Chapter 12

Isolation and Proteomic Analysis of Platelets by SELDI-TOF MS

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Abstract

Many growth factors, leukotrines, and biological ligands are not circulating free in plasma or serum, except in the case of late or disseminated disease. During early tumor growth and angiogenesis, platelets actively and selectively sequester regulators of angiogenesis and, as such, the platelet protein content can be used as a marker of early tumor growth or angiogenesis. With the recent increase in the clinical use of biologic modifiers in cancer and chronic disease therapy, the search for markers of early disease, therapeutic response, and/or recurrence has suggested that analysis of platelet proteins may be more relevant and accurate. We provide a guideline for the proteomic analysis of platelet proteome, placing specific emphasis on angiogenesis regulators, even though other platelet proteins may serve as markers of disease in the future. The analysis of serum/plasma has been fraught with difficulties because of the extraordinarily large number of proteins and because some of the proteins are contained in extraordinarily large amounts, masking the less abundant proteins. Thus, platelets may provide a much more biologically relevant analyte for biomarker discovery.

Key words: Platelet, Platelet proteomics, Angiogenesis, Markers of tumor growth, Vascular endothelial growth factor (VEGF), Basic fibroblast growth factor (bFGF), Platelet-derived growth factor (PDGF)

Abbreviations

Coag	Coagulation, referring to a test used to measure coagulation time of whole blood
EGF	Epidermal growth factor
Fbgn	Fibrinogen
bFGF	Basic fibroblast growth factor
PGE	Prostaglandin
PRP	Platelet rich plasma
PDGF	Platelet derived growth factor
PPP	Platelet poor plasma
SELDI-TOF MS	Surface enhanced laser desorption/ionization – time-of-flight mass spectrometry
SPA	Sinapinic acid
TFA	Trifluoroacetic acid
VEGF	Vascular endothelial growth factor

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1. Introduction

The introduction of biologic modifiers into cancer therapy, treatment of chronic inflammatory disorders, and acute care has led to an intense search for circulating biomarkers of disease progression and therapeutic response. Considerable effort is currently focused on methods for early cancer detection, including those involving: detection of specific proteins or proteomic profiles in the serum (1–4), DNA in stool samples (5–8), and gene expression profiles in lesional biopsies (9–12). The realization that angiogenesis is a critical part of tumor progression of solid (13) and liquid (14, 15) tumors, led over the past few decades to numerous attempts to correlate plasma and serum levels of angiogenesis-regulatory proteins with disease progression (16, 17). While helpful in the identification of patients with disseminated disease, the reliability of serum and plasma levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), or other angiogenesis related proteins in early-stage tumors remains uncertain (18, 19). The absence of biological ligands from plasma/serum is most likely due to the short half life of these proteins and the evolutionary disadvantage of retaining them free in circulation as many of them are toxic in high concentrations. High circulating levels of proteins that stimulate angiogenesis, such as VEGF or bFGF, have been shown to result in severe paraneoplastic syndrome (20–22). This syndrome, characterized by tachycardia, hypertension, nervousness, cachexia, and early death, has been well documented in disseminated malignancies, early occult neoplasms, as well as in severe inflammatory conditions and burns.

Early cancer invasion and injury are dependent on the spatial and temporal regulation of a local release of the relevant growth factors, cytokines, and other angiogenesis regulators. Similarly, platelets are crucial for the homing of endothelial cell progenitors (23) and inflammatory cells, thus regulating tissue re-generation and maintenance. The evolutionary necessity to localize inflammatory effectors and angiogenesis regulators also explains why we have not been able to detect elevated plasma/serum levels of these proteins during early disease initiation; and why we could not use the circulating levels of angiogenesis regulators as markers of early disease, disease recurrence, or progression. It has always been appreciated that the local concentration of angiogenesis regulators significantly exceeds that of plasma or serum (24) (Fig. 1), but the vehicle for the delivery of angiogenesis regulators to sites of activated endothelium within an early tumor, atherosclerotic lesion, wound, or a plague of endometriosis has only been described recently (25, 26).

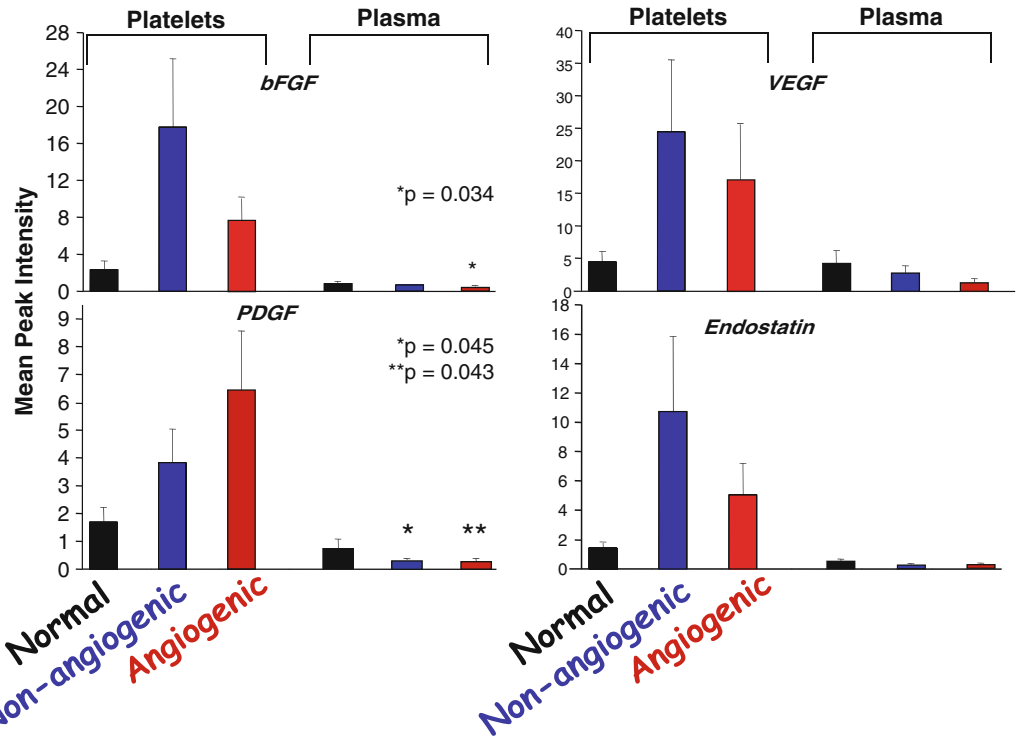

Fig. 1. Graphical representation of protein levels of angiogenesis regulators in plasma and platelets. We present four angiogenic regulators and their respective pattern of expression. The levels of pro-angiogenic growth factors (VEGF, bFGF, and PDGF) tend to be higher in the platelets, but not plasma, of tumor-bearing mice as compared to tumor-free sham operated mice. Although at 30 days nonangiogenic tumors are ~100 times smaller than their angiogenic counterparts, the platelet proteome in mice bearing nonangiogenic tumors shows detectable differences in the levels of angiogenesis regulators. In mice with nonangiogenic dormant tumors, the elevation of positive angiogenesis regulators tends to be counterbalanced by an elevation of the endogenous inhibitor endostatin. In contrast, in mice bearing the angiogenic clone, (*red*) platelets show a decrease in negative angiogenesis regulators such as endostatin. This suggests that, in the "angiogenic" tumors, the overall balance of angiogenesis regulators may be tipped toward a more pro-angiogenic phenotype in a manner reflected on the platelet proteome. Of note is the observation that all of the proteins are found at much higher concentrations in the platelets when compared to the plasma. The experiment was repeated on two separate occasions with five mice per each experiment, and the graph represents means ± standard error of means. This research was originally published in *Blood*. Klement GL, Yip TT, Cassiola F, Kikuchi L, et al. Platelets actively sequester angiogenesis regulators. *Blood*. 2009; 113:2835–2842. © American Society of Hematology.

We recently reported that platelets contain numerous proteins that regulate angiogenesis (27–29). Specifically, we had shown that the accumulation of angiogenesis regulators in platelets of animals bearing malignant tumors significantly exceeds their concentration in plasma or serum, as well as, their levels in platelets from non-tumor-bearing animals. This process is selective, as platelets do not take up a proportional amount of other plasma proteins (e.g. albumin), even though these may be present at higher concentrations (27, 30). We also found that VEGF-enriched Matrigel pellets implanted subcutaneously into mice, or the minute quantities of

VEGF secreted by microscopic subcutaneous tumors (0.5–1 mm^3), resulted in an elevation of VEGF levels in platelets, without any changes in plasma levels of the protein (27). The profile of other angiogenesis regulatory proteins (e.g. PDGF, bFGF) sequestered by platelets also reflected the presence of tumors in vivo before they were macroscopically evident. The ability of platelets to selectively take up angiogenesis regulators in cancer-bearing hosts may have implications for the diagnosis and management of many angiogenesis-related diseases, and provide a guide for anti-angiogenic therapies.

With the appreciation that platelets sequester, transport, and deliver angiogenesis regulators, the methodology for platelet isolation, purification, and analysis has gained in importance. In fact, with the increasing use of anti-angiogenic agents, growth factors, and other biologics, it is likely that the detection and quantification of relevant platelet proteins will continue to gain in importance. In addition, searches for protein biomarkers within the serum or plasma are hindered by the sheer number of proteins found within the blood. These proteins exist over an extremely large dynamic range spanning over 10 orders of magnitude (31), making the search for proteins in the plasma very difficult. A particular drawback has been the presence in serum/plasma of large amounts of albumin and immunoglobulins. These proteins, while present in platelets, are not actively sequestered by them and the levels of actively sequestered proteins are far easier to detect in platelets.

SELDI-TOF MS offers many advantages in the analysis of platelets over other methods of biomarker discovery because it allows you to quickly analyze sample sizes that are statistically relevant. Platelets are rich in proteins that regulate angiogenesis. Many of these proteins, if not all, are cationic with a $pI > 8$. The standard anion exchange fractionation procedure for SELDI-TOF MS separates these angiogenesis-regulating proteins from the bulk of proteins contained within platelets, especially albumin. The ability to analyze the "platelet angiogenesis proteome" partially purified from the remainder of the platelet proteome may provide distinct advantages in the search for novel biomarkers of disease and/or therapeutic response. While SELDI-TOF MS does not give you protein identification up front, purification and identification on the back-end of sample analysis is routine for many research laboratories.

2. Materials

2.1. Human Platelets

Human blood should only be drawn by a phlebotomist or trained medical professional. If the draw occurs within a phlebotomy clinic, or other medical professional office, no additional materials should

be required for platelet acquisition other than routine laboratory supplies. However, if one is asked to provide the appropriate blood collection tubes, citrated tubes routinely used for coagulation (coag) studies may be purchased (see Note 1).

1. BD Vacutainer® (buffered sodium citrate: (9NC) blood collection tubes) (BD Biosciences, San Jose, CA).

2.2. Mouse Platelets

Outside of routine animal care facilities and standard surgical procedures and equipment, the following materials are required for mouse blood withdrawal:

1. Sterile sodium citrate buffer (105 mM, pH 5), stored at room temperature.
2. Needles (23–21 gauge) (BD Biosciences).
3. Syringes (1 cc) (BD Biosciences).

2.3. Fractionation

Fractionation of samples by anionic exchange utilizing a step-wise pH gradient reduces the overall complexity of a protein mixture thus making it easier to observe potential changes in the concentration of less abundant proteins. Samples need not necessarily be fractionated and can be run neat; however, this may reduce the likelihood of finding meaningful differences.

1. 96-well plates (v-bottom, low protein binding preferred).
2. Aluminum plate sealers (preferable to plastic because aluminum withstands storage at –80°C better and because the aluminum plate sealers can be punctured by a pipette tip whereas the plastic cannot).

The following materials need to be obtained or prepared if a ProteinChip Serum Fraction Kit (Bio-Rad Laboratories, Inc., Hercules CA) will not be used for fractionation.

1. Anion exchange Q ceramic HyperD F sorbent, 1.
2. 96-well filtration plate.
3. 9 M urea, 2% CHAPS, 50 mM Tris–HCl, pH 9 (U9 buffer).
4. 50 mM Tris–HCl, 0.1% OGP, pH 9 (wash buffer 1).
5. 50 mM HEPES, 0.1% OGP, pH 7 (wash buffer 2).
6. 100 mM sodium acetate, 0.1% OGP, pH 5 (wash buffer 3).
7. 100 mM sodium acetate, 0.1% OGP, pH 4 (wash buffer 4).
8. 100 mM sodium acetate, 0.1% OGP, pH 3 (wash buffer 5).
9. 33.3% isopropanol, 16.7% acetonitrile, 0.1% trifluoroacetic acid (TFA) (wash buffer 6).

2.4. Profiling

Currently, ProteinChip Arrays can only be purchased from Bio-Rad as it is proprietary technology. We highly recommend that all binding

buffers also be purchased from Bio-Rad to avoid any quality control issues that may arise from researchers preparing their own buffers. While many chip chemistries are available, with the associated binding and wash buffers, we will concentrate on CM10 ProteinChip Arrays.

1. CM10 ProteinChip Arrays (Bio-Rad).
2. CM10 Low-Stringency Binding Buffer (Bio-Rad).
3. Acetonitrile.
4. 0.1% TFA.
5. Sinapinic acid (SPA).

3. Methods

The focus of this section is the collection and isolation of platelets from mice and humans as these two species offer examples of platelet collection from small and large organisms. The methods presented can be extrapolated to other experimental animal models. Platelets are delicate and, as such, a caveat for working with platelets is their sensitivity to external conditions and surfaces. Precautions need to be taken during collection and isolation to avoid platelet activation. Platelet activation causes changes in platelet morphology that ultimately lead to alterations in protein levels due to release of granular content (Fig. 2) (32). Due to the release of proteins during platelet contraction, proteomic analysis of platelets may not reflect the biological phenomenon being studied if care is not taken to prevent activation (see Note 2).

Platelet activation can be prevented by two means: (1) pharmacological pretreatment of the platelet preparations with calcium antagonist (verapamil), cyclooxygenase inhibitors (prostaglandin-2 (PGE_2)), or inhibiting fibrinogen (Fbgn) receptor activation using Fbgn binding peptide Gly-Pro-Arg-Pro (GPRP) (33–35) and (2) meticulous avoidance of activating surfaces and conditions. In deciding which pharmacological agent to use for the prevention of platelet activation, it is imperative that one considers their mechanism of action so as to not produce interference with the pathways of interest (see Note 3). An additional caveat to pharmacological pretreatment of platelets is the notion that the protein profile may change upon treatment. We have found that pretreatment with prostaglandin E significantly alters the level of a protein with an approximate mass of 14,620 Da (Fig. 3). To avoid activating surfaces and conditions, it is important to avoid: glass and polystyrene plastic surfaces, changes in temperature, dust, alterations in pH, shearing forces, and prolonged incubations without gentle agitation. In particular, putting whole blood or platelet preps on ice will

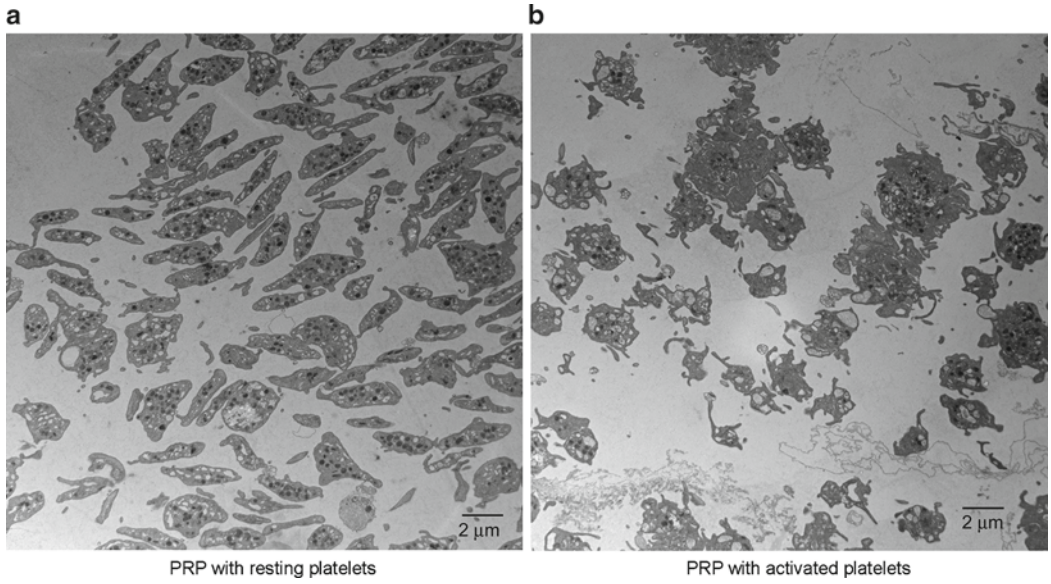

Fig. 2. Morphological changes in platelets due to activation. In a resting state, platelets are discoid in shape with two types of granules, alpha and dense granules (**a**). Platelets also contain lysosomes and mitochondria. The membrane of the platelet is interspersed with open canallicular system (OCS), which represents direct communications of the cytoplasm with the exterior of the platelet. This tenuous balance of the platelet membrane is crucial in the activation of platelets, which results in retraction of the granular content to the center of the platelet, widening of the OCS, and extension of filopodia, lamellipodia, and pseudopodia (**b**). Image is courtesy of Dr. Flavia Cassiola (27).

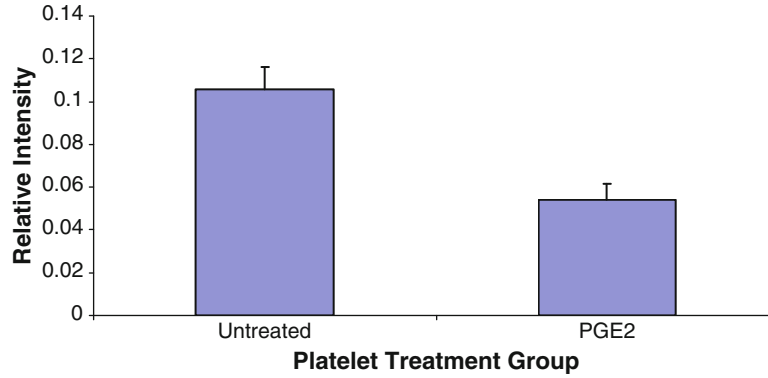

Fig. 3. Pretreatment of platelets with prostaglandin E alters the level of a protein with a mass of 14,620 Da. Platelets in PRP were incubated with PGE_2 for 1 h at 37°C or untreated, but given vehicle control. Following incubation, platelets were isolated, lysed, and analyzed by SELDI-TOF MS. Only the peak with a mass of 14,620 was found to be significantly altered by treatment with PGE_2 (p-value < 0.05). This is an important observation as pharmacological pretreatment of platelets to prevent activation may alter the protein content of the platelets.

cause irreversible damage to platelets and thus the proteomic profile. Pharmacological and methodological prevention of platelet activation should produce samples conducive to valid analysis of the protein content of platelets. We recommend that careful

methodological protocols be employed and that pharmacological methods be used sparingly as the effects of their use on platelet protein content is not fully understood.

3.1. Platelet Collection

3.1.1. Collection of Human Platelets

The collection of human platelets for platelet aggregation and coagulation studies has been well established in the majority of clinical laboratories around the world, and as such does not require a detailed description. Briefly, platelets are a component of whole blood and can be readily obtained from a routine blood draw. However, unlike other blood components, the collection of platelets requires careful management of calcium chelation by ensuring that the blood draw maintains a volume per volume solution of 10% sodium citrate. Collection of human blood should be performed by a trained phlebotomist or appropriate medical professional, into standard blue top coagulation Vacutainers® containing citrate and filled to the top in order to preserve the appropriate citrate dilution. Use of a syringe or long tubing should be avoided in order to avoid platelet activation. A large boar needle is preferred to avoid sheering resulting in hemolysis and platelet activation (see Note 4). It is desirable to process whole blood samples immediately, or within 3 h of blood draw as long as the collection tubes are placed on a gentle rocker for that time period. Once platelet-rich plasma is isolated, it can be preserved on a gentle rocker for up to 3 days. Platelets react very quickly to the changes in sample pH and as such the quality of the sample decreases with prolonged metabolic activity that accompanies delays in processing. Unlike situations where cooling of the sample may decrease the metabolic activity, platelets should never be placed on ice, or in a cold environment, as this results in platelet lysis. Room temperature will suffice for most platelet applications although 37°C would be optimal.

3.1.2. Collection of Mouse Platelets

The collection of mouse platelets varies significantly from laboratory to laboratory and this variation may be the source of the variability of results seen in the literature. While mouse platelets are thought to be less sensitive to activation and can be *spin washed* in contrast to human platelets, all of the aforementioned rules for the collection of human platelets should be observed (see Note 5). To summarize, the steps involved in collection of platelets from animal experimental subjects are as follows:

1. Prepare a 105 mM solution of sterile sodium citrate buffer, pH 5.
2. Prepare a 1 cc syringe per mouse containing 100 μL of the sodium citrate buffer. This amount of buffer will allow for a final concentration of 10% volume per volume dilution of the citrate as long as the syringe is filled with blood to a volume of 1 mL.

3. Of the three methods to obtain blood from mice (orbital bleed, tail vein bleed, and direct cardiac puncture bleed), only the direct cardiac puncture provides sufficient flow and minimization of the shearing forces that may disrupt or activate platelets (see Note 6). Therefore, blood should be collected via a terminal cardiac bleed, under anesthesia, using a 23–21 gauge needle directly attached to the 1 cc, pre-citrated syringe.
4. Remove the needle from the syringe and place the whole blood specimen into a 1.5 mL polypropylene tube.
5. Place tube on a gentle rocker at room temperature or 37°C to avoid platelet activation.

3.2. Platelet Isolation

Human blood contains approximately 100,000–400,000 platelets per microliter. As long as the appropriate precautions are taken, and those platelet counts are preserved, a platelet pellet obtained from 1 mL of platelet-rich plasma (PRP) will contain ~1–1.5 mg of total protein (data not shown).

3.2.1. Isolation of Human Platelets

1. Centrifuge the whole blood collected in Subheading 3.1.1 for 20 min at 150 × *g* using a swinging-bucket rotor at room temperature or 37°C. (Do not use a refrigerated centrifuge and do not place samples on ice).
2. Immediately following centrifugation, transfer the top phase, this is the platelet-rich plasma (PRP) (Fig. 4), to a fresh polypropylene tube. Care must be taken to avoid the buffy coat as this layer contains white blood cells that are nucleated and have a much greater protein content that will skew the proteomic analysis of platelets. For a standard 5 mL Vacutainer®, 5 mL of whole blood should yield ~2.5 mL of PRP (see Notes 7 and 8).
3. PRP should be divided into 1 mL aliquots, in order to facilitate standardization of the protein levels to the original platelet count. The goal is to have quantities of protein ultimately expressed as microgram of identified, differentially expressed protein per 100,000 platelets (see Note 9). A practical suggestion is to have this 1 mL aliquot in 1.5 mL polypropylene tubes labeled as "platelets" as this tube will end up containing the platelet pellet.
4. The 1.5 mL tubes containing the PRP are then centrifuged for 10 min at 900 × *g* at room temperature or 37°C to pellet the platelets. Please note, centrifuging the platelets at higher *g*-forces will cause activation due to the collision of platelets with the bottom of the tube.
5. Following centrifugation of the platelets, the plasma (aqueous phase) is transferred to a fresh 1.5 mL polypropylene tube labeled "plasma." These tubes now contain platelet poor plasma (PPP).

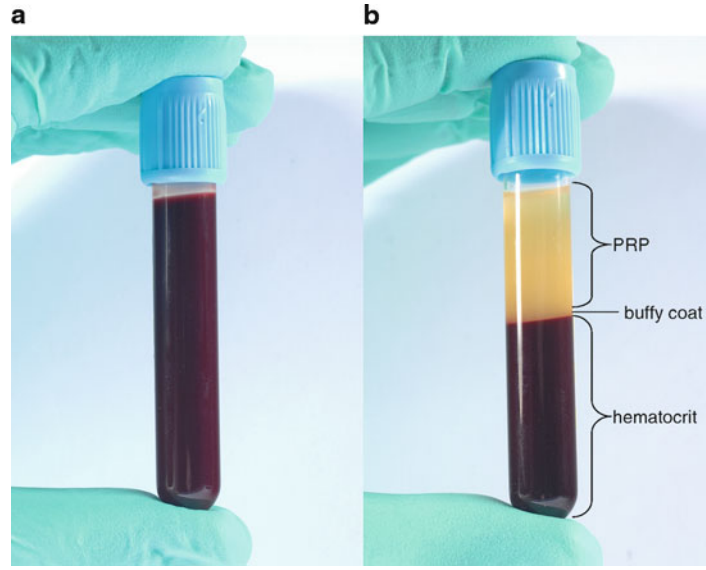

Fig. 4. Diagram of whole blood fractionation following centrifugation. Immediately following blood draw, the entire solution within the tube is homogenous and uniform in color (**a**). Following the first centrifugation at 150 × *g*, the whole blood separated into three distinct layers: PRP (*top layer*), buffy coat (*middle layer*), and the hematocrit (*bottom layer*). The hematocrit contains red blood cells, the buffy coat contains white blood cells, while the platelets are contained within the PRP. When removing the PRP, care should be taken to ensure that none of the buffy coat is taken up.

6. After removal of the PPP, residual plasma may still be present on the platelet pellet and tube wall. An additional step should be employed to remove this proteinacious material using non-fibrous filter paper to blot up the residuum. Note that plasma protein concentrations (in the 50 mg/mL range) will mask many of the less-abundant proteins in platelets unless this step is taken (see Note 9). Care should be taken to not disturb the platelet pellet.
7. Store plasma and platelet pellets at –80°C until further protein analysis.

3.2.2. Isolation of Mouse Platelets

Mouse blood contains approximately 500,000–1,000,000 platelets per microliter (36). Given that a smaller volume of whole blood is obtained, platelet pellets from mice are often difficult to observe upon centrifugation. Care should be taken to standardize the position of platelet pellets within microfuge tubes by always centrifuging tubes with the tabs of all tubes facing the same direction. Mouse platelets can be processed in a similar manner as human platelets; however, there are some differences in the isolation method that are worth noting.

1. Centrifuge whole blood in 1.5 mL tubes at 150 × *g* for 30 min at room temperature or 37°C.

2. Immediately following centrifugation, remove the top phase and transfer to a fresh 1.5 mL tube labeled "platelets." The fresh tube now contains PRP. Care should be taken to avoid the buffy coat and the hematocrit layers as they contain white and red blood cells, respectively, and will contaminate the proteomic analysis. The original ~1 mL of whole blood obtained from a mouse should yield ~400–500 µL of PRP.
3. Centrifuge the tubes containing the PRP at 900 × *g* for 30 min at room temperature or 37°C to pellet the platelets.
4. Remove the plasma and transfer to a new 1.5 mL tube. Label this tube "plasma" as it now contains the PPP.
5. Store plasma and platelet pellets at –80°C until further analysis.

3.3. Fractionation of Platelet Samples for SELDI-TOF MS

Standard SELDI-TOF procedures are utilized during platelet analysis with minor deviations from the manufacturer's protocols. Please reference all protocols from Bio-Rad Laboratories, Inc. for starting conditions and materials.

1. For analysis, lyse a pellet of human platelets obtained from 1 mL of PRP in 100 µL of U9 buffer (9 M urea, 2% CHAPS, 50 mM Tris–HCl, pH 9) and incubate at room temperature for at least 30 min. Mouse platelets obtained from ~0.5 mL of PRP should be lysed in 50 µL of U9 and incubated for at least 30 min. Platelet-poor plasma should be prepared according to standard SELDI-TOF methods for the analysis of serum. Optimally, 50 µL of plasma is incubated with 50 µL U9 and incubated for 30 min.
2. Following incubation in U9, add 100 µL U1 buffer (1 M urea, U9 buffer diluted 1:9) to the platelet lysate obtained from human platelets. For mouse platelets, 50 µL of U1 should be added. Add 100 µL U1 to denatured plasma samples.
3. Centrifuge samples at 13,000 × *g* for 1 min to pellet any solid material such as cell membranes that have not dissolved.
4. One hundred microliters of the diluted human platelet lysate should be loaded onto a standard SELDI-TOF anionic exchange column for fractionation (rehydrated anion exchange Q ceramic HyperD F sorbent, 1). The remaining 100 µL can be used later for protein identification or immunoassays. For mouse platelets, the entire 100 µL of dilute platelet lysate should be used for fractionation (see Note 10). Diluted plasma from both species should be added at 100 µL.
5. Place the fractionation plate onto a shaker for 60 min at room temperature.
6. Place the fractionation plate onto a collection plate and use a vacuum manifold to draw the solution through the filer on the bottom of the fractionation plate into the collection plate.

Alternatively, the fractionation and collection plates can be placed into a swing-bucket centrifuge at 200–400 × *g* for 1 min (speed will vary depending on how viscous the sample is and how well the resin has been mixed).

7. Add 100 µL of wash buffer 1 (50 mM Tris–HCL, 0.1% OGP, pH 9) to the column and incubate with shaking for 10 min at room temperature.
8. Repeat the filtration step by either vacuum manifold or centrifugation collecting the flow through into the previous collection plate. The collection plate now contains Fraction 1 and can be stored at –80°C until analyzed by SELDI-TOF.
9. Add 100 µL wash buffer 2 (50 mM HEPES, 0.1% OGP, pH 7) to the column and incubate with shaking for 10 min at room temperature.
10. Place fractionation plate onto a new collection plate and repeat filtration step by either vacuum manifold of centrifugation.
11. Add another 100 µL of wash buffer 2 to the column, incubate with shaking for 10 min at room temperature, and collect into the collection plate from step 10. This is now Fraction 2 that can be stored at –80°C until analyzed.
12. Repeat steps 9–11 with wash buffers 3–6 to generate Fractions 3–6. Wash buffer 3 contains 100 mM sodium acetate and 0.1% OGP at pH 5 (wash buffers 4 and 5 are similar with pH 4 and 3, respectively). Wash buffer 6 contains 33.3% isopropanol, 16.7% acetonitrile, and 0.1% TFA.
13. For SELDI-TOF analysis, 50 µL of a fraction is added to 100 µL of the appropriate binding buffer for each chip chemistry to be utilized. Laser intensity needs to be determined empirically for each SELDI-TOF machine as this will vary depending on laser strength and age of the laser and detector. A typical spectrum from Fraction 1 on CM10 is shown in Fig. 5.

3.4. Profiling

This section concentrates on the use of CM10 ProteinChip Arrays. There are other chemistries available such as Immobilized Metal Affinity Capture (IMAC30) and Hydrophobic (H50) and the corresponding buffers, available from Bio-Rad, should be used in conjunction with the other chemistries.

1. Equilibrate CM10 ProteinChip Arrays with 150 µL CM10 Low Stringency Buffer for 5 min with vigorous shaking at room temperature.
2. Remove buffer and add 100 µL CM10 Low Stringency Buffer to the arrays.
3. Add 50 µL of fractionated sample to the arrays and incubate for 30 min with vigorous shaking at room temperature.

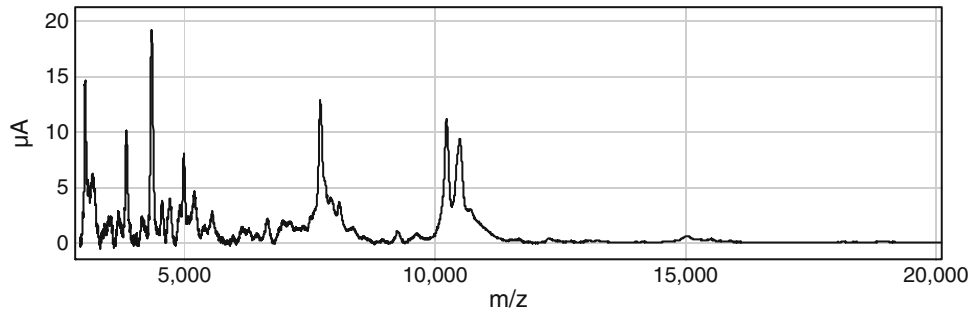

Fig. 5. Typical spectrum for Fraction 1 of a platelet lysate on CM10 surface arrays. A low laser energy was used to generate the spectrum resulting in mostly low molecular weight proteins being detected. One of the most abundant proteins in platelets is platelet factor 4 (PF-4) and this polypeptide is observed as the peak at approximately 7.8 kDa.

4. Remove samples from arrays.
5. Wash arrays three times with 150 μL CM10 Low Stringency Buffer for 5 min each wash with vigorous shaking at room temperature.
6. Quickly rinse arrays twice with 150 μL deionized water and allow to air dry.
7. Apply 1 μL of SPA to the arrays and allow to air dry.
8. Repeat step 7.
9. Read arrays on a SELDI-TOF MS at settings that are optimal for your samples and machine.

4. Notes

1. Collection for a coag study is a routine procedure performed in phlebotomy clinics. The procedure involves veni-puncture using a large bore needle and collection of blood directly into the citrated tube without the use of a syringe or excess tubing. For best results, a pre-clear of 1–2 mL of blood prior to sample withdrawal should be performed.
2. During activation, the platelet membrane retracts while extending pseudopodia and filopodia, the granular content is centered, and the open canalicular system enlarges (Fig. 2). These morphological and physiological alterations are associated with degranulation of the dense granules, calcium flux, and ATP release. Contrary to common belief, the release of proteins from alpha granules may be under different, as of yet undescribed, controls (27).
3. When choosing to pharmacologically inhibit platelet activation, care should be taken to ensure that the compound does not

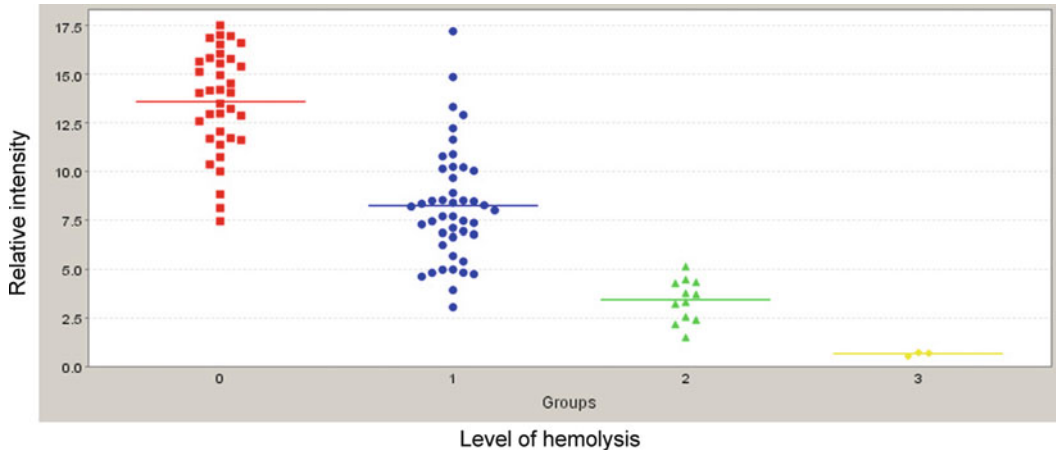

Fig. 6. Hemolysis alters the protein profile on CM10 arrays. Plasma samples obtained from 96 prostate cancer patients were segregated into four groups based on their level of hemolysis. Hemolysis was detected by eye and determined by the level of "redness." Group 0 indicates samples in which no detectable red color was observable and hence contains no hemolysis (36 samples). Group 1 contains samples that were slightly "pink" (45 samples), group 2 contains samples that were more orange in color (12 samples), and group 3 contains samples that were "blood red" (3 samples). A protein with an approximate mass of 66 kDa (assumed to be serum albumin) was significantly decreased with increasing hemolysis levels. This most likely was the result of increased hemoglobin levels out-competing albumin for binding sites on the CM10-binding surface.

interfere with the process under investigation. For example, the use of prostaglandin to inhibit platelet activation may not be conducive to studying the affects of aspirin on the platelet proteome.

4. Conditions that produce hemolysis in the sample should be avoided as anything that disrupts the plasma membrane of an erythrocyte will most likely affect the membrane of a platelet in a similar fashion. Furthermore, we have found that hemolysis has a profound impact on the proteins detected by SELDI-TOF MS. In our studies, we categorized plasma samples, by eye, from prostate cancer patients into four levels of hemolysis ranging from no hemolysis to extreme hemolysis (samples were blood red), with two intermediate levels of hemolysis being a slight pink color to a darker, slightly red color. In addition to the hemoglobin peaks found at ~15.2 and 15.9 kDa, several other peaks were able to distinguish between samples based on hemolysis levels (Fig. 6). Because hemolysis levels alter the protein content of the plasma, and potentially platelets, and this alteration of protein content leads to a differential protein detection by SELDI-TOF MS, we recommend that samples with hemolysis be discarded and not used for biomarker discovery or validation.
5. We do not recommend washing platelets. In our studies, we have found that washing of platelets is an uncontrollable variable

that causes partial activation of platelets, leading to an alteration of protein content, and results in a loss of approximately half of the platelets within the sample; making it difficult to normalize to platelet counts obtained from the original sample. However, it is important to remove as much as of the plasma as possible as described in Subheading 3.2 in order to avoid masking less abundant proteins.

6. We recommend terminal cardiac puncture bleeds because it provides sufficient flow and minimizes traumatic collection and hemolysis. Furthermore, terminal cardiac bleed allows for the collection of the greatest amount of blood when compared to orbital and tail vein blood draws. A typical blood volume for a mouse is 2 mL; terminal cardiac puncture generally provides 1 mL of blood while orbital and tail vein bleeds yield approximately 200 μL. In addition, orbital and tail vein bleeds produce tissue factor contact from the wound with the collected specimen. The production of a thrombus will alter the platelet number within the plasma and cause activation of platelets resulting in altered granular content of the platelets.
7. Following centrifugation, whole blood will separate into three phases: plasma (top phase), buffy coat (middle phase), and the hematocrit (bottom phase) (Fig. 4). In many cases, it is difficult to determine where the boundary between the plasma and buffy coat occurs. When removing the platelet-rich plasma from the separated whole blood, it is preferable to leave some of the platelet-rich plasma behind to ensure that none of the buffy coat is intermingled with the platelet-rich plasma as the buffy coat contains white blood cells.
8. The amount of plasma obtained from whole blood should be approximately half as long as the hematocrit is normal, i.e., 5 mL of whole blood should yield 2.5 mL of plasma. However, the amount of plasma will vary based on the hydration level of the individual from which the blood was drawn. Hence, a 5-mL draw of whole blood may give between 1.5 and 3 mL of plasma.
9. Normalization to external factors such as platelet count is often considered unnecessary in SELDI-TOF MS because SELDI-TOF MS utilizes a “differential display” and is often normalized to the total ion current. Some patients with severe disease or undergoing treatment with certain drugs can be thrombocytopenic (a condition marked by low platelet counts) making it difficult to compare these patients to a “normal” (healthy) population as differences may be due to the low number of platelets and, hence, be non-specific. We suggest that results from SELDI-TOF, or other standard biochemical assays, can be normalized to platelet count and expressed as a quantity of protein per 100,000 platelets. Although, as stated before,

normalization, other than to total ion current, may not be required with SELDI-TOF MS as normalization to platelet count does not appear to alter results by a significant amount (37). Alternatively to platelet count, other traditional normalization procedures used in Western blots, and other biochemical techniques, such as actin or GAPDH levels, may be employed as long as the platelet pellet is relatively free of white and red blood cells and the levels of the molecule used to normalize is reflective of the platelet count. If it has been determined that the platelet pellets are relatively free of other cell types, a cellular protein should be used for normalization to ensure that plasma proteins are not affecting the normalization procedure. No matter how much care is taken, a minute volume of plasma will be retained within the platelet pellet, thus total protein should not be used as a normalization factor. Plasma protein levels are ~50 mg/mL, therefore, if 20 µL of plasma is left on the platelet pellet, an increase of 1 mg of protein may be observed. This is a significant increase over the typical 1–1.5 mg of total protein typically obtained from the platelet pellet obtained from 1 mL of human PRP. It is our belief that platelet count is the optimal method for normalization in cases where external normalization is required.

10. The entire amount of mouse platelet lysate is used for analysis as collection from mouse to mouse can vary in the amount of PRP obtained, whereas in humans a consistent 1 mL of PRP is utilized for analysis. Often, only 200 µL of PRP can be obtained from a mouse. In order to ensure high quality spectra, the entire platelet lysate must be used.

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