provision of a global resource of well-characterised affinity reagents for the mapping of the human proteome (*e.g.*, *ProteomeBinders*, www.proteomebinders.org; *Affinomics*, www.affinomics.org; the *Clinical Proteomic Technologies Initiative*, proteomics.cancer.gov; and the *Antibody Factory*, www.antibody-factory.de). The *Human Proteome Atlas* activity (www.proteinatlas.org) is currently the most advanced initiative.[41] While availability of binders for all kinds of analysis purposes will take a long time still to be established, considering that there are probably more than a million of protein isoforms and modifications, a set of molecules for the detection of a "basic human proteome" of some 22 000 proteins (assuming one protein per human gene) will be available soon.

Applications of antibody microarrays in secretome analysis

There are numerous reports about antibody microarray analyses, ranging from small concept studies to actual proteome expression profiling efforts with several hundred antibodies, using a large variety of platforms of home-made or commercial design. Apart from many technically oriented reports, the objectives were manifold, including the identification of disease associated biomarkers,[42] cell phenotyping,[43] bacterial serotyping,[44] oncoproteomic analyses,[7] investigation of drug abuse,[45] or the definition of signatures of hereditary diseases.[46] The secretomes from many sources were studied, such as the protein content of plasma or serum, urine, cerebrospinal fluid, tears, saliva and of conditioned media of cultured cells. Investigations of human plasma samples represent the largest group of reports. They have been reviewed before,[47,48] however, and are not discussed herein. Instead, this review focuses on the profiling of cellular secretomes and body fluids other than serum or plasma.

Table 1 Applications of antibody microarrays in cellular secretome analyses

Cells (stimulus)	Antibody targets	Biomarkers	Ref.
Stem cells			
Rat bone marrow-derived mesenchymal progenitor cells	Cytokines	TIMP-1, MCP-1, VEGF-164, CINC-2	49
Human embryonic stem cell-derived mesenchymal stem cells HuES9.E1	Cytokines	IGFBP2, TIMP1 and TIMP2	50
Adipose tissue-derived stem cells	Cytokines	CXCL5	51
Epithelial cells			
Normal ovarian surface cells	Cytokines	LIF, IL-10 and IL-4	52
Human bronchial epithelial cell line BEAS-2B	Cytokines	MCP-1, IL-8, RANTES, ENA-78, GROα, VEGF, CXCL16, MMP-9	53
BEAS-2B (carbon nanotubes)	Cytokines	IL-6, IL-8 and MIF	54
Human prostate cells (IFN-γ, IL-1β and IL-2)	Cytokines	IL-6, IL-8, GROα, ENA-78, CXCL-16 and MCP-1	55
Retinal pigment cells	Angiogenesis	IL-6, IL-8, MCP-1, TIMP-1, TIMP-2, VEGF	56
Lens epithelial cells	Cytokines	TGF-β2, IL-4 and VEGF	57
Thymic epithelial cells	Cytokines	IL-6, IL-8, GRO, GRO-α and MCP-1	58
Other non-cancer cells			
Prostatic stromal cells	Cytokines	MCP-1	55
Trabecular meshwork cells	Cytokines	TGF-β2, IL-4 and VEGF	57
Periodontal ligament fibroblasts	Cytokines	IL-1, -6, -8, -10, MCP-1, -2, -3, GDNF, VEGF and IGFBP-2	58
Human bone marrow mononuclear cells (HBMC)	Cytokines	IL-6, IL-8, GROα, ENA-78 and CXCL-16	59
Human umbilical cord blood derived mononuclear cells (EGF, FGF, all-trans retinoic acid)	Cytokines	IL-1α, IL-1β, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-16, Ang, VEGF, BDNF, GDNF, NT-3, NT-4, PDGF-B, EGF, HGF, MCP-1, MCP-4, MIP-1β, MIP-3α, SDF-1, Etx-2, PARC, MIG and GRO	60
Human stromal cell line HS-5 (drug treatment with Ara-C, Dau, Dox and Vin)	Cytokines	CKβ, IL-12, IL-13, IGFBP-2, MCP-1, MCP-3, MCP-4, MDC, MIP-1β and MIP-1σ	61
Astrocytes and primary cortical neurons (Huntingtin protein)	Cytokines	CCL5 and RANTES	62
Monocyte derived macrophages U937, human gingival and pulp fibroblasts (triethylene glycol dimethacrylate)	Cytokines	MCP-1	63
Ganglion neuron SC-DRG (cryo-shock)	Cytokines	IL-1α, MIP-4, MIP-5, leptin, IL-15, ICAM-1, TNFRI and TNFRII	64
Human dermal fibroblasts, human dermal microvascular endothelial cells, WM1158 melanoma cell line	Cytokines	IL-8, MCP-1 and TIMP-2	65
Human umbilical vein endothelial cells	Cytokines	VEGF, IL-8 and amphiregulin	66
Annulus cells (prostaglandin E1)	Cytokines	IL-6 and EGFs	67
Human myeloma ILKM-3	Cytokines	IL-1α, IL-6, IL-8, RANTES, TNFα-receptor 1, VEGF and CTLA	68
Macrophage (bacterial material)	Cytokines	TNF-α, IL-6, IL-17, MCP-1, RANTES and IFN-γ	69
Carcinoma associated fibroblast	Cytokines	VEGF and IL-1α	70
Major pelvic ganglia, penile smooth muscle cells	Cytokines	CXCL5	51
Preantral mouse follicles (hMG and rFSH)	Cytokines	TECK, sTNFRI and SDF-1α	71
Dental pulp and odontoblasts (TGF-β1)	Cytokines	IL-1α, -1β, -2, -6, -7 and -12	72
Neurons (glucose/oxygen/serum deprivation)	Cytokines	TGF-β1, GDNF, NT-3 and leptin	73
Endothelial cells (Angiotensin-II)	Other selection	IP-10	74
Cancer cells			
Breast cancer cell lines MCF-7, MDA-MB231	Cytokines, angiogenesis	VEGF, IL-8, amphiregulin. MCP-1 and IL-6	66, 75
MCF-7 (leptin)	Cytokines	FGF9, TNFβ, MCSF, IGFBP-3 and TGF-b3	76
Non-small cell lung cancer cell lines A549, H1299	Cytokines	MCP-1, IL-8, RANTES, ENA-78, GROα, VEGF, C–X–C motif, CXCL16 and MMP-9	53
Myeloid leukaemia cell line K562	Cytokines	CKβ, IL-12, IL-13, IGFBP-2, MCP-1, MCP-3, MCP-4, MDC, MIP-1β and MIP-1σ	61
Epithelial ovarian carcinoma	Cytokines	LIF, IL-10 and IL-4	52
Prostate cancer cell lines PC-3, LNCaP, C4-2B	Cytokines	IL-1α, MIP-4, MIP-5, leptin, IL-15, ICAM-1, TNFRI, TNFRII, MCP-1, IL-6, IL-8, GROα, ENA-78 and CXCL-16	59, 64
Prostate cancer cell line (quercetin and kaempferol)	Cytokines	GM-CSF	77
Primary renal cell carcinoma A-498 cells (IL-4, TNF-α)	Cytokines	IL-4, TNF-α, IL-8, TIMP-1, GRO, IL-6, MCP-1, M-CSF, GDNF, HGF and RANTES	78
Thymic carcinoma cell line ThyL-6	Cytokines	IL-1α, IL-6, IL-8, RANTES, soluble TNFα-receptor 1, VEGF and CTLA	68
Oral squamous cell carcinoma cell lines Ca9.22, YD-38 (CCL7)	Cytokines	VEGF and IL-1α	70

Cellular secretomics

Most investigations on conditioned media from cultured cells used a sandwich-assay format for detection, which has the advantage of being able to deal with low protein concentrations, since no labelling is required, but has the disadvantage that the degree of multiplexing is very limited. Therefore, only few molecules can be studied at a time, rendering the whole approach of a highly parallel assay format less useful. In most studies, arrays from commercial sources with antibodies against cytokines were applied; only few studies aimed at other target molecules (Table 1). This restriction to a particular, although important class of proteins is strongly limiting the amount of information that could be gathered from the studies. In addition, the analyses done to date are individually motivated experiments; no concerted activity has yet been organised.

The cells used in the studies were of diverse origin (Table 1). Next to stem cells and stem cell-like cells, also a large body of other cell types has been utilised. Quite a few of them were epithelial cells. In addition, conditioned media from different cancer cell lines were subjected to an analysis on antibody microarrays as part of a molecular assessment of several types of cancer such as lung, breast, ovarian, renal, prostate, thymic and oral cancer as well as leukemia. Some of the cellular systems looked at were not cell lines but had been obtained directly from animals and human tissues.

Co-culturing cells with conditioned media is an interesting scheme for analysing the effects of secreted proteins on microenvironment and cell-to-cell communication. Furthermore, a wide range of secretomics expression analyses was performed after various types of stimulus or induction (Table 1). This ranged from cryotherapy effects and glucose/oxygen/serum deprivation to inductions with factors such as hormones, growth factors, cytokines, chemicals, proteins or bacterial extracts. In addition, secretome profiling was used to explore biological events like cell differentiation, prospective therapy, angiogenesis, neurotrophic action, infantile aphakic glaucoma, prostatic enlargement, inflammation, bone resorption and cytotoxicity.

Body fluid secretomics

As it is obvious that cellular secrets will be transferred to the body fluids, secretome profiling was also performed on various body fluids (Table 2). Unlike the platforms applied to analysing cellular secretomes, the array content used in these studies varied a lot in terms of the number of printed antibodies and the kind of proteins targeted by the antibodies. There have been only relatively few publications about such analyses to date, which is surprising given the fact that body fluids can be obtained easily and may provide in part an even non-invasive means of diagnostics. Maybe the relatively trivial fact that a high protein concentration is needed for a successful labelling has hampered progress. Since water itself interferes with the standard ester reactions that are frequently used for attaching directly a fluorescence label to the proteins,

Table 2 Applications of antibody microarrays in body fluid analyses

Body fluid	Disease/condition	Antibody targets	Biomarkers	Ref.
Urine	Pancreatic cancer	Other selection	TSN8, TBB5, TRI22, AKA12, TEP1, MLP3B and RBM3	23
	Systemic juvenile idiopathic arthritis	Other selection	TIMP1, IL-18, P-Selectin, MMP9 and L-Selectin.	79
	Prostate cancer	Cytokines	IL-18BP	80
	Chronic kidney disease	Other selection	MIG, IP-10, MIP-1delta, osteoprotegerin	81
		Cytokines	LIX, MCP-1, beta-NGF and TIMP-1	82
	Chronic kidney disease	Cytokines	MCP-1, RANTES, TIMP-1, TNF-alpha, VEGF, E-selectin, Fas, IL-2, MMP-2, TGFβ	83
Tears	Contact lenses	Other selection	Cystatin, secretoglobin, lysozyme and S100 A8	84
	Vernal kerato conjunctivitis	Stationary phase proteins	IL-4, IL-5, IL-8, IL-10, bFGF, HB-EGF, VEGF, HGF, MMP-1, MMP-2, MMP-3, MMP-9 and MMP-10	85
	Allergy	Stationary phase proteins	IL-8	86
	Giant papillary conjunctivitis	Other selection	Eotaxin, eotaxin-2, IL-4, IL-6, IL-6sR, IL-7, IL-11, MCP-1, MIP-1delta, MIG, TIMP-2 and M-CSF	87
	Allergy	Membrane-bound proteins	EGF, MCP-1, VEGF, IL-8, TIMP-1, -2, ANG, IP-10, GRO, ENA-78 and MIP-3α	88
Cerebrospinal fluid	Idiopathic intracranial hypertension	Cytokines	CCL2	89
	Spinal cord injury	Cytokines	IL-6, -8, MCP-1, NAP-2, ICAM-1, soluble Fas, TIMP-1 and MMP-2, -9	90
Synovial fluid	Rheumatoid arthritis and osteoarthritis	Chemokines	MDC, CTACK, ENA78, SDF1α, TECK, IP10, XCL1, MCP1, Eotaxin2, NAP2	91
	Arthritis	Other selection	Citrullinated fibrinogen	92
Bronchoalveolar lavage fluid	Hyperoxia	Cytokines	LIX, sTNF-R1, MIP-1γ, IL-6, MCP-1	93
Wound fluid	Chronic venous leg ulcers	Cytokines	IL-1α, β, MIP-1delta, IL-8, MIP-1α, Lcn-2, TLR-2 and TLR-4	94
Prostatic fluid	Prostate disease	Cytokines	HGF and IL18Bpa	95
Gingival crevicular fluid	Chronic periodontitis	Cytokines	TIMP-2, TNF-beta, GRO, IP-10, Ang, VEGF,IGFBP-3, OPG, EGF, GDNF, PARC,OSM, FGF-4, IL-16, LIGHT and PlGF	96
Saliva	Chronic obstructive pulmonary disease	Cytokines	IL-8, TIMP-1, EGF, MCP-1 and IP-10.	97

achieving a high protein concentration prior to labelling is crucial for success.

Conclusions and perspectives

The non-reductionist approach in assessing biological phenomena has opened a new era in the detection, management and monitoring of diseases. Deciphering the human genetic code triggered a chain reaction toward addressing at a similar level other molecule classes, in particular proteins and the proteome as a whole. The intimate involvement of proteins in basically all biological processes and their importance in regulating activities in cells and tissues made them already the prime target for drug administration. Also, there is a continuous increase of knowledge and subsequent utilisation with respect to protein-based molecular diagnostics. Secreted proteins represent a significant fraction of the proteome, are relatively easily accessible and are likely to be a promising source of biomarkers due to the numerous variations already observed between the protein profiles under normal and diseased conditions.

The technology, although new and still hampered by the relatively small number of available binder molecules, has demonstrated its potential for an increasing number of applications in secretome profiling. Particularly the ability to scale the degree of multiplicity not only with the number of analytes that should be studied but also with biologically relevant aspects, such as protein structure, amount and interaction, is a factor that is crucial for eventually successful application. At the same time, immunoassays are processes that are well known and established in many fields, including the most demanding pharmacological and clinical settings.

While there are enormous differences in the performance parameters of the reported systems, depending on the protocols and materials used, systems have been described that surpass standard assays such as ELISA by far in throughput, sensitivity, selectivity and cost and are clearly of a quality sufficient for applications. In these cases, the actual sensitivity, specificity and selectivity depend on the binder molecules only. The major challenge left for really comprehensive analyses is therefore getting more and better antibodies or other binders in sufficient numbers.

Acknowledgements

The work of the authors was or is financially supported by the European Union as part of the *DropTop*, *ProteomeBinders*, *AffinityProteome* and *Affinomics* projects. S.A.M. and M.S.S.A. are gratefully acknowledging the support by long-term fellowships of the Deutscher Akademischer Austausch Dienst (DAAD).

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