Clin. Lab. 2008;54:67-79

REVIEW

Plasma-Derived Microparticles for Biomarker Discovery

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SUMMARY

The field of mass spectrometry-based proteomics has been transformed over the last decade due to advances in technology, sample preparation, bioinformatics, and computational tools. While this has led to a dramatic increase in research related to biomarker discovery, the promise of finding a significant number of new biomarkers has not yet materialized. Current proteomic technology is able to detect and analyze extremely small amounts of proteins (picomole to attomole level), but has difficulty detecting and quantifying proteins present at 2- to 3-orders of magnitude lower than the more abundant proteins. This is referred to as the dynamic range problem. Normal biological fluids used for biomarker discovery, such as plasma or urine, contain a small number of proteins present at much higher amounts than the remaining proteins. For example, in the plasma, albumin and immunoglobulins are present at milligrams per milliliter, while proteins of interest for biomarker discovery may be present at micrograms to picograms per ml. This has led us to investigate the microparticle subproteome which has a high likelihood of containing potential biomarkers. While this subproteome makes up less than 0.01% of the total plasma proteome, it is rich in proteins altered under a variety of pathological conditions.
(Clin. Lab. 2008;54:67-79)

KEY WORDS

Microparticles, exosomes, biomarker discovery, ectosomes, proteomics, apoptosis

Abbreviations:

ESI	-Electrospray ionization
ETD	-Electron-transfer dissociation
FT-ICR	-Fourier transform ion cyclotron resonance
ICAT	-Isotope coded affinity tag
ITRAQ	-Isobaric tag for relative and absolute quantitation
LC-MS/MS	-Liquid chromatography-tandem mass spectrometry
MALDI	-Matrix-assisted laser desorption/ionization
MS	-Mass spectrometry
MP	-Microparticles m/z mass-to-charge ratio
PAGE	-Polyacrylamide gel electrophoresis
SILAC	-Stable isotope labeling by amino acids in cell culture
TOF	-Time-of-flight
VWF	-Von Willebrand factor

INTRODUCTION

Current State of Mass Spectrometry (MS)-Based proteomics

A large number of technological advances have revolutionized the field of MS-based proteomics over the last twenty years. MS determines the mass-to-charge (m/z) ratio for gaseous ions. In the late 1980's, two methods were developed that generate gaseous ions from proteins and peptides without causing significant degradation of these molecules. These ion sources, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), had such an impact in the field of proteomics that their inventors shared the Nobel Prize in Chemistry in 2002 (1). In MALDI, the protein

Manuscript accepted April 21, 2008

or peptide is embedded in a matrix material which protects the protein from being destroyed and aids in its vaporization when excited with a laser. In ESI, the proteins or peptides in an aqueous acidic buffer are sprayed directly into an electrical field so that charged droplets are formed. As the solvent gradually evaporates from these droplets, the ionized protein molecules remains as a gaseous ion.

Additional advances concern sample preparation, bioinformatics and computational tools, and instrumentation. Advances in sample preparation techniques include methods to better separate proteins and peptides, often by coupling older techniques such as cell fractionation to MS-based proteomics. In particular, the development and commercialization of affinity columns which remove the 6 to 10 most abundant proteins has greatly improved our ability to examine the proteins present in plasma (2). The advances of bioinformatics tools include the development of algorithms which automatically interpret the spectra (3, 4) aided by the sequencing of the genomes for many species including humans, mice and rats. These approaches enable the reliable detection of hundreds of proteins in a given MS analysis in days, which would otherwise have taken months. In addition, the improvements and increased availability of MS instrumentation cannot be overstated. MS instruments today are much more robust, have better sensitivity, higher resolution, and quicker analysis time than those of just several years ago.

Improvements in resolution came with the advent and commercialization of a variety of different instruments including the quadropole TOF (5), Fourier Transform Ion Cyclotron Resonance (FT-ICR) (6), and the Orbitrap (7) Mass Spectrometers. The commercialization of ETD (Electron Transfer Dissociation) facilitates the examination of longer peptides and posttranslational modifications much more easily (8). Advances in all these areas are continuing at a robust rate, and while no single invention will likely have the impact of MALDI and ESI, their cumulative effects will lead to a growth in proteomics that may rival the advances which occurred in genomics over the last twenty years.

Difficulties in Biomarker Discovery – Relative Quantitation.

Currently, there are two major difficulties in MS-based proteomics. The first is in obtaining quantitative results for complex mixtures.

While it is relatively easy to identify hundreds of proteins in a given sample, it is much more difficult to determine the amount present. Even when comparing two similar samples, it is difficult to estimate the relative amount of a given protein. Most approaches being developed to generate relative quantitation of complex protein mixtures can be classified as either unlabelled or labelled. For unlabeled approaches, the classic and still widely used method to determine differences is simply to compare the relative signal (ion intensities) for peptides of a given protein (9). This has been used for decades to accurately estimate the levels of small molecules in complex matrices, such as blood.

However, with small molecules, one uses an isotopically labeled standard. This is more difficult with protein mixtures where one wants to determine the relative amount of many proteins in the same sample. While, in theory, this should provide a good estimate of relative abundance, this approach has been used with limited success and only for the more abundant proteins in a complex mixture. Whether this is due to a lack of reproducibility in the chromatography, problems with the data processing, suppression of signals, instability of the ESI, the sampling algorithm used in data-dependent searching, or other factors is unclear. Another approach is based on spectral counting. In this method, the number of times a peptide or protein is selected for MS/MS analysis or the score from that analysis is used as a relative estimate of abundance (10). For example, in our recent study, we detected differences in 22 proteins by this method (9). These are very crude estimates of relative abundance and statistical methods to compare peptide abundance based on spectral counts are not available. The unlabeled approaches can provide qualitative differences between two samples, but are less likely to detect even 2-fold changes in a given protein. However, advantages inherent in unlabeled approaches include excellent sensitivity and the ability to compare more than two samples directly. In the labeled approaches, the peptides or proteins from two complex mixtures are differentially labelled with a tag containing either a heavy or light isotope. These include commercially available tags such as ICAT (11), iTRAQ^{TM} (12), using heavy/light water ($H_2{}^{18}O/H_2{}^{16}O$) during protein digests (13), or using stable isotope la-belling by amino acids in cell culture (SILAC) (14). In the past, we have compared two relatively complex protein mixtures using an Isotope Coded Affinity Tag (ICAT) (Figure1) (9). This tag contains a cleavable linker molecule attached to biotin. In one sample, all the cysteines are labeled with a "light" isotope tag and in a second sample with a "heavy" tag. The samples were mixed, delipidated, and then digested with trypsin to generate peptides. The peptides were extracted, and enriched using an avidin column which selectively enriches for the tagged peptides. The biotin section of the ICAT reagent is cleaved off and the resulting peptides are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). All proteins which are not enriched had similar ion intensities for the light and heavy labeled peptides. Differentially labelled peptides had different ion intensities. Other isotopic labeling techniques have their own strengths and weaknesses. For example, eight different labels for iTRAQ will soon be commercially available. They label all peptides, not just the ones containing cysteine. These tags are isobaric, meaning that the labeled peptides will only generate one peak during MS. When the peptides are fragmented, the relative abundance of the peptides from the various samples can be quantified from their fragmentation spectra. Since all

peptides are labeled, posttranslationally modified peptides can be examined. ITRAQ may be less useful for examining a complex mixture because ICAT only looks at a small number of peptides from a given protein.
Another disadvantage of iTRAQ is that differences in any processing prior to labeling can result in false positives. With ICAT, proteins are labeled prior to most processing steps and then mixed. This eliminates false differences due to variability in processing. Other factors, such as the type of mass spectrometer being used to analyze the sample, source of the sample, resources, and expertise of the scientist(s) may dictate which labeling technique is the most appropriate for a given study. While labeled methods can produce more quantitative results than the unlabeled approaches, they have drawbacks including the inability to compare more than 2 samples (8 for iTRAQ), poor or uneven labeling producing misleading results, and increased expense. In our own studies, we detected differences in the presence of many more proteins using the spectral count (unlabeled) approach than with ICAT labelling.

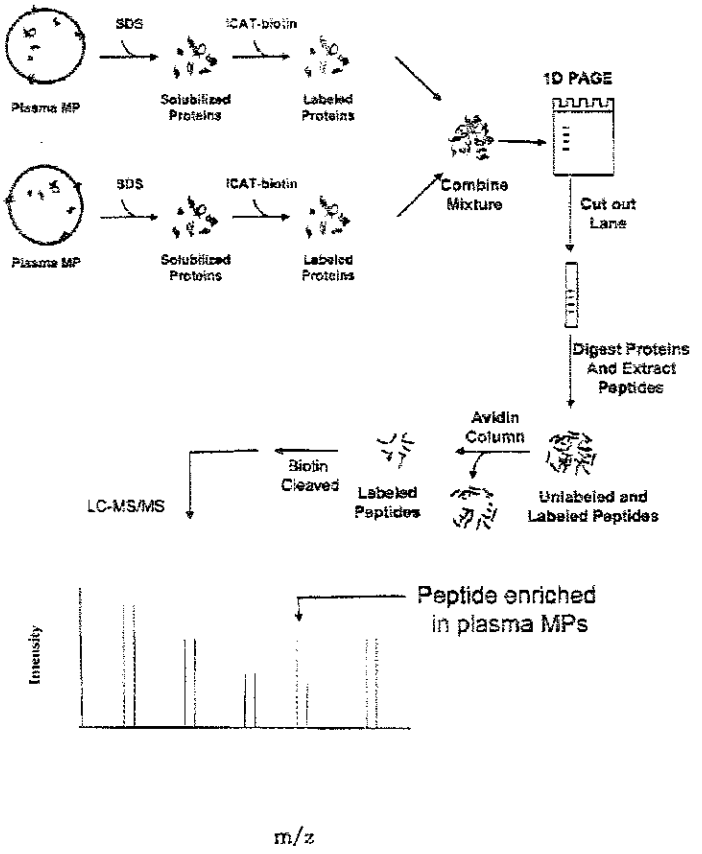

Figure 1: Strategy for relative quantitation of plasma MPs and platelet MPs using ICAT. The relative abundance of proteins in two samples is compared using a labeled approach. The two MP proteomes are solubilized using SDS and the cysteine residues are labeled with either a "heavy" (blue) or "light"(red) ICAT-label which differ in mass by 9 daltons. Both ICAT labels contain a cleavable biotin arm. The samples are mixed, delipidated and SDS is removed by 1D PAGE. The proteins are digested with trypsin to generate peptides which are extracted from the gel and labelled peptides are separated from unlabeled peptides using an avidin column. The biotin arm is cleaved and the sample is analyzed by LC-MS/MS. If the relative protein abundance is similar in both samples, the relative ion intensities of the two peaks should be identical. If not, the ratios will differ.

The Dynamic Range Problem in Mass Spectrometry

The second major obstacle with MS-based proteomics is related to the poor dynamic range of MS based instrumentation. It is very difficult to detect proteins that are present at concentrations several orders of magnitude lower than the more abundant proteins. For example, we have performed a simple analysis of human plasma which is believed to contain over 50,000 different gene products and up to an estimated half a million distinct proteins due to the generation of alternatively spliced products, differences in post-translational modifications, and cleavage products (15). Blood was collected from a healthy individual in the presence of an anticoagulant. Plasma was isolated following centrifugation, and the plasma proteins were digested with trypsin to produce peptides. A simple 3 hr LC gradient was used to separate the peptides, coupled to an ESI source of an ion trap tandem MS. The instrument was set to perform one MS scan followed by 10 MS/MS scans of the 10 peaks with the highest intensity in a "data-dependent" analysis. To avoid continuously selecting the same peptides, once a particular m/z was selected for MS/MS analysis, that particular m/z was not allowed to be selected for one minute. We detected 1485 MS/MS spectra which matched to various human proteins. Of these, 305 matched to 55 unique peptides from albumin. Two or more peptides for a given protein were only detected for 66 proteins (Table 1), a minimal standard used in many proteomic experiments to establish the presence of a given protein. The more abundant proteins in plasma are present in the range of mg/ml, which include albumin (4 mg/ml), immunoglobulins (3 mg/ml) and fibrinogen (2.5 mg/ml). This is in contrast to many others present in micro-, nano-, or even picograms per milliliter (Figure 2). As of 2002, MS had only identified 287 plasma proteins (15). Within 2 years around 1175 had been identified (16). More recently, 12,300 peptides from 4567 unique gene products have been identified in a single sample of mouse plasma in one study (17). While these recent results are impressive, they only begin to scratch the surface of the plasma proteome. In addition, although current MS-based proteomics can identify proteins in a complex mixture, relative quantitation is extremely difficult for all but the most abundant proteins.

Analytical Fractionation

One of the ways scientists have been able to identify more proteins is by prefractionating the sample using analytical approaches. The basic scheme performed in many MS-based discovery laboratories is depicted in Figure 3. First, the plasma is depleted of its 6 to 10 most abundant proteins using a commercially available affinity column (2). While depletion kits are very effective, small differences in their efficiencies can lead to misleading results and they generally only increase the dynamic range by about one order of magnitude. In addition, a large number of peptides and smaller proteins may bind to the columns either directly or indirectly (2). Next, either the proteins are fractionated and then digested or the proteins are digested and then the peptides fractionated. The separation techniques being utilized include gel filtration, ion exchange chromatogramphy, isoelectric focusing, and capillary electrophoresis. Next, all the fractions are analyzed by LC-MS/MS. In theory, this approach should identify many differentially expressed proteins. However, there are two major problems. The first is that each fractionation step results in many more samples to analyze by LC-MS/MS. For example, if the peptide mixture is separated into 10 fractions, each one of these has to be analyzed. Using the standard tandem MS instrumentation today, MS-based proteomics is a relatively low throughput operation, and increasing the number of samples 10-fold will decrease throughput proportionally, but will increase the dynamic range by only one order of magnitude. To identify proteins in the ng/ml and µg/ml ranges is essentially impossible. The second problem with this model is that all fractionation steps must be performed in a very reproducible manner. If a given protein (or peptide) is in a given fraction one time and the next fraction the next, it may lead to many false positives. Therefore, extreme care must be taken with all fractionation steps.

Instead of using these analytical approaches with all their inherent problems, some investigators have taken a different approach to this problem. Instead of trying to analyze all proteins in the proteome of plasma (or other easily accessible sample), they have tried to select a subproteome within the sample which has a high likelyhood of containing biomarkers. We have developed and used one such approach.

Table 1: Sixty-six proteins detected by two or more peptides from an analysis of plasma digest using LC-MS/MS. Spectra have not been individually inspected and a number of proteins may be erroneous or duplicates. Accession numbers are from NCBI.

Accession	Protein	unique peptides	spectral counts
4502027	Albumin	55	305
4557385	complement component 3	49	99
4557225	alpha-2-macroglobulin	26	47
4557871	Transferrin	25	62
21361198	serine (or cysteine) proteinase inhibitor	18	43
4502501	complement component 4B	17	17
4557321	apolipoprotein A-I	16	25
11761629	fibrinogen, alpha chain	14	20

4502153	apolipoprotein B	13	13
11761631	fibrinogen, beta chain	11	21
4557485	ceruloplasmin (ferroxidase)	10	15
32483410	vitamin D-binding protein	10	10
45580723	haptoglobin-related protein	9	16
11761633	fibrinogen, gamma chain	9	12
4504783	inter-alpha (globulin) inhibitor H2	9	9
11321561	hemopexin	8	13
4557327	beta-2-glycoprotein I	8	8
4826762	haptoglobin	7	9
4502261	serine (or cysteine) proteinase inhibitor	7	7
4557379	complement component 3	6	7
4502149	apolipoprotein A-II	5	11
31542984	inter-alpha (globulin) inhibitor H4	5	7
4504781	inter-alpha (globulin) inhibitor H1	5	7
38044288	Gelsolin	5	5
4502005	alpha-2-HS-glycoprotein	5	5
4502151	apolipoprotein A-IV	5	5
4502397	complement factor B	5	5
4502503	complement component 4 binding protein, alpha	5	5
4504893	kininogen 1	5	5
4505881	Plasminogen	5	5
4507725	Transthyretin	5	5
4506355	pregnancy-zone protein	4	9
4504489	histidine-rich glycoprotein	4	8
4505529	orosomucoid 2	4	5
9257232	orosomucoid 1	4	5
21071030	alpha 1B-glycoprotein	4	4
4501843	alpha-1-antichymotrypsin	4	4
4504375	complement factor H	4	4
4504579	I factor (complement)	4	4
11321587	complement factor H-related 1	3	3
5803139	RBP4 gene product	2	4
51458237	RIKEN cDNA A930039G15 gene product	2	3
10947052	ankyrin 2	2	2
13540612	hypothetical protein DKFZp434F0318	2	2
13699811	WHSC1L1 protein	2	2
19747267	titin	2	2
19923106	paraoxonase 1	2	2
22538387	A-kinase anchor protein 9	2	2
23097308	nesprin 1	2	2
29568109	dedicator of cytokinesis 4	2	2
29725607	smooth muscle cell associated protein-1	2	2
30794488	kinesin family member 27	2	2
39725934	serine (or cysteine) proteinase inhibitor	2	2
41281453	serine/threonine kinase 2	2	2
41393602	complement component 1	2	2
4501987	afamin	2	2
4502067	alpha-1-microglobulin	2	2
4502511	complement component 9	2	2
4503629	coagulation factor XII	2	2
4503635	coagulation factor II	2	2
4507831	unc-51-like kinase 1	2	2
4557287	angiotensinogen	2	2
4557323	apolipoprotein C-III	2	2
4557325	apolipoprotein E	2	2
51493205	chromosome 14 open reading frame 78	2	2
9506655	hypothetical protein FLJ20054	2	2

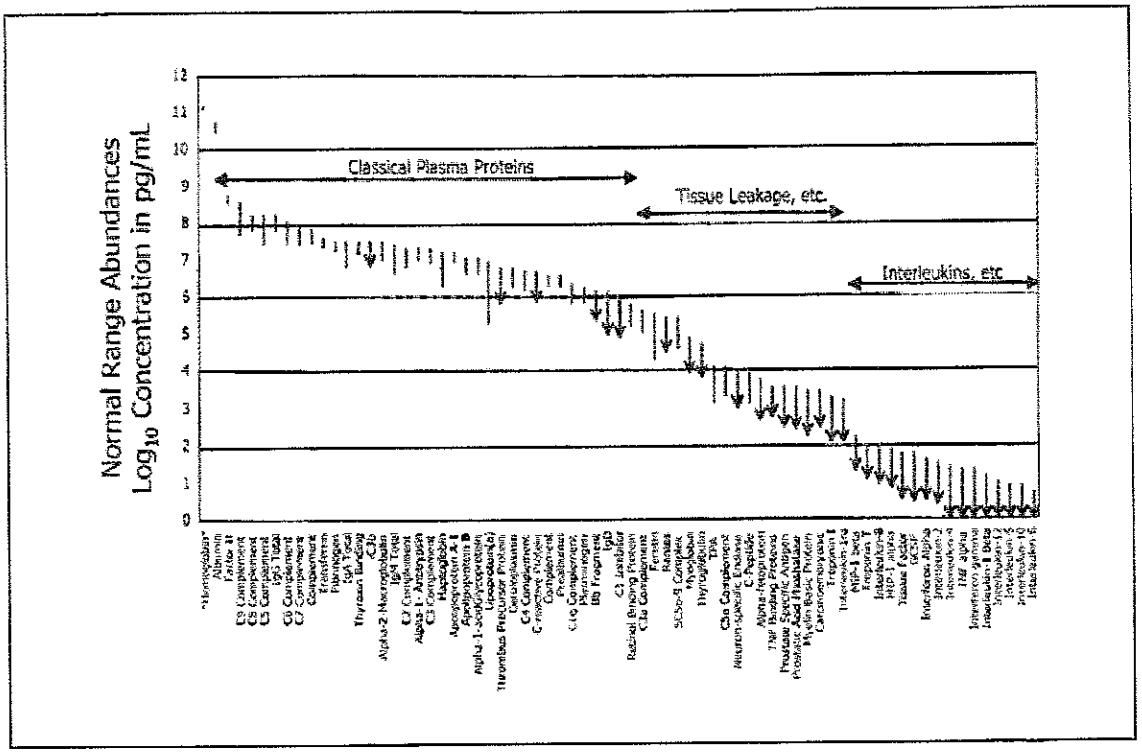

Figure 2: Dynamic Range of Plasma. Relative abundance of approximately 70 proteins in the plasma. More abundant proteins, such as albumin and immunoglobulins are present at milligram per milliliter levels. Others are present at many orders of magnitude lower concentrations. From Anderson NL. 2002; Mol. Cell. Proteomics 1: 845-867.

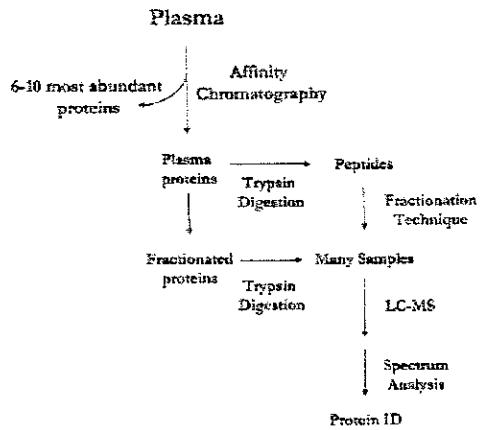

Figure 3. Currently used scheme to examine the plasma proteome more extensively. Many biomarker discovery efforts to examine plasma first remove the six to ten most highly abundant proteins by affinity chromatography. Then, either the proteins are digested to generate peptides and the peptides are separated or the proteins are separated and then digested. All samples are typically analyzed by LC-MS/MS.

Microparticles

Microparticles (MPs) are small subcellular membranous vesicles released by essentially all cell types, especially when activated or under stress. They include ectosomes, generated from the ectocytosis (or blebbing) of the plasma membrane and exosomes, released by fusion of intracellular multivesicular endosomes with the cell surface (Figure 4) (18-19). Ectosomes have a wide range of sizes even when they are from the same cell type and can be up to 1.5 µm in diameter while exosomes are uniform in size and range from 50 to 90 nm depending on cellular origin. In plasma, MPs were discovered as a component of the blood which promote coagulation due to the presence of anionic phospholipids on their outer surface (19). These anionic phospholipids, later determined to be mostly phosphatidylserine, are now widely used to detect MPs from blood samples using flow cytometry, based on their affinity for fluorescently labeled annexin V. The cellular source of the microparticles is determined by cell-specific markers detected by flow cytometry (20).

For example, MPs with CD41 (glycoprotein IIb) expression are believed to be generated from platelets. Using this method, microparticles from erythrocytes, endothelial cells, neutrophils, lymphocytes and even smooth muscle cells have been detected in the plasma. However, in healthy individuals, over 90% of plasma microparticles originate from platelets (20).

The number of microparticles and their cellular origin appears to be a very sensitive marker of vascular health. Under a whole host of different pathological conditions the microparticle number and composition is altered (Table 2). Conditions include sepsis (21), diabetes (22), and sickle cell disease (23, 24). The proteins present in microparticles are dependent on the agonist used to activate the cells (25, 26). We therefore hypothesized that microparticles may contain a very useful plasma subproteome for biomarker discovery.

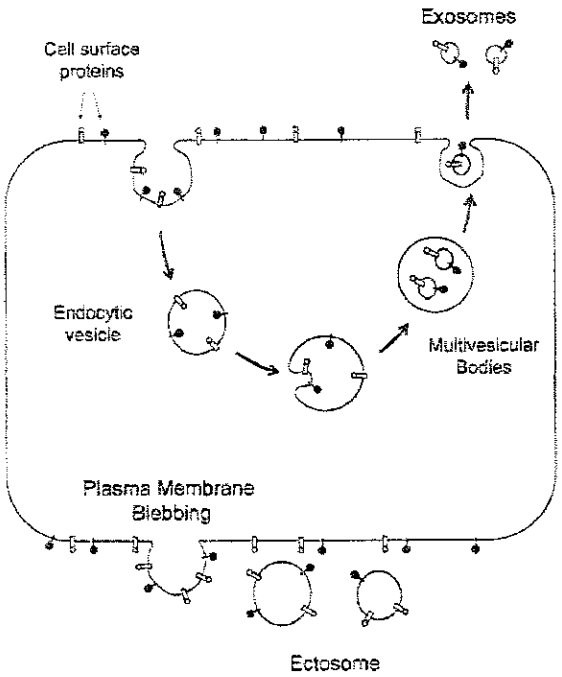

Figure 4: Pathways for Microparticle production from cells. The two major sources of plasma microparticles are by ectocytosis and exocytosis. Top: Exosome originate from endocytic vesicles which are created from invagination of the cell membrane to form a pocket. This then pinches off into the cell to form an endocytic vesicle. Sections of the membrane pinch off to form exosomes inside the vesicle which are then released from the cell. Generally exosomes are uniform in size between 50 and 90 nm. Bottom: Ectosomes are created directly from the outward blebbing of the plasma membrane. Their size varies up to 1.5 µm in diameter even from the same cellular source.

Table 2: Pathological Conditions Associated with changes in the number and/or cellular source of MPs. All entries refer to changes in plasma MPs except when noted.

Disease/Condition	Cellular Origin of MPs	Reference
Vascular and Cardiovascular Related		
Coronary Artery Disease or ACS	Platelet, Endothelial	(36-39)
Myocardial Infarction	Platelet	(37)
Peripheral Arterial Disease	Platelet	(37)
Acute and Chronic Vasculitis	Neutrophil	(40)
Venous Thromboembolism	Endothelial	(41)
Hypertension	Endothelial, Platelet	(42)
Acute Ischemic Stroke	Endothelial	(43)
Cisplatin-induced stroke	Endothelial and Platelet	(44)
Atherosclerotic (in plaques)	Leukocytes	(45)
Raynaud's Phenomenon	Platelets	(20)
Nonvalvular Atrial Fibrillation	Platelet	(46)
Deterioration of Artery Elasticity	Endothelial	(47)
Stent-induced Vascular Inflammation	Platelet	(25)
Atherothrombotic Events	Platelet	(48)
Aortic Prosthetic Valve Surgery	Platelet	(49)
Transient Ischemic Attacks	Platelets	(50)
Lacunar Infarcts	Platelets	(50)
Multiinfarct Dementias	Platelets	(50)
Metabolic Disorders		
Type 1 Diabetes	Endothelial, Platelet	(51)
Type II diabetes	Endothelial	(51-53)
Diabetic Retinopathy	Platelet, monocytes	(54, 55)
Metabolic Syndrome	Endothelial	(56)
Fabry Disorders	Endothelial	(57)
Infection Related		
Ebola Hemorrhagic Fever (macaques)	Tissue Factor positive	(58)
Meningococcal Sepsis	Platelets or Granulocytes	(21)
Infectious Colitis	Total	(59)
SIRS	Endothelial	(60)
Sepsis	Endothelial, Platelet	(61)
Blood Diseases		
Sickle Cell Disease	Endothelial, Monocytes, Platelets	(23, 24)
Thalassaemia	Platelet	(62)
Obesity	Total	(63)
Systemic Lupus Erythematosus	Platelet	(64)
Thrombotic Thrombocytopenic Purpura	Endothelial	(65-67)
Paroxysmal Nocturnal Haemoglobinuria	Endothelial	(68)
Inflammatory Disease		
Rheumatoid Arthritis	Platelet	(69)
Ulcerative Colitis	Platelet	(70)
Crohn's Disease	Total	(59)
Interstitial Nephritis	Neutrophil	(40)
Other		
Erectile Dysfunction	Endothelial	(71)
High fat meals	Total	(72)
Preeclampsia	Syncytiotrophoblasts	(73)
Pregnancy	Syncytiotrophoblasts	(73)
End-stage Renal Disease	Endothelial	(74)
Colorectal Cancer	Tissue Factor positive	(75)
Multiple Sclerosis	Endothelial	(76)
Hematopoietic stem cell transplants	Endothelial	(77)
Chronic Renal Failure	Endothelial	(78)

Our first goal was to determine if we could analyze the proteome of microparticles. We initially investigated the proteome of platelet-derived microparticles in order to establish the methods necessary for proteome analysis. Platelets were isolated from a healthy individual and activated with ADP to generate MPs. The MPs were isolated, lysed with SDS, delipidated and the proteins were partially separated by 1D PAGE. The gel lane was cut into 26 sections. For each section, the proteins were digested with trypsin to generate peptides, which were extracted from the gel and analyzed by LC-MS/MS. We detected 578 proteins with high confidence based on the detection of 5 or more spectra from at least 2 different peptides per protein (27). This was approximately twice the number of proteins identified in any single report of the proteome of platelets.

Next, we analyzed the proteome of microparticles isolated from plasma (plasma MPs). Initially, we used the conventional method for isolating MPs. Platelet poor plasma was centrifuged at high speeds for 1 to 2 hours to isolate MPs. We performed this type of analysis and found that the protein profile by 1D PAGE appeared to be very similar to that of plasma (Fig. 5), suggesting an overwhelming contamination with plasma proteins.

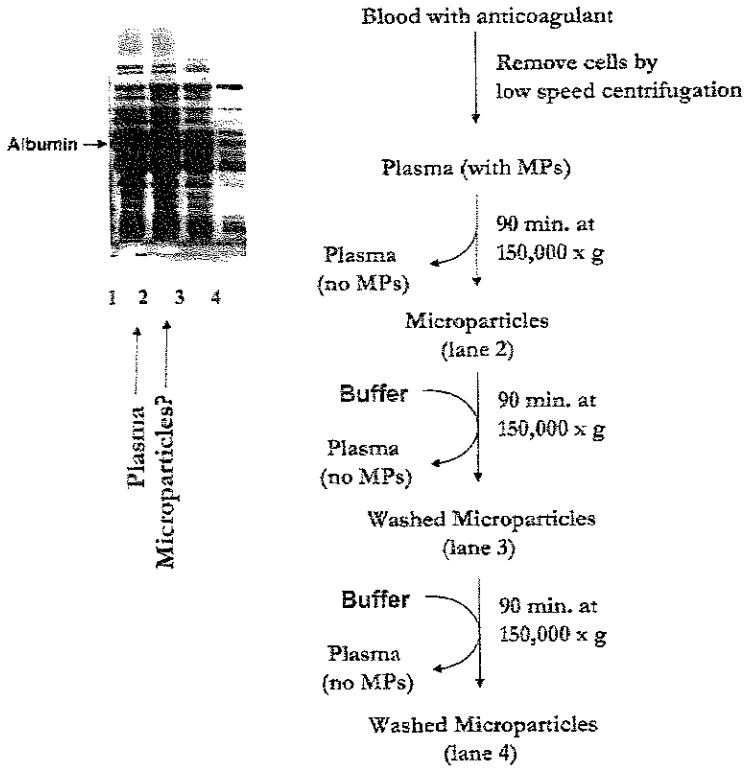

Figure 5: Isolation of Plasma Microparticles from Plasma using Ultracentrifugation alone. Initial attempts to isolate plasma MPs is illustrated. The protein profile of MPs as determined by PAGE followed by visualization by silver stain was similar to that of plasma (lane 1 versus lane 2). Following two washings, the protein profile appeared different, but the major proteins still included the predominant plasma proteins such as albumin.

After washing the pellet several times, the protein profile began to change but analysis of the gel indicates that plasma proteins were still a major problem.

Jin, et al., performed similar studies and found that the top 5 proteins were all major plasma proteins (28). Therefore, we developed a different approach to isolate the plasma MPs, combining gel filtration with ultracentrifugation. We compared the proteome of plasma MPs with that of MPs generated from purified platelets (9). We found a total of 21 proteins that were almost exclusively found in the plasma MPs, and another 2 that were greatly enriched. Von Willebrand factor (vWF) was the most abundant protein (based on spectral counts), and has been linked to the generation of platelet MPs (29). The proteins found almost exclusively in plasma MPs can be classified as involved with apoptosis (CD5-like antigen, galectin 3 binding protein, several complement components), iron transport (transferrin, transferrin receptor, haptoglobin), immune response (complement components, immunoglobulin J and kappa chains), and the coagulation process (protein S, coagulation factor VIII). It appears that these proteins arise from a variety of cell types. For example, CD5 antigen-like protein, also called SP α, is probably from monocytes and macrophages (30). Transferrin receptor is known to be downergulated from the surface of erythrocytes by internalization followed by exocytosis (31).

Typically, we isolate about two micrograms of plasma MP protein per ml of plasma. This is in contrast to plasma, which has about 80 mg of protein per ml. Having established these systems in our laboratory, we are currently using this approach to examine changes in the MP composition in a variety of disease states in hopes of discovering disease biomarkers.

Urine is another sample source which has been used extensively in biomarker discovery. Like plasma, it suffers from the same dynamic range problems, but to a lesser degree. One established marker in urine is water channel aquaporin-2 for several water-balance disorders (32). This is a membrane protein which can be isolated in the fraction of urine containing small, low-density membrane vesicles. Pisitkun et al, hypothesized that this protein and other putative membrane proteins are excreted into the urine as part of exosomes (33). They confirmed this hypothesis by isolating and examining these microparticles. Due to the lower amount of protein in the urine, about 1/1000 of that in blood, it was sufficient to isolate these by ultracentrifugation, followed by a single wash. Their initial report identified 295 proteins in urine exosomes which originate predominantly from the kidney distal tubules. They went on to examine the sample storage and processing requirements for use of this method in biomarker discovery (34). More recently, they compared the urine exosome proteome in both an animal model and in urine obtained from individuals with acute kidney injury and discovered fetuin-A as a potential biomarker for this ailment (35).

CONCLUSION

The field of mass spectrometry-based proteomics has been transformed over the last decade due to advances in technology, sample preparation, bioinformatics, and computational tools. While one might expect this would lead to a dramatic increase in the discovery of new biomarkers, the number of new biomarkers has not increased. One of the reasons for this is that potential biomarkers are probably present in the plasma at submicrogram per ml levels, while more predominant proteins are present at milligrams per ml. Using normal proteomic-based approaches, the signal from potential biomarkers is drowned out by noise from abundant plasma proteins. This has led us to investigate the plasma microparticle subproteome which we believe has a high likelihood of containing potential biomarkers.

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