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Review

Pre-analytical and methodological challenges in red blood cell microparticle proteomics

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ABSTRACT

Microparticles are phospholipid vesicles shed mostly in biological fluids, such as blood or urine, by various types of cells, such as red blood cells (RBCs), platelets, lymphocytes, endothelial cells. These microparticles contain a subset of the proteome of their parent cell, and their ready availability in biological fluid has raised strong interest in their study, as they might be markers of cell damage. However, their small size as well as their particular physico-chemical properties makes them hard to detect, size, count and study by proteome analysis. In this review, we report the pre-analytical and methodological caveats that we have faced in our own research about red blood cell microparticles in the context of transfusion science, as well as examples from the literature on the proteomics of various kinds of microparticles.

Contents

1. Introduction

By its importance for living organisms, blood is often called "fluid of life". Transfusion is indeed vital and indicated in numerous clinical situations such as severe haemorrhage, anaemia or hypovolemia, in which case red blood cell (RBC) concentrates are administered to sustain the oxygenation of tissues, haemostasis imbalance or disorders, in which case platelet concentrates are administered, or deficiency of coagulation factors (transfusion of fresh frozen plasma). Since the beginning of transfusion, numerous efforts have been made to secure blood products and gain knowledge about their molecular structures. With millions of blood product transfused worldwide every year, each incremental piece of progress has a potential broad effect on a great number of lives. Nevertheless, there is still a risk of side effects associated to transfusion such as fever, inflammation, and iron overload or autoantibody formation [1,2].

Erythrocyte concentrates (ECs), platelet concentrates (PCs) and fresh frozen plasma (FFP) are the three main labile blood products and have to be stored according to their particular components.

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Those components are subjected to modifications or degradations during storage, a process known as the "storage lesion" [3]. RBC ageing in blood banking conditions differs from physiological *in vivo* ageing. Indeed, conditions to which RBCs are exposed during storage, such as temperature and nature of the medium, are dramatically different from physiological conditions. Several preservative solutions such as citrate–phosphate–dextrose–adenine (CPDA), saline–adenine–glucose–mannitol (SAGM) or phosphate–adenine–glucose–guanosine–saline–mannitol (PAGGSM) are available in routine [4]. These additive solutions allow to store red blood cell concentrates from 35 days up to 49 days in accordance with the European transfusion standards [5], requiring that at least 75% of erythrocytes must survive *in vivo* 24-h after transfusion (see Table 1). During storage many physiological and biochemical alterations occur in the supernatant of ECs including an increase in the concentration of lipids, MPs, free haemoglobin and a pH reduction. Red blood cells also undergo several changes such as loss of potassium, adenosine triphosphate or 2,3-diphosphoglycerate. Their membranes become more rigid, there is a disruption of phospholipid asymmetry, lipid raft rearrangement, loss of fragments or even release of MPs [6]. The effects of storage lesions, including the shedding of MPs, on transfusion efficiency and potential side effects are not clearly understood. Among other approaches, proteomics allows the investigation of important issues in blood research [7] and transfusion science [8] in order to gain better knowledge of the blood products delivered to patients as well as gain insight into the mechanisms at work in transfusion side effects.

This review focuses on methodological and analytical challenges in the proteomic analysis of red blood cell-derived microparticles. However, examples from other microparticle types (platelet, endothelial,. . .) are discussed as well to highlight some analytical or methodological aspects. Moreover, whereas flow cytometry is a central tool for the analysis of microparticles, it is beyond the scope of this review to discuss the various techniques used to analyze microparticles by flow cytometry. Excellent reviews have been published [9], and we discuss here only challenges and recent development in flow cytometry.

2. What are microparticles?

MPs are plasma membrane vesicles shed in blood flow by various types of cells such as platelets, red and white blood cells, or endothelial cells. Those MPs, also known as microvesicles [10] or in some cases as ectosomes [11] have a size of less than 1 μm [12] and contain a subset of proteins derived from their original cells as well as surface receptors allowing the identification their origin. Most studies generally agree that MPs are heterogeneous and vary in size, concentration, phospholipid composition, surface antigens and protein content. Release of MPs is thought to be a highly controlled process prompted by various stimuli such as shear stress, complement attack, pro-apoptotic stimulation or damage [13]. Although still subject to discussion, a model of vesiculation has been established. This model brings in translocases, lipid rafts, various protein modifications and irreversible membrane rearrangements. [14]. Recently, an association between erythrocytes aging processes and MPs formation has been proposed as a part of an apoptosis-like form in erythrocytes [15]. This "aging" process of red blood cells has also been observed during storage in blood bank condition [16].

MPs have long been considered as cell fragments or "dust" without any biological role. Although their functions are still largely unknown, there are more and more evidences that MPs are involved in a broad spectrum of biological activities [14] such as haemostasis [17], thrombosis [12], inflammation [17], transfer of surface proteins [18] or even angiogenesis [19].

Table 1
The development of RBC storage solutions. ACD, acid citrate dextrose; AS-3, additive solution 3; CPD, citrate phosphate dextrose; CP2D, high dextrose CPD; CPDA-1, CPD plus adenine; CPDA-2, CPD with adenine and extra dextrose; ½ CPD, half volume CPD; PAGGSM, phosphate, adenine, glucose, guanosine, saline and mannitol; RAS-2, research additive solution 2; SAG, saline adenine glucose; SAGM, SAG plus mannitol. Adapted from Ref. [4].

	Typical recovery	Haemolysis	Vesicles
Three-week storage			
ACD	75% [58]	0.1% [58]	
CPD	79% [58]	0.1% [58]	
Five-week storage			
CPDA-1	72% [59]	0.5% [59]	70% [60]
CPD/SAG	83% [61]	0.6% [61]	
Six-week storage			
CPDA-2	80% [62]		
CPD/SAGM	78–84% [61]	0.4% [61]	25% [63]
CP 2D/AS-3	78–84% [61]	1.0% [61]	
Seven-week storage			
CPD/PAGGSM	74%[64]	0.5%[64]	
1/2CPD/RAS2	78%[65]	0.5%[65]	
Eight-week storage			
CPD/EAS-81	85% [63]	0.4% [63]	10% [63]

In the case of haemostasis, MPs provide an additional negative phospholipid surface for the assembly of the tenase enzymatic complex involved in the coagulation cascade [20]. Moreover, a study demonstrated that platelets MPs have from 50 up to 100-times more procoagulant activity than platelets [21]. Nonetheless, not only platelet MPs are involved in this process, indeed, erythrocyte MPs and other MPs have a procoagulant activity as well [22]. In addition, a recent paper by Furie and Furie [20] mentions a "microparticles accumulation pathway" as a part of the coagulation process. Briefly, according to this model, there are constitutive MPs at low concentration expressing inactive tissue factor (TF). These MPs are then captured in a developing thrombus and their accumulation in the injury site leads to activation of MPs TF and helps to amplify coagulation.

Another impelling example is the implication of MPs in erythrocyte ageing process [23]. Indeed, during their 120 days of lifespan, red blood cells lose between 15% and 20% of their volume and haemoglobin concentration increase by 14% [24]. Thus, microvesiculation would be a mean for red blood cells to eliminate denatured haemoglobin which could be toxic [25], and besides, microparticles release would also be a mean for red blood cells to get rid of specific membrane proteins which could prevent or induce their removal from blood flow according to the situation. In a protective role, MPs may help to clear away the C5–9 complement attack complex, band 3 neoantigen, IgG or other harmful agent from the membrane when the red blood cell is still viable [13,25–28], and thus prevent early removal from blood flow. In contrast, MPs could promote removal of erythrocytes: CD47 is an integral membrane protein present on erythrocytes surface, acting as a marker for self [23]. Thanks to CD47, normal red blood cells are recognized as self by the macrophages (through their signal regulatory protein α) and phagocytosis is inhibited. Senescent or damaged red blood cells whose CD47 expression is reduced by shedding of MPs enriched in CD47 would be no longer recognized as self and thus be eliminated by macrophages.

Although the presence of MPs in blood is common in healthy individual, an increase in the concentration of MPs in plasma has been demonstrated under various pathological conditions such as thrombocytopenic disorder [29], cardio vascular disease [30], diabetes [31] or sepsis [32]. It is also important to refer that a few

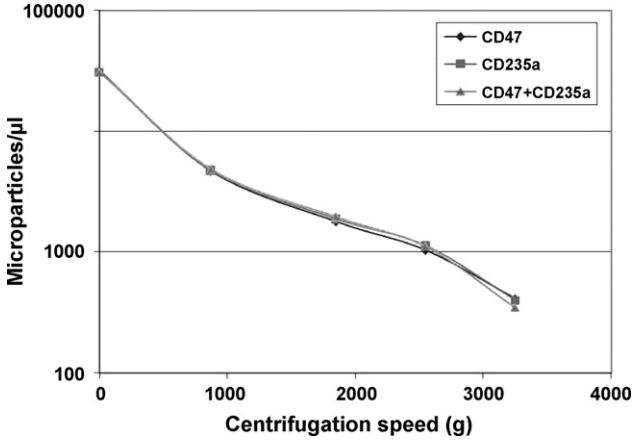

Fig. 1. Count of microparticles in supernatant of ECs. The number of microparticles measured decreased with increasing centrifugation speed (test on one ECs stored for 38 days). Note that the numbers measured were identical with the two antibodies used in this study (anti-human CD235a or CD47). Adapted from [34].

pathologies such as Scott syndrome [33] are linked to a decrease in MPs concentration in plasma.

In ECs and platelet concentrates, an increase in the number of MPs during storage has been evidenced [34,35]. Although MPs have been detected in FFP too, in contrast to ECs and PCs containing cells, there is no increase in MPs during storage. It has been proposed that MPs are implicated in different vascular pathologies. For example, a recent paper by Lawrie et al. [36] has demonstrated the effect of MP presence on clotting time of FFP. Thus, as MPs affect the clotting time, they could alter the quality of the blood product. Whereas the overall impact of MP presence on the blood product value is still largely unknown, there is little chance that their presence be totally innocuous.

3. Methods of microparticle analysis

The increasing interest in MPs lies in the fact that as they circulate in blood flow, they could constitute hallmarks of cellular activation or damage. Therefore new efficient methods have to be developed with the aim to obtain qualitative or/and quantitative data on MPs. It is important to add that there is no standardized method for MPs analysis, making any comparison difficult between different studies. In order to analyze MPs, several approaches are available in the literature such as electron microscopy, ELISA, proteomic methods and flow cytometry [9,37].

3.1. Isolation of MPs from biological medium

Prior to analyze MPs, isolation or concentration of MPs from samples (whole blood, platelet or red blood cells concentrate, fresh frozen plasma) could be needed according to the experiment. Indeed, in most studies, classical differential centrifugation (see Table 2) is principally employed. Notice that the references in Table 2 deal with platelet MPs, nevertheless it is a good illustration of the plentiful isolation methods available. For centrifugation, there are two main steps for MP isolation. First, low speed centrifugation (from 200 to 13,000 × g) removes intact cells, which leaves a MP-rich supernatant that can be directly analyzed. Alternatively, a second high-speed centrifugation (18,000–100,000 × g) can be used to pellet microparticles from the supernatant. Worth noticing is that most studies deal with platelet MPs, and that centrifugation conditions have to be adapted from one cell type to the other. Fig. 1 shows the optimisation of the centrifugation conditions to remove RBCs from ECs while keeping the MPs in the supernatant. In our experience, the best centrifugation conditions were

Table 2
Classical methods for MP isolation reported in the literature. Adapted from Ref. [37].

Main technique	Quantitation	Anti-coagulant	Prepare PPP	MP pelleting	Generic MP detection	Cell-specific identifications		
						Platelet	Endothelial	Leukocytes
Flow cytometry	Counts	Citrate	1550 × g, 20 min	18,000 × g, 30 min	Annexin V	CD62P, CD61, CD63	CD31, CD62E, CD144	CD4, CD8,. . .
Flow cytometry	Counts	Citrate	1550 × g, 15 min13,000 × g, 2 min		Annexin V	CD	CD51, CD144, CD146	CD45
Solid-phase capture	Prothrombinase capture	Citrate	1500 × g, 15 min13,000 × g, 2 min		Annexin V, tissue factor	CD62P, GPIba,	CD31, CD62E	CD45
Flow cytometry	Counts	Citrate	200 × g, 10 min1500 × g, 7 min			CD41, CD42b, CD31	CD31 +, CD42-, CD62E	CD45
ELISA	Standard PMP	EDTA	1500 × g, 20 min			GP IX (capture), CD62P, CD40L		
Flow cytometry	Counts	Citrate	13,000 × g, 10 min	100,000 × g, 60 min	Annexin V	CD41a	CD144	CD14

determined to be two centrifugations at 1850 × *g*, 4 °C, 20 min, to remove all RBCs from the supernatant. In these conditions, there is a 15-fold loss of microparticles compared to untreated EC, but there are no detectable RBCs in the supernatant. In cases when concentrated microparticles are necessary (for example for proteomic studies), an additional ultracentrifugation is usually performed to pellet MPs from the supernatant; this is usually accomplished by centrifuging the MP-rich supernatant at 100,000 × *g* to obtain a MP-free supernatant. The main drawback of this approach is that the MP pellet might also contain a lot of contaminants. It is our experience that the RBC supernatant after low speed centrifugation contains mainly MPs that can be detected and counted by flow cytometry with cell-specific markers (such as antibodies against CD47 or CD235a) or markers of phosphatidylserine externalisation such as Annexin V, with very little background noise. On the contrary, when microvesiculation is induced *in vitro* by challenging RBCs with calcium ionophore or calcium alone, there is a huge increase in the number of small fragments of the same size as MPs (at least as can be estimated from flow cytometry, see below), but that are negative to any labeling with anti-CD47, anti-CD325a and Annexin V. When pelleting the MPs by ultracentrifugation, there is no doubt that a large part of these fragments are pelleted as well and may obscure the biologically relevant information about MPs.

3.2. MP counting

As mentioned above, an increase in the number of MPs in blood is linked to various pathologies, hence the idea of using MPs as biomarker and thus the need to develop method allowing to quantify MPs. Flow cytometry is often considered as the method of choice to analyze MPs, indeed it allows analyzing thousands of MPs in one sample of whole blood or in a fraction with determination of many different markers at the same time. Additionally, flow cytometry enables not only qualitative but also semi-quantitative analysis. However, as MPs have a size smaller than 1 μm, this requires working at the inferior limits of the instrument, which have for consequences a loss of precision and/or accuracy. In addition, flow cytometry is not able to distinguish MPs, small cell debris and aggregates of MPs. This observation is confirmed by electron microscopy of erythrocyte MPs from EC: their size is around 0.15 μm and they tend to form clumps consisting of 10–15 MPs with an aggregate size of ~1 μm (see Fig. 2). Note that whereas in Fig. 2, MPs are issued from stored erythrocytes, however similar observations have been made in sample from treated red blood cells [38].

In our hands, flow cytometry analysis was performed with TruCount tubes (with a precise number of fluorescent beads to determine the number of MPs in a sample) and fluorescent cell-specific antibodies (either anti-CD47 or anti-CD235a) or phosphatidylserine-specific Annexin V. It was found that during storage of ECs in blood bank conditions, the number of MPs increased from around 3300 ± 1200 MPs/μl at day 5 of storage and it increases up to 64,000 ± 37,000 MPs/μl after 50 days of storage (the storage limit being of 42 or 49 days depending on the additive solution used, see Table 1).

Interestingly, a wide variability between the different concentrates was observed (see Fig. 3). The variation is likely due to the physico-chemical changes that occur in blood units during storage. Those changes affecting erythrocyte's viability reinforce and accelerate haemolysis and the associated release of MPs. Changes take place at different storage time, more or less rapidly according to the blood donor; indeed this might be caused by intrinsic factor such as sex, age, health, genotype, or diet, due to the fact that each sample was identically processed.

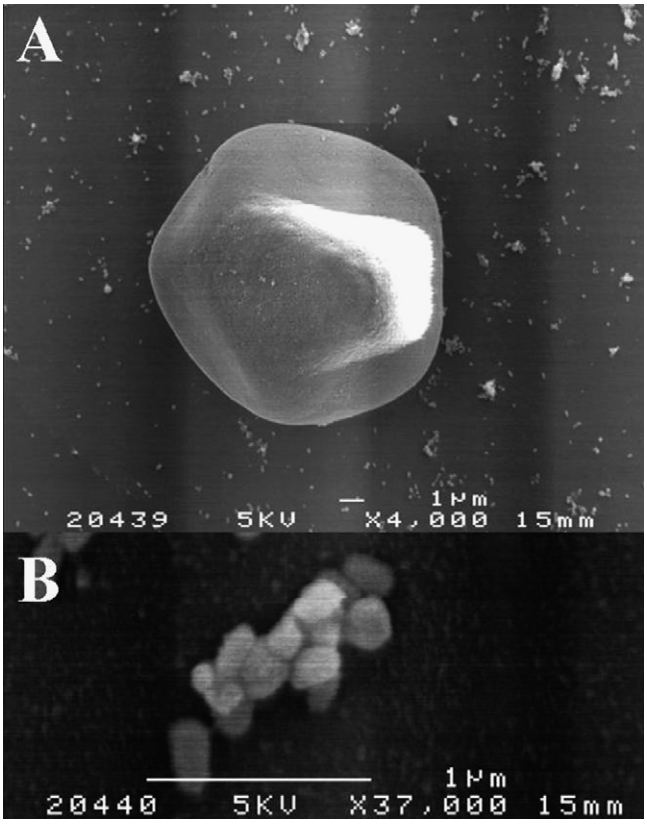

Fig. 2. Pictures of erythrocyte microparticles taken by scanning electron microscopy. (A) Magnification of 4000×, MPs surrounding an erythrocyte. (B) Magnification of 37,000×, heap of MPs.

3.3. Pre-analytical factors

It has been observed that factors such as temperature, shaking, or the dilution buffer influence the number of MPs counted; it is not totally clear from the literature and our experience if these factors affect the MPs counts by favouring MP *in vitro* aggregation, for example, or if they indeed induce artifactual microvesiculation of RBCs.

The impact of sample manipulation out of the cold room was tested with a fresh EC split in 2 similar blood bags. One was simply stored at 4 °C for 40 days while the other one was put out of the cold room for 1 h twice a week (i.e. 10 times during 40 days). MPs were

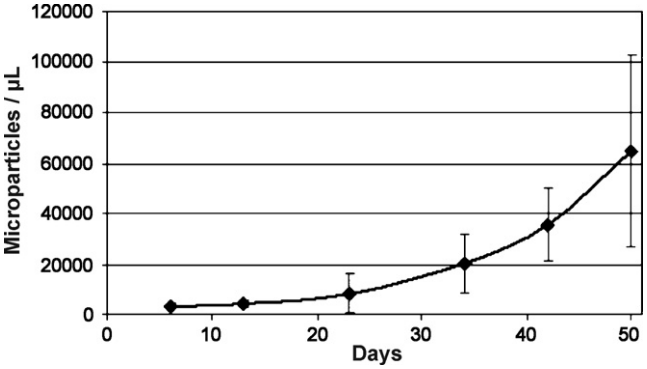

Fig. 3. Count of microparticles directly in erythrocyte concentrates during storage (without centrifugation). Data are expressed as the mean ± SD experiment (*N* = 7). At day 5, 3371 ± 1188 microparticles/μl were counted, whereas at day 50, their concentration was 64,858 ± 37,846 microparticles/μl. Anti-human CD47 was used to label MPs [34].

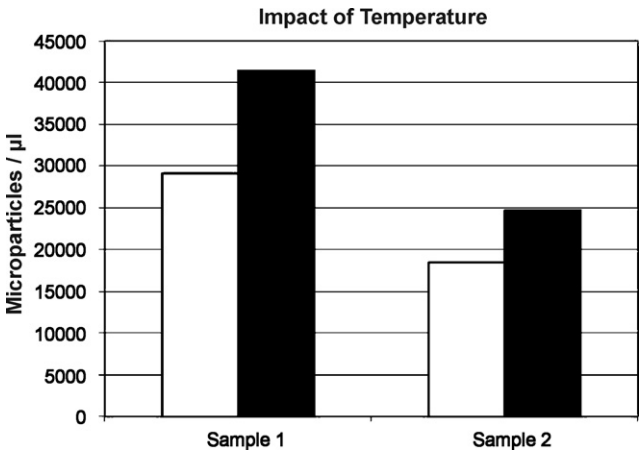

Fig. 4. Effect of cold room/RT cycles on MP counts. White bars represent samples continuously kept at 4 °C, black bars represent samples exposed 10 times to room temperature during storage.

then counted after 40 days according to the methods described by Rubin et al. [34]. The experiment was performed on two different concentrates and in both, much more MPs were observed in bags that underwent 10 cycles of 4 °C–room temperature (see Fig. 4). Another experiment to test the impact of working temperature was done with two different ECs, one stored for 15 days and the other one for 38 days in standard blood banking conditions. Few millilitres of each EC were incubated for 1 h at three different temperatures (4, 24 and 37 °C, respectively). Samples were then centrifuged and MPs were counted in the supernatant by flow cytometry. Fig. 5 shows the variation in MP concentration for the two different samples handled at 4, 24 and 37 °C. Not surprisingly, the EC stored for 38 days contains more MPs than the one stored for 15 days, but both samples show that increasing handling temperature induces a higher MP concentration.

Moreover, the solution used to dilute MP samples just before flow cytometry analysis also has an influence on MP counts. Here, three different ECs, stored for 2, 8 and 43 days in standard blood banking conditions, were diluted in different solutions: PBS, NaCl 0.9% and FACS Flow (FF) solution (BD Biosciences, Franklin Lakes, NJ). Flow cytometry analysis was performed as quickly as possible after the dilution of the sample. While the variation in the number of MPs was relatively small in concentrates stored for 1 and 8 days according to the buffer, a large increase of MPs was observed in the samples diluted in PBS compared to other buffers, as shown in Fig. 6.

An experiment to test if freezing/thawing sample of MPs free of cells affects MP counts has been conducted. Indeed, comparisons between MPs count in fresh supernatant and in supernatant frozen once have been done on ECs stored from 9 to 45 days (both supernatant were issued after two centrifugations of erythrocytes concentrates). Briefly, MPs were labeled and then counted by flow cytometry according to the protocol designed by Rubin et al. [34]. Notice that freezing sample could only be done after centrifugation, on cell-free samples. The difference between fresh and frozen supernatants does not exceed the standard variation coefficient of our standard experiment, which usually is from 3% to 13%. To give an example, in a 16 days stored sample, the difference between thawed and non-frozen sample was around 3%. So, MP counting, sample freezing did not affect the number of MPs detected.

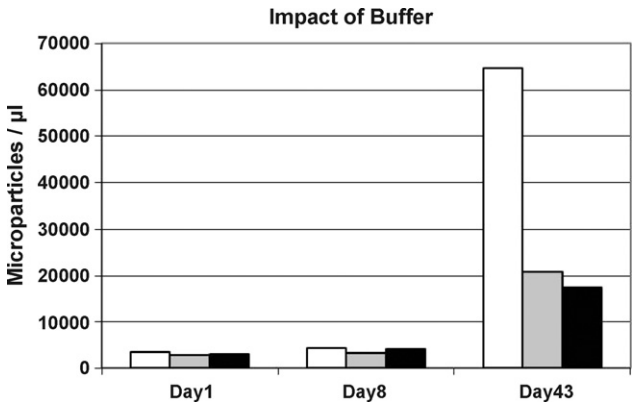

Fig. 6. Effect of the dilution solution used for flow cytometry analysis on the MP count after 2, 8 or 43 days of storage in standard blood banking conditions. White bars correspond to PBS, grey bars to NaCl 0.9%, black bars to FACS Flow.

Finally, the impact of shaking on MP count was checked: few millilitres of two different erythrocyte concentrates (the first one stored for 8 days and the other one stored for 42 days) were shaken using a Vortex mixer for different times (5, 10 and 20 s). Flow cytometry analysis was finally performed in the concentrate with TruCount tubes to count MPs beforehand stained with anti-CD47. Fig. 7 shows the increase in microparticle concentration after various vortexing times.

These four examples illustrate the impact of sample handling, dilution, and manipulation on the measured microparticle concentration. Whereas no clear rationale exists for the observed effects, some guidelines can be drawn from these experiments:

(1) The measured MP concentration does not depend on the detection system used (be it cell-specific antibodies or phosphatidylserine-specific Annexin V), as shown in Fig. 3.
(2) All other factors might have an influence on the measured MP concentration, and best efforts should be made to standard-

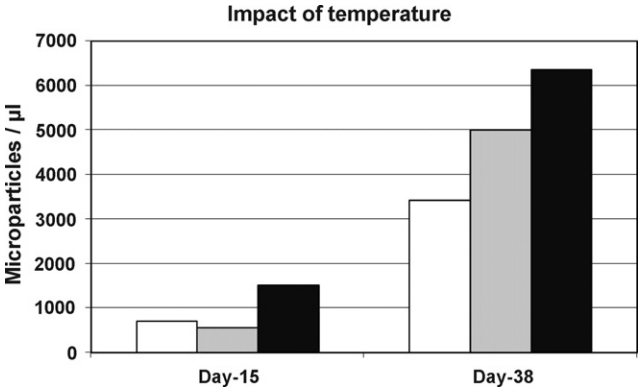

Fig. 5. Effect of handling temperature on MP concentration (white bars represent samples handled at 4 °C, grey bars at 24 °C, and black bars at 37 °C).

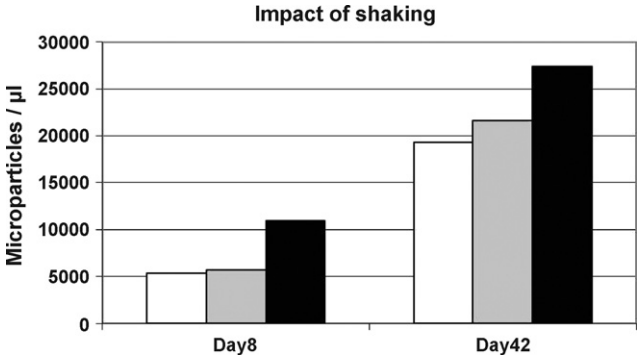

Fig. 7. Effect of vortexing on the MP count. White bars correspond to a 5 s vortexing, grey bars to a 10 s vortexing, and dark bars to a 10 s vortexing.

ize sample handling and preparation as much as possible; this is especially true for the temperature to which sample are exposed, the solution used to dilute the sample before flow cytometry analysis, and the way solutions are mixed.

3.4. Microparticle counting and sizing

To return to MPs count, a solid-phase method was developed by Nomura and co-workers [39] allowing indirect quantification of MPs. Briefly, Annexin V coated plates are used to capture phosphatidylserine-exposing microparticles. After washing, the (indirect) procoagulant activity of the bounded MPs can be determined using a prothrombinase assay. The main drawback of this approach is that the results are expressed as an area of accessible phosphatidylserine and not as a microparticle concentration, which makes the comparison with flow cytometry results hardly feasible [40,29]. Alternatively, a specific antibody can be added to quantify a cell-specific MP type. Although this assay is very sensitive to detect weakly expressed antigens, the relative non-specificity and non-sensitivity of Annexin V binding for MPs makes the technique difficult to use in routine.

Though standard flow cytometers have been extensively used to detect, count, and probe antigens at the surface of microparticles, it has to be clear that scattered light cannot be used to size microparticles. Most flow cytometers use light sources of wavelengths of 488 nm for example, which places the diffraction limit at half a micron. In practice, it can be difficult on most instruments to discriminate platelets from background noise based solely on scattered light, and it is hardly possible to size even smaller objects such as microparticles. Flow cytometric analysis of microparticles has thus to rely on specific probes and labels to discriminate relevant objects from background noise, but very little can be said about the size of the detected objects. A definitive demonstration of this difficulty has been provided by Becker et al. who analyzed a calibration bead mixtures ranging from 3 to 8 μm and showed that the scatter response of commercially available flow cytometers is not necessarily monotonous for smaller objects [41].

In this context, the laboratory of Bruce Furie modified a commercial flow cytometer that embedded a coulter counter, the Cell Lab Quanta SC from Beckman Coulter, to be able to measure objects of a few hundreds of nanometers. Fig. 8 shows the dot plot analysis of 780 nm fluorescent beads, and authors claim they were able to accurately size particles down to half a micron. This modified flow cytometer had been applied to the study of tissue factor-bearing microparticles in cancer [42,43]. Unfortunately, to the best of our knowledge, this instrument remains a unique prototype, whose technical specifications and modifications have not been disclosed, making the evaluation of its possibilities hard to truly evaluate.

More recently, Lawrie et al. [44] tested two commercially available dynamic light scattering instruments (the Zetasizer Nano S from Malvern Instruments Ltd., and the N5 submicron particle Size Analyser from Beckman Coulter) to size microparticles from fresh frozen plasma. Whereas both instruments were able to correctly size calibration beads, they showed ambiguous results when applied to microparticles from fresh frozen plasma: it seems that these instruments are more adapted to study objects with

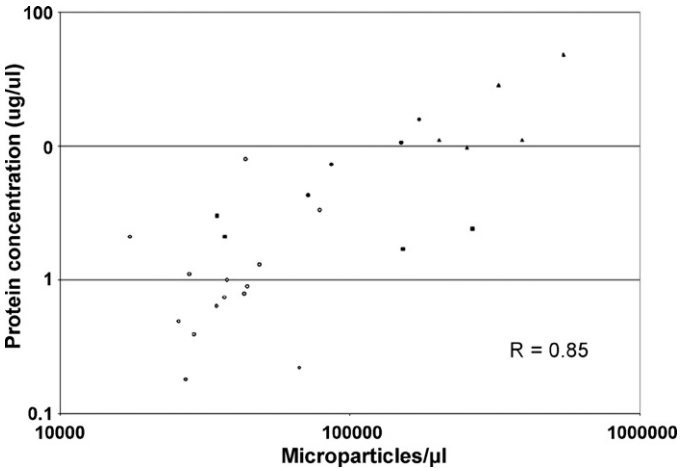

Fig. 8. Correlation between protein titration and microparticle concentration. Different markers correspond to different samples, the coefficient of correlation according to Bravais–Pearson test is $R = 0.85$.

AC	Entry name	Protein name	MW (Da)	RBC (sequence coverage %)	RBC (identified peptide)	MPs (sequence coverage %)	MPs (identified peptide)
P31946	1433B_HUMAN	14-3-3 protein β/α	28 082	25·2	7	16·7	4
P62258	1433E_HUMAN	14-3-3 protein epsilon	29 174	34·9	11	NO	NO
P61981	1433G_HUMAN	14-3-3 protein γ	28 303	NO	NO	13·4	4
P27348	1433T_HUMAN	14-3-3 protein θ	27 764	14·3	5	NO	NO
P63104	1433Z_HUMAN	14-3-3 protein ζ/δ	27 745	44·5	11	8·2	2
P02730	B3AT_HUMAN	Band 3 anion transport protein	101 792	NO	NO	6·8	3
Q4TW87	Q4TW87_HUMAN	β-Globin chain (Fragment)	11 487	93·3	15	NO	NO
P07738	PMGE_HUMAN	Bisphosphoglycerate mutase	30 005	45·9	13	37·8	10
P00915	CAH1_HUMAN	Carbonic anhydrase 1	28 870	72·8	27	65·5	20
P00918	CAH2_HUMAN	Carbonic anhydrase 2	29 246	72·7	25	71·9	15
P07451	CAH3_HUMAN	Carbonic anhydrase 3	29 557	36·2	8	17·7	4
A0N071	A0N071_HUMAN	δ-Globin chain (haemoglobin δ)	16 055	55·1	10	55·1	9
P27105	STOM_HUMAN	Erythrocyte band 7 integral membrane protein	31 731	14·9	4	76·4	43
P17931	LEG3_HUMAN	Galectin-3	26 188	11·6	3	NO	NO
P78417	GST01_HUMAN	Glutathione transferase ω-1	27 566	33·2	10	7·9	2
P69905	HBA_HUMAN	Haemoglobin subunit α	15 258	71·1	9	71·1	9
P68871	HBB_HUMAN	Haemoglobin subunit β	15 998	83·0	16	93·9	15
Q16775	GLO2_HUMAN	Hydroxyacylglutathione hydrolase	28 860	8·8	2	NO	NO
P30041	PRDX6_HUMAN	Peroxiredoxin-6	25 035	25·9	6	16·1	4
P18669	PGAM1_HUMAN	Phosphoglycerate mutase 1	28 804	8·3	2	NO	NO
Q06323	PSME1_HUMAN	Proteasome activator complex subunit 1	28 723	16·5	4	NO	NO
P25788	PSA3_HUMAN	Proteasome subunit α type 3	28 433	6·7	2	NO	NO
P25789	PSA4_HUMAN	Proteasome subunit α type 4	29 484	12·3	4	NO	NO
P60900	PSA6_HUMAN	Proteasome subunit α type 6	27 399	9·3	2	NO	NO
O14818	PSA7_HUMAN	Proteasome subunit α type 7	27 887	21·4	4	NO	NO
P00491	PNPH_HUMAN	Purine nucleoside phosphorylase	32 118	59·5	16	17·6	4
Q0KG01	Q0KG01_HUMAN	RhD protein	45 052	NO	NO	4·1	2

Fig. 9. Proteins identified from a 25–35 kDa region of a 1D-GE of RBC membranes and RBC microparticles by MALDI-TOF/TOF mass spectrometry. Greyed proteins have a molecular weight that does not correspond to their position on the gel.

sharp monodisperse size distributions than biological material with broad and overlapping size distributions. Nevertheless, the availability of such instruments as well as their lower costs compared to high-resolution flow cytometers might make them interesting complementary tools in microparticle research.

3.5. Microparticle proteomics

Recent advances in proteomics and new available techniques could play an important role in order to elucidate the exact roles of MPs studying their protein content. It is beyond the scope of this review to detail the particular biological results obtained by different studies, which are highly dependent on the cell type under study. In this section, we would rather insist on methodological aspects and difficulties that are shared by all investigators working on microparticle proteomics. Numerous publications describe microparticle proteomic studies [45–49]. To the best of our knowledge, most studies have adopted the same workflow for proteomic analysis of microparticles. First microparticles are pelleted by ultracentrifugation as discussed above, then diluted in a standard buffer (e.g. PBS) for protein titration by classical assays such as Bradford. In our hands, there appeared to be a correlation between protein concentration and microparticle concentration (coefficient of correlation (Bravais–Pearson): $R = 0.85$), as shown in Fig. 8, indicating that in terms of protein quantity, microparticles of various RBC origins (e.g. from RBCs stored in different additive solutions, or of different storage duration) are relatively homogeneous.

Because microparticles are not amenable to straightforward lyses such as hypotonic shocks or freeze/thaw cycles, most studies have adopted the same protein solubilization protocol: microparticles are directly solubilized in one-dimensional gel electrophoresis (1D-GE) loading solutions such as Laemmli, containing SDS and a reducer such as dithiothreitol, and proteins are separated by 1D-GE, protein bands are cut, and further digested by a proteolytic enzyme (usually trypsin) and identified by various mass spectrometry techniques. The clear-cut advantage of this technique compared to gel-free approaches is that to some extent 1D-GE is compatible with the analysis of hydrophobic proteins. This feature is important since microparticles, due to their formation mechanism and surface-to-volume ratio are expected to contain more membrane proteins with respect to their total proteome compared to their parent cells. For example, using this approach, Miguet et al. found 34% of plasma membrane proteins in the microparticles derived from malignant lymphocytes, which is twice as much as in their parent cells [48]. Additionally, in our experience, 1D-GE provides another level of information that is complementary to the MS/MS identification of proteins based on a few peptides: the molecular weight of the identified protein can indeed be deduced from its position on the gel, which can sometime provide some indications about protein processing. For example, in a slice of a RBC microparticle separated by 1D-GE, we identified proteins such as Band 3 and RhD protein (see Fig. 9) that are much larger than the gel band in which they were found, suggesting that truncation or cleavage has occurred. Conversely, we also found proteins, such as haemoglobin subunits, that are much lighter than the gel band in which they were found, which is consistent with the fact that haemoglobin tends to be cross-linked to cytoskeleton proteins, especially under stress conditions [50]. The observation of such variance between observed and expected molecular weights has already been repeatedly reported in RBC proteomics: for example, Pasini et al. reported that such proteins originate either from organellar proteins, in which case they are most likely to originate from degradation products processed during cell maturation (case for proteins with lower molecular weight than expected), from ubiquitinated proteins targeted for degradation (case for proteins with higher molecular weight than expected), or from detergent- and reducing agent-resistant macromolecular assemblies, such as cytoskeletal assemblies [51].

Interestingly, following the same approach Bosman et al. differentially analyzed so-called microvesicles, isolated from the supernatant of RBCs at $40{,}000 \times g$, and so-called nanovesicles, further isolated by centrifugation at $100{,}000 \times g$ [6]. Though this differential isolation relies on a somehow arbitrary criterion, the differential proteomic analysis performed by the authors showed marked differences between micro- and nanovesicles: main protein categories found in microvesicles are membrane and cytoskeletal proteins as well as metabolic enzymes, whereas nanovesicles are particularly enriched in uncategorized proteins. On the contrary, whereas immune proteins such as immunoglobulins and complement proteins are almost absent from RBC membranes and microvesicles, they are clearly identified in nanovesicles. This differential protein sorting between micro- and nanovesicles might indicate that the two populations are generated by different mechanisms and play different roles in RBC ageing and stress response.

Following the observation that microparticle proteins are not necessarily found at the right position on a 1D gel, and thus that some important protein processing occurs at the microparticle level, we undertook a systematic effort to separate the microparticle proteome at the protein level by two-dimensional gel electrophoresis (2D-GE) so as to be able to analyze differentially processed proteins. Unfortunately, 2D-GE is poorly amenable to the analysis of membrane and hydrophobic proteins. Hence following the work by Rabilloud et al. about membrane proteins solubilization for 2D-GE [52,53], we solubilized a MP pellet in urea, thiourea, DTE, pharmalyte™ and detergent (either CHAPS, ASB-14 or Brij 35). The protein mixture was then fractioned by OFFGEL electrophoresis and the fractions corresponding to pH 3–7 were pooled, so that haemoglobin was left in the most basic fractions. Following this haemoglobin depletion step, proteins were separated by 2D-GE with the detergent of choice. A typical 2D gel is shown in Fig. 10 (where Brij 35 was used to solubilize membrane proteins). Whereas

Fig. 10. 2D-GE separation of ~100 μg erythrocyte microparticles proteins beforehand depleted in haemoglobin by OFFGEL electrophoresis. The first dimension is a 4–7 linear pH gradient, second dimension is a 4–12% gradient polyacylamide gel. In the present gel, Brij-35 detergent was used to solubilize membrane proteins.

some haemoglobin can still be observed at the lower right corner of the gel, as pointed by several authors [54–57], haemoglobin depletion helps resolving more spots compared to the unfractioned sample. Moreover, the picture obtained by this differential solubilization strategy is dramatically different from the one obtained by using classical protocols (e.g. CHAPS solubilization).

4. Conclusion

There has been increased interest in the recent years in microparticle proteomics, mainly because microparticles are shed in biological fluids (in blow flow or in urine) by their parent cells and are thus readily accessible for analysis. Furthermore, they are in many situations hallmarks of cell lesion, and their proteomic analysis promise to shed some light on the biology of their parent cell, which may ultimately provide some valuable biomedical information. Somewhat differently, in the field of transfusion medicine, RBC and platelet microparticles are inherent parts of the blood product delivered to patients, and there is little chance that their presence be totally innocuous. However, proteomic analysis of microparticles turned out to be much more difficult than expected, mainly because of pre-analytical caveats. As we have attempted to demonstrate in this review, every single manipulation of the sample might have a direct influence on its microparticle content. Factors such as temperature, the dilution solution used, the way solutions are mixed, are crucial in the determination of microparticle concentration, and thus in the subsequent proteomic analysis. Additionally, the small size of microparticles makes their detection and sizing very difficult by standard techniques such as flow cytometry. The numerous caveats we tried to exemplify in this paper make the comparison between different studies almost impossible, and this is the reason why so much debate exists in the literature about the microparticle properties and functions. However, there is no doubt that proper control of the pre-analytical factors, as well as proper reporting of the methods used, will prompt sound and valuable insights into RBC biology through proteomic analysis.

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