Nanosized blood microparticles

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CHAPTER 2

Pre-analytical and analytical issues in the analysis of blood microparticles

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Thrombosis Haemostasis 2010 Dec 21;105(3):396-408

Abstract

Results of plasma microparticles (MPs) measurements reported in the literature vary widely. This is clearly not only related to the lack of well-standardized MP assays, but also to variations in pre-analytical conditions. In this review we will discuss the pre-analytical variables related to plasma and MP preparation which may affect MP analysis. Additionally we will address several analytical issues in commonly used MP assays and briefly discuss some novel approaches for the detection and characterization of MPs.

Ideally MP measurements should be performed in plasma, freshly prepared directly after blood withdrawal. As platelet contamination seems to be one of the major pre-analytical problems in processing plasma for MP measurement, the use of platelet-free plasma may be preferred. When frozen-thawed plasma is used, especially PMP and annexinV-positive MP counts should be interpreted with caution.

When flow cytometry is chosen as a method for quantification of MPs, some analytical conditions should be standardized, e.g. settings of the flow cytometer, quality of the antibodies, and use of counting beads. Fluorescence-nanoparticle tracking analysis and atomic force microscopy can accurately count nanosized MPs, but unfortunately the operational procedures of both methods are still time consuming and they give no information on the functional properties of MPs. The MP-TF activity assay provides information on MPs carrying active TF, regardless of their parental origin.

Ultimately, standardisation of pre-analytical procedures and the introduction of reliable and rapid methods for the measurement of MPs are urgently needed to facilitate their use as biomarker in the pathophysiology of diseases.

Introduction

Since Wolf in 1967 (1) showed that platelet shedding results in the formation of 'platelet-dust', the shedding of microparticles (MPs) in blood has been recognized as a regulated process during cell activation and early apoptosis (2;3). MPs formed by membrane vesiculation of cells are generally heterogeneous in size (<1 µm). They differ from exosomes with respect to their origin, protein content, and size distribution. Exosomes originate from multivesicular bodies and are usually more homogeneous and smaller in size (<0.1 µm) (4-7). However, there is no consensus on the precise cut off in the size distributions of these two populations. This is not only due to limitations of the methods used for the characterization of these nanoparticles, but also by pre-analytical variables like anticoagulant, centrifugation, and storage.

The large majority of MPs detected in blood originates from platelets, but other blood cells such as leucocytes, erythrocytes, endothelial cells and even malignant cells also may shed MPs. These MPs still bear antigens of their parental cells so that their origin can be determined by using specific antibodies against these antigens (4;8). Furthermore, blood MPs may carry the negatively charged phosphatidylserine (PS) on their surface as a result of plasma membrane remodeling during their formation from their parental cells (2;4;5). The exposure of PS on MPs provides a catalytic surface which promotes the assembly of procoagulant proteins and stimulates coagulation reactions (2;9-11). The PS content of MPs may vary according to their cellular origin, the mechanism by which they have been formed and several pre-analytical conditions (9-14).

The presence of MPs in the blood of healthy persons is well documented and elevated numbers of specific subsets of MPs have been reported in vascular disorders, cancer, and auto-immune diseases. Therefore, there is a growing interest to study the role of MPs in physiology and pathology (for recent reviews see (4;9;12-16)). However, individual studies use a wide variation of pre-analytical and analytical procedures, which all may affect the outcome of the MP measurement (17) (Table 1). Therefore, the Vascular Biology group of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) decided during their meeting in 2003, to address issues related to the detection, measurement, function, and clinical significance of membrane MPs derived from blood and vascular cells.

At present, standardization of pre-analytical and analytical methods for the measurement of MPs remains a challenge. In this review we will discuss the pre-analytical variables related to plasma and MP preparation which may affect the

characterization and enumeration of MPs. In addition we will address several analytical issues in commonly used MP assays and briefly discuss some novel approaches for the detection and characterization of MPs. Finally, we will make some general recommendations on pre-analytical and analytical conditions in MP assays.

Pre-analytical conditions

Blood collection

Needles ranging from 19 to 22 Gauge (G) are commonly used for venepuncture in haemostasis laboratories (18). Prolonged placement of a tourniquet is avoided during venepuncture (19). Theoretically, when a small-diameter needle (high G number) is connected to an evacuated tube, the vacuum force applied to the blood is large. Such shear stress and excessive pressure on the vein during blood withdrawal might cause *in vitro* haemolysis (20) and possibly formation of erythrocyte MPs (ErMPs) (21). A system using a small-diameter butterfly needle is commonly used on small children and patients with difficult venous access (22). This system reduces the blood flow and consequently increases the risk of platelet activation (18). During blood collection, the first 3-7.5 mL of blood is discarded to avoid the effects of the vascular damage caused by the venepuncture (23;24).

Several anticoagulants are used to collect blood for MP analysis (Table 1). It is important to consider how these anticoagulants may help to limit platelet activation during blood collection and plasma preparation, because platelet activation will result in release of alpha and dense granules and platelet MPs (PMPs) (25-27). Sodium citrate with a concentration of 0.105 or 0.129 mol/L (3.2% or 3.8%) is the most widely used anticoagulant (8). Other anticoagulants that have been used are Acid-Citrate-Dextrose (ACD) (28-30), ethylenediaminetetraacetic acid (EDTA) (8;31-33), a strong chelator of calcium ions, and heparin (34-36), which will preserve extracellular calcium. However, blood collected in heparin gave significantly higher levels of annexin V-positive MPs than blood collected in sodium citrate (37). Connor et al (38) reported that the number of annexin V-positive MP and the concentration of procoagulant phospholipid increased more strongly over a 60 min period following blood collection in sodium citrate-anticoagulated samples than in EDTA-anticoagulated samples. They suggested that when blood is not immediately processed, MP measurement should be performed on EDTA-anticoagulated samples. Use of citrate theophylline adenosine dipyridamole (CTAD) has been shown to inhibit *in vitro* platelet activation by increasing the cytosolic AMP concentration without affecting platelet function (18;25;39). During a period of 3 hours after blood collection PMPs counts increased 2-fold in plasma

from citrated blood but were relatively stable in plasma isolated from CTAD-blood (40). Unfortunately, there is no study which directly compares the effects of citrate, CTAD and EDTA on plasma MP counts.

Plasma isolation

Platelets need to be removed from the plasma in order to avoid cellular activation leading to inadvertent production of MPs. For this purpose, a proper centrifugal speed should be applied for plasma preparation. According to the Clinical and Laboratory Standards Institute (CLSI) (41), plasma with a platelet count less than $10x10^9$/L is obtained by centrifuging the blood at 1,500g for 15 minutes at room temperature (RT). To obtain platelet-poor plasma (PPP) CLSI recommends re-centrifuging the plasma for another 10 minutes at 1,500g. We observed that PPP isolated by centrifuging blood once at 1,550g for 20 minutes still contained $2.8x10^9$/L platelets. However, when the same blood sample was used to isolate PPP by performing a two-step centrifugation, each at 2,000g for 10 minutes, there were only $6.1x10^8$/L platelets present in the plasma.

Platelet-free plasma (PFP) can be isolated by a centrifugation using 1,500g for 20 minutes followed by a high speed centrifugation at 13,000g for 2 minutes (8). Some studies (23;42;43) where flow cytometry was used to measure MPs, indicate that MP counts are lower in PFP than in PPP. The reason is still unclear. Probably, the last centrifugation step used in the preparation of PFP is vigorous and depletes platelets and also some MPs.

Cold activation of citrated blood samples has been reported to result in falsely elevated levels of (activated) factor VII (FVII) and factor VIII (FVIII) (44;44). Therefore, the temperature is generally kept at RT (20-25℃) for centrifuging citrated blood (8). However, the effect of centrifugation temperature on MPs has not yet been thoroughly investigated.

Furthermore, during centrifugation the use of breaks should be avoided as much as possible and plasma should be carefully collected without disturbing the platelet layer to prevent remixing of the plasma with the platelets.

Storage

Ideally, plasma is prepared immediately after blood collection. To avoid changes in MP number and characteristics the use of freshly isolated plasma is recommended for MP measurements (8). However, the logistic of blood sampling, transport time

to the in-house laboratory or even to laboratories elsewhere are all variables which are difficult to control and keep uniform across different centres.

According to CLSI guidelines (41) storage of uncentrifuged blood on ice is not recommended because of the possibility of cold activation of FVII and FVIII, loss of von Willebrand Factor (vWF), and platelet disruption (45). During storage blood samples should be maintained at room temperature while mechanical agitation is avoided (46).

In multi-centre studies and prospective trials it is often inevitable to freeze and store the plasma samples before performing the assay. However, validated protocols are not yet available for freezing and thawing of plasma to be used for MP analysis (47). In some publications it is described that plasma is first snap frozen in liquid nitrogen before it is stored at -80°C (23;47-50) while others directly freeze the plasma directly at -80°C (51-53).

Frozen-thawed PPP prepared by single centrifugation at 2,000g for 30 minutes contained 10-fold more PMPs counted by flow cytometry than fresh PPP (54). Conversely, PMP and annexin V-positive counts were not increased in frozen-thawed PFP prepared by a two-step centrifugation procedure (1,500g for 20 minutes and 13,000g for 2 minutes) (48). Probably some platelets present in PPP are fragmented during freezing and thawing leading to an increase in the number of PMPs and annexin V-positive MPs. Weber et al reported in an abstract that methods applied for cryopreservation of MPs, even by using established chemicals for cell cryo-conservation such as DMSO, trehalose, and paraformaldehyde, could not prevent the 10-fold increase in the number of annexin V-positive MPs caused by freezing and thawing (43).

However, most studies which investigated the effect of storage on MPs used flow cytometry for the measurement of MPs. A conventional flow cytometer uses a 488-nm laser and as MPs are heterogeneous and can have sizes below 488 nm, the sizing and identification of MPs is not accurate ((55), see also section ‘Flow cytometry’ for a more detailed discussion).

MPs isolation

Direct measurement of MPs in plasma is preferred to prevent loss of MPs during the isolation procedure and to preserve their morphology (51). For this purpose, flow cytometry and capture-based assays can be employed (31;32;51-53;56;57). Other methods require a MP isolation step prior to the MP measurements, such as fluorescence or electron microscopy, proteomic analysis and some functional MP

assays (29;34;49;58;59). In the next section these analytical methods will be discussed.

The advantages of using isolated MPs are less interference of plasma proteins and an increase in MP concentration. On the other hand, isolation procedures will cause inevitably loss of some MPs, especially when multiple washing steps are included. Most importantly, it is not known which centrifugation speed is optimal to pellet (all) plasma MPs.

Table 2 compares the results of several studies using different centrifugation protocols to prepare plasma and MPs for the measurement of PMPs by flow cytometry. Results are those obtained for citrated blood of healthy subjects. It seems that when fresh plasma is used, more PMPs can be detected directly in plasma than in the isolated MP fraction. Furthermore, the number of PMPs is about 3-10-fold higher in MPs isolated from frozen-thawed PPP than in MPs isolated from fresh PPP, especially when single-centrifuged PPP has been used.

Recommendations

During blood collection, it is necessary to prevent *ex vivo* activation of blood leading to inadvertent production of ErMPs or PMPs. Therefore, blood should be withdrawn by using a large diameter needle, for example a 21-G needle, and a tourniquet is only applied for locating the vein. In general, sodium citrate, EDTA, and CTAD anticoagulants can be used for blood collection. However, more information is needed on the relative stability of MP in blood collected in these anticoagulants.

After blood withdrawal, blood should be immediately centrifuged to isolate plasma. The centrifugation speed applied is crucial for the removal of platelets from plasma. Because PPP still contains some platelets, use of PFP may be preferred. However, it should be considered that some MPs might be lost during the high-speed centrifugation step. MPs measurements should be performed preferably directly in fresh plasma to reduce MP loss and to preserve MP characteristics. When frozen-thawed plasma is used for MP measurements, especially PMP and annexin V-positive MP counts should be interpreted with caution.

Ultimately, it is important to set up guidelines for preparing samples for MP measurements which include the choice of anticoagulant used for blood collection, the centrifugation speed to isolate plasma and MPs, and the preferred storage of plasma.

Table 1. Pre analytical variables and analytical methods to detect and characterize MPs.

Blood collection			Plasma preparation			Storage	MP isolation			Detection method	Ref.
Needle (G)	Anticoagulant	Type	Speed(g)	Time(min)	T (°C)	(°C)	Speed (g)	Time (min)	T (°C)		
19	EDTA	PFP	1st: 1,200 2nd: 12,000	15 12	20 20	-70	2x100,000	60	20	• Flow cytometry • EM • 1B10 antibody capture assay combined with MP-TF assay	(34)
21	Citrate	PPP	2,600	15	4	-	-	-	-	▪ Flow cytometry	(33)
n.s.	Citrate	PPP	1,550	20	20	-80	18,890	30	20	▪ Flow cytometry ▪ MP-TF assay ▪ Fluorescence confocal microscopy	(49)
21	Citrate	PFP	1st. 1,500 2nd: 13,000	15 2	RT RT	-80	-	-	-	▪ Flow cytometry	(51)
19 (butterfly needle)	Citrate	PPP	3,000	15	n.s.	-40	20,000	30	n.s.	▪ Flow cytometry ▪ EM ▪ Prothrombinase assay	(80)
n.s.	Citrate	PPP	2,000	15	8	-80	-	-	-	▪ TF-ELISA ▪ MP-TF assay	(52)
21	Citrate	PFP	1st: 200 2nd: 20,000	15 15	20 20	-80	-	-	-	▪ EM ▪ MP-TF assay	(86)
n.s.	Citrate	PFP	1st: 1,500 2nd: 13,400	15 2	n.s.	-80	-	-	-	▪ Annexin V-capture assay	(53)
21	EDTA-ACD	PPP	8,000	5	RT	-40	-	-	-	▪ PMP-capture assay	(31)
21	ACD	[a]PPP [b]PFP	[a] 2x 1,550 [b] 1st: 2x 1,550 2nd: 10,000	20 20 10	n.s.	-80	-	-	-	▪ Flow cytometry	(23)
n.s	Citrate	PPP	2x 2,100	20	n.s.	-80	-	-	-	▪ Impedance-based flow cytometry	(75)
21	Citrate	PPP	2x 2,000	10	20	-	18,890	30	20	▪ Flow cytometry ▪ AFM	(58)

-: not done; n.s: not specified; RT: room temperature

Table 2. The effect of different centrifugation steps and storage on flow cytometry detection of PMP in citrated plasma of healthy subjects. MPs were measured either directly in plasma or after an additional centrifugation step to isolate the MPs from plasma (fresh or frozen/thawed).

Healthy Volunteers		Plasma Preparation				Frozen* / Thawing	MP Isolation			PMP/10^6 L		Ref.
N	Sex	Type	Speed (g)	Time (min)	T (°C)		Speed (g)	Time (min)	T (°C)	Mean	Range	
10	ns	PPP	1,550	15	RT	-	-	-	-	922	(365-1,802)	(40)
15	m: 15	PPP	1,550	20	20	-	17,570	30	20	237	(116-565)	(110)
7	m, f: 7	PPP	2x2,000	2x10	20	-	18,890	30	20	141	(63-291)	(58)
60	m: 30 f: 30	PFP	1st: 1,500 2nd: 13,000	15 2	RT RT	-80°C/37°C	-	-	-	656 1,775	(407–962) (1,014–3,039)	(51)
47	m: 18 f: 29	PFP	1st: 1,500 2nd: 13,000	15 2	RT RT	-80°C/n.s.	-	-	-	625 ± 493		(111)
45	m: 29 f: 16	PPP	2x3,000	2x15	RT	-80°C/37°C	-	-	-	945	(659–1,393)	(112)
20	m: 4 f: 16	PPP	2x2,000	2x30	4	-70°C/n.s.	20,800	45	10	517 ± 72		(113)
37	m: 16 f: 21	PPP	1,550	20	20	-80°C/on melting ice	18,890	30	20	1,500	(700-7,100)	(49)
20	m: 16 f: 4	PPP	1,550	20	20	-80°C/on melting ice	17,570	30	20		~4,500-6,250	(50)
10	m: 4 f: 6	PPP	1,550	20	20	-80°C/on melting ice	18,890	30	20	3,200	(2,500-4,100)	(21)

-: not done; n.s. : not specified; RT: room temperature; m: male; f: female; *: storage

Analytical methods for MP measurements

Common MP assays

Flow cytometry

Flow cytometry is commonly used for the measurement of MPs. It is fast and allows both the enumeration of MPs and the assessment of their cellular origin (4;8). MPs pass a laser in a flowing sample stream and are counted by flow cytometer. The enumeration of MPs is based on the light that is scattered by each MP in forward direction (roughly proportional to their size) and in side direction (dependent on to granularity and structural complexity). However, the laser used by the flow cytometer excites at 488 nm. As MPs are heterogeneous and can have sizes below 488 nm, this wavelength is not suitable as a trigger or discriminator for MP detection (55;58;60).

To provide the concentration or absolute count of MPs in a sample, counting beads of a known concentration are used. These are calibrated microsphere-polypropylene/latex beads (mean diameter 7-9 or ~10 µm) that are brightly fluorescent across a wide range of excitation and emission wavelengths. When they are used as internal standard, the beads are added to each sample before the flow cytometry measurement. When used as external standard, the counting beads are processed as a sample at the same flow cytometry settings/conditions used for the samples. The flow cytometer counts the number of beads and/or MPs in the sample until the acquisition time is reached. With these numbers, the concentration of MPs in plasma can be calculated (50;51;58;61). As to the flow cytometry settings, the MP population is defined by using size calibration beads. Beads of 1 µm diameter are used to define the upper limit of the MP population and to discriminate the MP population from platelets (34;40). Another possibility is to use a mixture of fluorescent beads with diameters of 0.5, 0.9, and 3 µm (Megamix beads) to cover the MP (0.5 and 0.9 µm) and platelet populations (0.9 and 3 µm) ranges (51;62). However, as the refractive index of the plastic beads is different than that of MPs, also these beads remain an imperfect model for defining the cut-off of MP population.

The cellular origin of MP can be assessed by staining MPs with fluorescently labelled antibodies. In a flow cytometer, the fluorescent light passes a series of filters and appropriate fluorescence detectors allowing the simultaneous detection of more than two antigens on one MP (63). The correlation of the fluorescence signal to the light scatter of all measured events is used to identify and count a certain subset of MPs. The downside of this approach is that the fluorescence

signal depends on the specificity and affinity of the antibody to the target antigen and on the number of antigens present on the MP surface.

Although not all MPs expose PS (34;61;64-67), fluorescently labelled annexin V is commonly used to measure the total number of MPs with flow cytometry. The binding of annexin V to MPs is influenced by the calcium concentration and the membrane PS content (64;68). Consequently, the concentration of calcium in the buffer should be titrated for optimal staining of MPs in citrate- and EDTA-anticoagulated plasma samples. Staining with lactadherin, a milk-derived protein that also binds PS, has been shown to give similar results as obtained with annexin V staining (69). Lactadherin has been claimed to be more sensitive to small changes in PS expression than annexin V (66) and it detects PS-positive MPs in a calcium-free environment, such as citrate- or EDTA-anticoagulated plasma samples. Other markers such as bio-maleimide, phalloidin, and calcein acetoxymethyl ester (calcein AM) have been recently used to improve the detection of MPs. The staining with bio-maleimide, a thiol-reactive probe with spectral characteristics very similar to fluorescein, was found to give similar results as staining with annexin V (70). Staining with phalloidin and calcein AM seems to be useful to avoid false positive counts related to platelet or other cellular fragments. Phalloidin, a cyclic peptide which binds f-actin with high affinity, only stains platelet fragments discriminating these from PMPs (54). Calcein AM, a non-fluorescent marker that becomes fluorescent upon cleavage by cytosolic esterases only stains intact MP/cells and is not reactive with cellular fragments (65;71;72)

Flow cytometry uses fluorescently labelled specific antibodies to identify the cellular origin of MPs. These antibodies can distinguish MPs derived from platelets, leucocytes, erythrocytes, and endothelial cells. A list of such antibodies can be found in the publication of the Forum “Measuring circulating cell derived microparticles” (8) and some recent reviews (4;73;74). In addition, there are antibodies that have been used for the detection of MPs derived from malignant cells such as MUC1 for breast and pancreatic cancer cells (49;75), FasL for melanoma cells (76), EGFRvIII for glioblastoma cells (77), and CXCR-4 for leukemic cells (78).

The results of flow cytometry measurements depend on the type of instrument, instrument settings, and resolution. Recently, the ISTH SSC Working Group on Vascular Biology proposed the use of calibrated beads with sizes of 0.5, 0.9 and 3 µm (Megamix beads) to adjust the instrument setting and increase the resolution of the flow cytometer. The primary goal was to standardize the enumeration of PMPs >0.5 µm by using flow cytometry (51). Forty laboratories from 14 countries participated in the study. Firstly, these laboratories had to establish the resolution

and the level of background noise of their cytometers and to set the window of MP analysis by using the same batch of Megamix beads. Secondly, to define the inter-instrument reproducibility they received the same frozen PFP with three different concentrations of PMPs and used the same batch of phycoerythrin-labelled anti-CD41/IgG1 and fluorescein isothiocyanate-labelled annexin V from BioCytex (Marseille, France) for PMP staining. Several types of flow cytometer were used by these laboratories, such as FACSCalibur, FACSCanto II, and LSRII (all from Becton Dickinson) and EPICS XL, Gallios and FC500 (all from Beckman Coulter). The results of this study have been reported recently (62). Among 59 registered instruments from 40 laboratories, 49 were tested and 33 (67%) from 29 laboratories were qualified for subsequent analysis of PMP samples indicating that the strategy of using calibrated megamix beads is useful for the standardisation of PMP measurements by flow cytomety in multi-centre studies.

The enumeration of MPs by flow cytometer will not only be influenced by the analytical factors (e.g. laser, antibody, type of instrument, instrument calibration), but also on factors related to the sample preparation and storage (see Table 2). Especially the presence of residual platelets, storage of the blood/plasma, freezing/thawing of the plasma and centrifugation can influence MP counts.

Electron and fluorescence confocal laser scan microscopy

Electron microscopy (EM) can be used to study the morphology and membrane composition of MPs. In addition information on the antigenic composition of isolated MPs can be obtained by using immunogold-labelled antibodies (34;65;79). Heijnen et al (79) firstly reported that there were two types of membrane particles released by platelets after stimulation with thrombin receptor agonist peptide (TRAP). They concluded that the larger particles (diameters of 0.1-1 µm) were MPs because these particles were positively stained by immunogold-labelled antibodies against annexin V, GPIb, Pecam-1, integrins αIIb-β3, and two tetraspanins, Peta-3 and CD9. In contrast, from the smaller particles (diameters of 40-100 nm and defined as exosomes) less than 30% were stained by these antibodies, while about 50% were stained by gold labelled anti-CD63. Aras et al (34) performed EM imaging of MPs (diameters of 0.1-0.5 µm) isolated from PFP of a volunteer receiving endotoxin and showed that MPs have vesicular structures, but no cellular organelles. Interestingly, some of these MPs expressed CD14 and/or TF antigen as shown by immunogold labelling. By using EM, Bernimoulin et al. (65) demonstrated that MPs derived from THP-1 cells contain cytoskeletal proteins organized into a complex cytoskeletal network. As to the enumeration of MPs, EM can be used to make a rough estimate of the relative number of MPs in preparations subjected to flow cytometric analysis (54;80).

EM operational procedures require a vacuum environment and dehydration of the sample. These conditions may affect particle/MP morphology and are also not suitable for direct detection of MPs in plasma. The standard diameter of EM grids, 3.05 mm, can only hold a small sample volume (~2-4 µL). The sample material used to perform a conventional on-grid negative staining by using uranyl acetate preferably contains 0.1 to 1.0 mg protein/mL in aqueous solution (81). Therefore, plasma MPs need to be isolated and concentrated by centrifugation. In addition, EM produces a two-dimensional image of a sample, which gives an inaccurate estimation of the MP size.

Prior to detection by confocal laser microscopy, MPs are stained with fluorescently labelled antibodies to enable the identification of subsets of MPs based on their antigen expression. Confocal laser microscopy has been used by Dale et al (82) to characterize PMPs. These PMPs were 0.3–0.5 µm in diameter and positive for glycoprotein IIb/IIIa, glycoprotein Ib, CD9, and PS, but negative for fibrinogen and thrombospondin. Interestingly, confocal laser microscopy also can be used to identify fusion particles. For instance, Tesselaar et al (49) used fluorescence confocal laser microscopy to demonstrate that part of the MUC1-positive MPs isolated from the plasma of a metastatic breast carcinoma patient, co-expressed the platelet specific antigen, CD61. The fluorescence intensity profiles of these MPs corresponded with a MP diameter of approximately 0.15-0.2 µm. However, this estimation of MP diameter was based on a two-dimensional image and therefore inaccurate. Unfortunately it is not possible to use this method directly in plasma because of the high background caused by the interference of plasma proteins.

Like other optical methods that are based on the detection of a fluorescence signal, the results obtained by confocal laser microscopy also depend on the specificity and affinity of the antibody to the target antigen and on the density of the antigens on the MP surface.

EM and fluorescence confocal laser scan microscopy give information on morphological features of MPs (e.g. size, membrane structure, cytoskeleton). The operation of both methods requires several hours and they are not suitable for direct detection and quantification of MPs in plasma. However, they are useful for visualizing MPs and for validation of other MP measurements.

Capture-based assays

For capture-based assays, wells are coated with a probe (antibody or annexin V) that specifically binds a subset of MPs from plasma. Captured MPs can then be

quantified/characterized by using a second probe (e.g., peroxidase conjugated-antibody) (31;32) or by using a functional property of the captured microparticles (e.g. their procoagulant activity) (53;56;57;83). At present there is limited experience with this type of approach, while there are still many questions related to the kinetics of MP capture. Nevertheless, we will briefly describe some of the test designs that have been proposed in the literature. A clear advantage of these assays is that they will allow the measurement of MPs directly in plasma.

Nomura et al (32) and Ueba et al (31) described a method to measure the number of PMPs in EDTA-ACD PPP. Wells were coated with a monoclonal antibody against glycoprotein CD42a, while anti-CD42b, conjugated to a peroxidase, was used as secondary antibody. The concentration of PMPs is reported as U/mL where one U/mL is defined as the number of PMPs obtained from 24,000 solubilised platelets/mL.

Aupeix et al (84) first reported the use of annexin V-coated wells for the quantification of procoagulant MPs. The immobilized annexin V captures PS containing MPs from recalcified PFP. The procoagulant surface of these MPs is then quantified by measuring the prothrombinase activity after adding excess FXa-FVa. A direct relationship exists between the procoagulant phospholipid concentration and the amount of thrombin generated. Hugel et al (8) described this assay in detail in a publication of the Forum “Measuring circulating cell derived microparticles”. Liposomes of known concentration containing 33% PS/67% PC (mol/mol) were used as reference material. The MP concentration is reported as nM PS equivalent.

Meanwhile two commercially available variants of this assay are available (Actichrome® MP activity from American Diagnostica, Connecticut, USA and Zymuphen MP activity from Hyphen Biomed, Neuville-sur-Oise, France). These assays use a washed and lysed platelet concentrate as calibrator. Results are reported as nM PS.

Annexin V captures PS containing MPs in the presence of calcium ions. Therefore, the free calcium concentration in (diluted) plasma is an important determinant, which may influence the capture efficiency. As PFP is used for these measurement, the type of anticoagulant (EDTA, 3.2% citrate, 3.8% citrate) used for blood collection may influence the test result. The need to recalcify the plasma also raises the possibility of activation of the clotting system. This should be prevented by adding specific inhibitors to the (diluted) plasma. A final analytical issue is that the calculation of the MP concentration in plasma is based on comparison with a calibrator which does not have the same characteristics/behaviour as plasma MPs.

A limitation of this approach is the need to use fresh plasma, because freezing thawing will result in an increase in the number of annexin V-positive MPs (43;47)

Instead of biotinylated annexin V, biotinylated antibodies (e.g. anti-CD42b for platelets, anti-CD31 and anti-CD105 for endothelial cells, anti-CD3 and/or CD4 for T lymphocytes and anti-CD11a for leucocytes) have been used to capture specific subsets of MPs and then measure the prothrombinase activity as in the annexin V capture-based assay (8;83). As a control, irrelevant IgG is used for capturing MPs. The MP concentration in this antibody capture-based assay is expressed in nM PS as in the annexin V capture-based assays. A problem with the interpretation of the results of these tests is that part of the captured MPs might not express PS and that the specificity of the antibodies is broader than generally assumed (e.g., anti CD31 and anti CD105).

Aras et al (34) described the use of an immunoglobulin M monocolonal mouse anti human fibroblast surface protein (1B10) to capture MPs from PFP. Captured MPs were analysed for the presence of active TF using a two-step chromogenic assay. The TF-dependent FXa generation (pM/hour) was determined after subtracting the amount of FXa generated in the presence of polyclonal anti-human TF antibody prior to addition of FVIIa and FX. The 1B10 antibody apparently recognizes antigens on the surface of monocytes, granulocytes, endothelial cells, smooth muscle cells and fibroblasts, but not on platelets and epithelial cells. Consequently, MPs originating from platelets and epithelial cells are most likely not measured in this assay.

These capture-based assays enable a high-throughput measurement of subsets of MPs but will give no information on the size and total number of these MPs in plasma. Specific problems related to the antibody capture-based assays are not only the eventual presence of soluble antigen together with MP bound-antigen (42), but also the specificity and affinity of the antibody for the target antigen, and the amount of antibody coated on the well. Together they will determine the number of MPs captured in the assay.

Apart from the analytical factors that influence the capture-based assays, pre-analytical steps such as blood collection, plasma isolation and sample storage are also important. Although frozen-thawed plasma is frequently used in capture-based assays, there is not much information on how freezing and thawing of plasma will affect the concentration of MP in these assays.

Functional assays on isolated MPs

The analysis of certain functional properties of MPs can be achieved by separating MPs from the plasma proteins. An example of this is the measurement of TF activity in isolated MPs. The isolated MPs are supplemented with excess FVII(a) and FX in the presence of calcium, after which the formation of FXa is measured with a specific chromogenic substrate using either a one-stage (49;85) or two-stage approach (86;87). The TF activity of MP (MP-TF activity) is defined as the FVII dependent and anti-TF sensitive formation of FXa. MP-TF activity and has been expressed as fM Xa/min (no external calibrator) or as pg/mL (using relipidated recombinant human TF as a calibrator). The one-stage assay uses excess negatively charged phospholipids in the assay (49;85;88), while the users of the two-stage assay do not add phospholipids. In our own experience the presence of these phospholipids does not importantly affects the TF activity of isolated MPs. Interestingly no difference in MP-TF activity was observed between MPs isolated from fresh plasma and MPs isolated from frozen thawed plasma (49). A commercial two-stage chromogenic assay (Actichrome® TF, American Diagnostica, CT, USA) has also been used to quantify TF activity in the isolated MP fraction. Although this assay has also been used to measure TF activity in plasma (57;89), the observed FXa generation was found to be independent of exogenously added FVIIa and/or TF, and not inhibited by anti-FVIIa (90).

One limitation of all available TF-dependent FXa generation assays is the lack of an accepted standard for TF. For this reason, a working group on TF standardization in cancer was presented by the SSC at ISTH 2005. This group aimed and proposed to compare measurement of TF with a variety of methods in different laboratories and to improve intra- and inter-laboratory reproducibility by developing standardized protocols, appropriate reagents, and reference materials.

New approaches in MP assays

Proteomics analysis

Mass spectrometry (MS)-based proteomic analysis provides an opportunity to characterize the protein composition of MPs. Generally this method requires extraction, separation, trypsin digestion, MS analysis, and identification of proteins. Cell-surface antigens are generally of high molecular weight and hydrophobic (91;92). Therefore, an ionic/non-ionic detergent (e.g. sodium dodecyl sulfate, Triton X-100, NP-40) or a high-pH solvent (sodium carbonate pH 11) is used for the extraction of the membrane proteins prior to the gel separation and MS analysis

(92;93). Finally, each MS spectrum is used to search the protein database for matched peptides (91;94).

Proteomic analysis has been used by several groups to investigate the protein content of PMPs (29;30). For instance, PMPs isolated from the supernatant of ADP stimulated-platelets contained membrane surface proteins derived from platelets (GPIIIa, GPIIb, and P-selectin), chemokine ligand 4 (CXCL4), pro-platelet basic protein (CXCL7), and RANTES (CCL5). Proteins from the alpha granules (fibrinogen, vWF, FV and FXIII, thrombospondin, and protein S) were also found (30). In a different study (29), several proteins were detected in MPs isolated from plasma that were not found in PMPs isolated from the supernatant of ADP stimulated-platelets. These included proteins associated with apoptosis (CD5-like antigen, galectin 3-binding protein, and several complement components), iron transport (transferrin, transferrin receptor, haptoglobin), immune response (immunoglobulin J and Kappa chains), and blood coagulation (protein S and FVII). Some of these might represent contamination of the MP with plasma proteins.

The supernatant of TRAP-induced platelets contains mainly proteins from alpha granules (28). Some proteins potentially involved in angiogenesis, such as platelet-derived endothelial cells growth factor (PD-ECGF), angiopoietin-1, and galectin-3 binding protein are also found in this fraction. Importantly, four different isoforms of protein disulfide isomerase (PDIA3, P4HB, PDIA4 and PDIA6) were found, proteins that have been identified as critical mediators of wound healing (95) and assist in the de-encryption of an inactive form of TF into its active form (96;97). Recently, the presence of PDI on the surface of PMPs was also reported (98).

Proteomic analysis allows identification of various proteins in a complex mixture. However, quantification of proteins is difficult. The contamination of MPs with abundant plasma proteins like albumin and immunoglobulins might affect the results of proteomic analysis (94).

Impedance-based flow cytometry

Unlike the conventional flow cytometer, impedance-based flow cytometry is based on the Coulter principle to detect changes in electrical impedance produced by particles in suspension. These changes can be measured as a voltage pulse or a current pulse. The pulse height is proportional to the volume of the sensed particles (99). For MP measurement, this system is calibrated using fluorescent polystyrene microspheres of uniform size. Subsequently, MPs can be detected directly in PPP by using fluorescently labelled antibodies.

Zwicker et al. (75) have compared the measurement of PMPs, calibrated microspheres (0.78 µm), and platelets in frozen-thawed PPP, both by impedance- and light scatter-based flow cytometry. The size of platelets and PMPs is poorly characterized by the light-scatter based flow cytometry and both populations overlap with the 0.78 µm-microspheres. Conversely, the impedance-based flow cytometer could resolve the populations of PMPs, calibrated microspheres, and platelets. The same group (75) used impedance-based flow cytometry to measure the number of TF-bearing MPs in PPP of pancreatic cancer patients and found 3 to 4 orders of magnitude more MPs than previously reported by Tesselaar et al (49) who used light scatter-based flow cytometry. This might confirm that light scattering is not suitable for the enumeration of TF-bearing MPs. Impedance-based flow cytometry is presently used to quantify TF bearing MPs in the MicroTEC study, a randomized, multi-centre trial to evaluate the benefit of primary thrombophylaxis in cancer patients with high levels of TF bearing MPs (100).

In impedance-based flow cytometry, the size detection limit of the measurement depends on the diameter of the flow cell. Postmarket installation of smaller flow cells with diameters of 25 and 40 µm is required to analyze particles between 0.3 and 1.0 µm in diameter (75). Thus, MPs below 0.3 µm in diameter might not be detected by using these flow cells. Using fluorescently labelled antibody, the size distribution of MPs can theoretically be determined from the amplitude of the fluorescent signal under the assumption that the signal is either proportional to the volume or the surface of the particles (in the case of surface labeling). In addition the size distribution is calculated assuming a spherical shape. In the case of fluorescent labeling one generally assumes that the labeling efficiency is independent of the particle shape and (surface) composition.

Dynamic light scattering (DLS) and Nanoparticle tracking analysis (NTA)

Dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) measure Brownian motion of MPs in liquid suspension and from this movement the particle size can be calculated using the Stokes-Einstein equation (101). NTA measures the movement of each particle through image tracking analysis, whereas DLS measures all particles at the same time and produces an average particle size.

Two DLS systems, the Zetasizer Nano S (Malvern Instruments Ltd, Worcestershire, UK) and the N5 Submicron Particle Size Analyser (Beckman Coulter, CA, USA) were used to examine the size distribution of MPs in frozen-thawed plasma (102). It was found that MPs with sizes ranging from 50-1000 nm could be detected by the N5 instrument utilizing a 30.1° angle of measurement, whereas the Zetasizer detected some particles considerably larger than those

identified by the N5. The main disadvantage of DLS for measuring MPs is that it performs best in studying monodisperse particles while cell-derived MPs are clearly polydisperse. This makes that the size distribution of MPs measured by DLS is biased towards the presence of small numbers of large particles or contaminants as they scatter light more intensely than the smaller particles. The Zetasizer Nano S uses the back scattered light to increase the sensitivity of the method, while the N5 system can use up to 6 different scattering angles. The scattering angles of both instruments do not overlap.

This problem seems not to occur with the NTA approach because a microscope and a CCD camera track and visualize the individual particles by using particle tracking image analysis software. Harrison, et al. (103) used an NTA system, known as Nanosight LM10 (Nanosight, Amesbury, UK) to measure MP distributions in isolated MPs and in PPP diluted in PBS (1:40-1:60). They found that MPs have a polydisperse distribution up to 1,000 nm, but with a predominant population from lower than 50 nm to above 300 nm. They estimated that PPP contains ~200-260x10^9 MPs/L which is 1,000-fold higher than previous estimates based on using flow cytometry.

As mentioned before, NTA can accurately size particles in a sample, but unfortunately the presence of a few larger particles will reduce the number of small particles detected by the software. This has been demonstrated by Filipe et al (104) by mixing 60-nm/100-nm beads in a 4:1 ratio. The NTA analysis of this mixture showed two distinct size populations, but it detected more 100-nm beads than 60-nm beads due to the masking effect of the larger beads over the smaller beads.

For MP measurements the NTA approach has some disadvantages when compared to the DLS approach. The operation of NTA is not yet as user friendly as that of DLS. This is due to the fact that several parameters need to be adjusted carefully by a skilled operator prior to the NTA measurement. These parameters include the settings for the video capture and analysis, which are essential to obtain accurate and reproducible measurement results. As the Brownian motion of a particle in a solution depends on the viscosity and temperature of the solution, these parameters should also be defined prior to MP measurement in (diluted) plasma.

NTA and DLS may be unable to differentiate MPs from other particles present in plasma like (lipids/lipoproteins). Recently, Nanosight has launched a fluorescence-NTA, the NS500. This instrument enables the detection of individual quantum dots, fluorescent nanoparticles with a diameter of 2-10 nm, with minimal background

interference of other particles in the solution. This will hopefully enable the detection of specific subsets of MPs, for example by using quantum dot-labelled antibodies.

Atomic force microscopy

Scanning probe microscopy, especially atomic force microscopy (AFM), has opened new perspectives in biomedical research for the investigation of (bio)particles (105). AFM probes the surface of a sample with an ultra-sharp tip, a couple of microns long and often less than 100 Å in diameter. The tip is located at the free end of a cantilever which is 100-200 µm long. The interaction force between the tip and the sample depends on their distance. A detector, often with the use of a laser beam, measures the cantilever deflection when the tip moves across the sample or when the sample is moved under the tip. The measured cantilever deflections allow a computer to generate a three dimensional (3D)-surface topography (x, y and z dimensions) (106). The detection is so sensitive that forces can be detected as small as a few picoNewton. The advantage of employing AFM in the study of MP is that AFM can be operated in fluid tapping mode. The AFM tip is oscillated in liquid to create intermittent contact with the sample surface allowing the preservation of sample properties in their physiological state (Figure 1). A flat surface is needed for MP attachment, in order to distinguish individual MPs from the roughness of the supporting surface. Muscovite mica is a commonly used surface for AFM imaging because this non conductive mineral consists of layers which can be easily cleaved to produce clean and atomically flat surfaces (107). After cleaving, the mica surface can be modified to covalently attach a protein or antibody.

Recently, Yuana et al (58) have used AFM operated in fluid tapping mode for the sensitive detection of a defined subset of MPs isolated from double-centrifuged PPP. Using AFM and mica-coupled anti-CD41 1,000-fold more CD41-positive MPs were detected than by using conventional flow cytometry. These MPs have sizes ranging between 10-475 nm with a peak at 67.5 nm which is clearly below the detection limit of conventional flow cytometry. After freezing and thawing a decrease in the number of CD41-positive MPs was found by AFM, but the size distribution of these MPs remained the same. AFM also was employed to investigate the formation of PMPs by Siedlecki et al. (108). PMPs were generated by using surface-activated and thrombin-stimulated platelets deposited on glass and were found to have a similar size distribution as reported by Yuana et al (58). Siedlecki's group also showed that PMPs are clustered in close proximity to adherent platelets and localized in the platelet periphery and in some cases appeared to originate from platelet pseudopodia.

Baran et al (109) used AFM to detect MPs from frozen-thawed PFP isolated from EDTA blood of gastric cancer patients. AFM detected MPs mainly with sizes around 10 nm. This result was confirmed by using other techniques such as transmission EM and DLS. However, they did not operate AFM in fluid, so the characteristic of MPs might have changed.

AFM can be operated in fluid to detect MPs with a minimal force, thereby preserving their natural state, but it also enables the detection of the individual MP and simultaneously measures the 3D-size of MPs. Furthermore, the numbers of MPs attached to the surface can be quantified by using image processing software. When a specific antibody is used to coat a surface, a specific subset of MPs can be captured (58). However, as MPs need to be isolated from plasma and concentrated prior to AFM measurement, the isolation step might influence the morphology and the number of MPs. At present, the detection of MPs by AFM is low-throughput (typically 2 hours per sample). Therefore, modifications that will allow faster detection of specific populations of MPs in plasma will be essential.

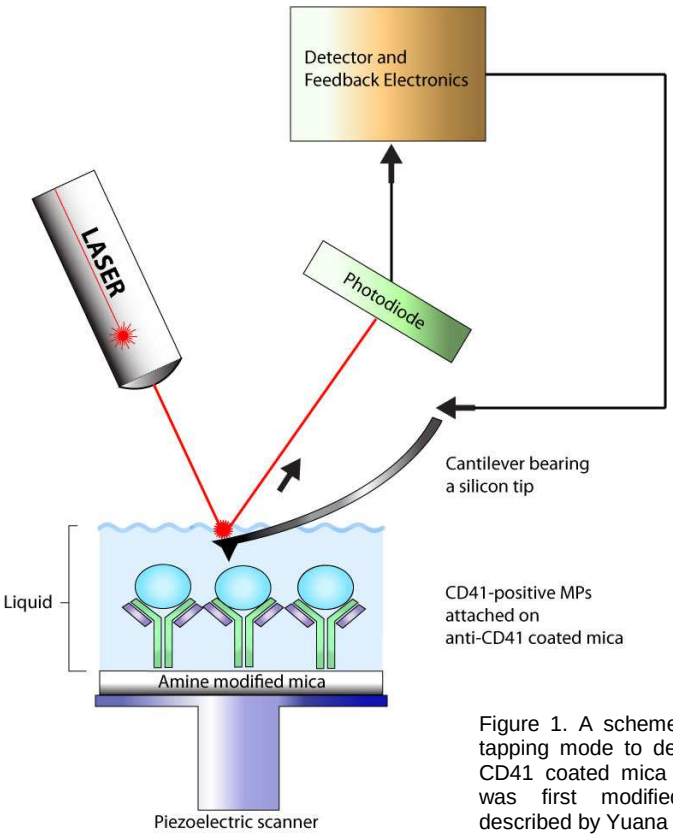

Figure 1. A scheme showing AFM operated in tapping mode to detect MPs attached on anti-CD41 coated mica surface. This mica surface was first modified with ethanolamine as described by Yuana et al. (58).

Recommendations

Results of plasma MP measurements reported in the literature vary widely. This is clearly not only related to the lack of well-standardized high-throughput MP assays, but also to variations in pre-analytical conditions. Some examples of these variations are listed in Table 1. Therefore, standardisation of both pre-analytical procedures and assay protocols is urgently needed.

When flow cytometry is chosen as a method for quantification of MPs, some analytical conditions should be standardized. Before measurement of the samples, the setting of the flow cytometer should be adjusted by using calibrated size standard beads, for example Megamix beads, to define the threshold (>0.5 µm and <0.9 µm) of MP detection. Counting beads should be used to measure the concentration or absolute count of MPs in a sample. For the characterization and enumeration of subsets of MPs the choice of antibody for labelling and capturing MPs is crucial. The antibody should be specific and have a high affinity for the target antigen.

Since not all plasma MPs expose annexin V and the percentage of annexin V-positive MPs can differ importantly depending on preanalytical conditions, the total number of MPs should not only be determined by binding of annexin V to MPs. In flow cytometry measurements and annexin V-capture assays binding of annexin V to MPs is influenced by the calcium concentration and the membrane PS content. Thus, it is important to find/develop a marker for direct detection of total number of MPs in a calcium-free environment.

Fluorescence-NTA and AFM can accurately count the nanosized MPs, but unfortunately both do not give information on the functional properties of MPs. Before employed in the clinic these methods need to be further validated. Similar like other methods which rely on the use of an antibody for the detection of MPs, specific and high affinity antibodies are required for the fluorescence-NTA and AFM.

Remaining Issues

Ideally MP measurements should be performed in plasma, freshly prepared directly after blood withdrawal. Platelet contamination seems to be one of the major pre-analytical problems in processing plasma for MP measurement. Platelets might be activated and/or fragmented resulting in an increase in the number of PMPs. Therefore, the choice of the anticoagulant used for blood collection and the centrifugation speed used for plasma preparation is critical. Freezing/thawing of

PPP unfortunately results in changes in the number of PMPs and in the number of PS-exposing MPs. There is therefore a great need for internationally accepted recommendations for the preparation and storage of plasma for MP measurements and for the isolation of MP from plasma. In addition, standardization of protocols, including the use of appropriate reagents and reference materials, is needed to improve intra- and inter-laboratory reproducibility in MP measurements.

At this moment we still have very limited information on what type of MPs (e.g. origin, protein content, and morphology) play a role in the development and progression of vascular disorders, cancer, and auto-immuno diseases. This is mostly due to the limitations of the methodology presently used for the enumeration of specific subsets of plasma MP. Some new methods (Impedance-based flow cytometry, DLS, NTA, and AFM) have been developed in the past years that might enable the accurate measurement of these nanosized MPs, but unfortunately these methods do not give information on the functional properties of MPs. Moreover, at this moment these methods are impractical for use in the clinic as they are low throughput and labour-intensive.

Acknowledgments

This work is supported by the Dutch Cancer Society (KWF UL 2006-3618). The authors thank Dr. T.H. Oosterkamp and Dr. M.E. Kuil for their critical reading of the manuscript.

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