HLT02 METVES

EMRP JRP – HLT 02 MetVes

List of stakeholder requirements for the use of method for dimensional characterisation of microvesicles in medical centres

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Abbreviations

DLS	Dynamic light scattering detection
FF	Freeze-fracture
FSC	Forward scatter
LALS	Large angle light scattering
MV	Microvesicles
NP	Nanopore
NTA	Nanoparticle tracking analysis
PS	Polystyrene
RPS	Resistive pulse sensing
SAXS	Small-angle X-ray scattering
SALS	Small angle light scattering
SEC	Size exclusion chromatography
SiO_2	Silicon dioxide
SSC	Side scatter
TEM	Transmission electron microscopy

I. Introduction

Within the European Metrology Research Programme (EMRP), the 3 year project “Metrological characterisation of microvesicles from body fluids as non-invasive diagnostic biomarkers” (METVES) has started in June 2012. The aim of the project is to develop traceable measurement techniques for the characterisation of MV as biomarkers.

The publishable Joint Research Project summary report can be found here:

http://www.euramet.org/index.php?id=emrp_call_2011#c10983

The project has 6 work packages (WPs). This questionnaire is part of WP 2 task 2.3: Comparison of the dimensional characterization of MV. The stakeholders committee for HLT02 has been contacted to take part in the questionnaire. Information obtained from the questionnaire is used to produce a list of stakeholder requirements with regard to the use of methods for dimensional characterization of MV in medical centers. This list contains the outcome of the survey.

The collected questionnaire contains existing information about:

1. Methods used or intended to be used for characterisation of MV in medical centres
2. Resolution, detection limit, and measurement time of the used methods
3. Outcomes from MV measured by the used methods
4. Reference materials used to calibrate size distribution and concentration of MV

About 70% completed questionnaires were received. This report compiles the information obtained from the stakeholders.

The original questionnaire is attached in appendix A (page 15).

II. Contributors

The following persons and institutions completed the questionnaire and contributed with their profound experience to the list presented in the report.

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III. List of applied methods and specifications

No.	Method	Resolution (nm)	Detection Limit (nm)	Analysis time (min)	Concentration calibration	Size calibration	Shape	Biochemical composition
1	Conventional flow cytometer							
1.1	BD FACSCalibur	~20 (PS beads by SSC)	~200 (PS beads) ~300-600 (urine MV)	1	Volumetric flow rate	Calibration of scattering intensity by NIST traceable PS beads from Thermoscientific and Mie theory	No	Fluorescent labelled antibodies
1.2	BD LSRII	n.d.	300 nm (placental MV)	5	PS beads	PS beads	No	Fluorescent labelled antibodies
2	New generation flow cytometer							
2.1	Apogee flow systems, A50-Micro	~5 (PS beads by LALS)	~100 nm (PS beads) ~150-200 nm (urine MV)	2	Injection with (acquired) syringe pump	Calibration of scattering intensity by NIST traceable PS beads from Thermoscientific and Mie theory	No	Fluorescent labelled antibodies
2.2	Stratedigm S1000Ex	40 (PS beads by SSC)	100-160 nm (PS beads by SSC)	1	Cytocount Counting beads	Megamix beads	No	Fluorescent labelled antibodies
2.3	Customized flow cytometer (Prof. Nolan's group)	n.d.	70 nm (Liposomes and plasma MV)	1	Volumetric flow rate	Surface area-dependent staining of liposomes	No	Fluorogenic lipid probe, fluorescent antibodies or other ligands

3	NTA, Nanosight, NS500	Ratio 1.3-1.5 (PS beads) [7]	~50 [a] (PS beads) ~70 – 100 (urine MV)	5-10	SiO_2 beads from Corpuscular	Automatically determined by the software integrated in NTA (based on Stokes-Einstein relation)	No	Fluorescence (405 nm laser)
4	RPS, Izon, qNano	~10 (PS beads by NP100) ~30 (PS beads by NP200 and 400)	~70 (urine MV by NP100) ~100 (plasma MV by NP200) ~200 (PS beads by NP400)	30	Izon calibration beads	Izon calibration beads	No	Zeta potential
5	TEM, Philips, CM-10	~1	~10-20 [c]	>60	n.a.	n.a.	Yes[b]	Immunogold labeling

Note

- n.a. :not applicable; n.d.: not determined
- [a]depends on the type of the instrument and particle refractive index; [b]depends on the preparation of the sample such as fixation, drying, and staining which might affect the morphology of MV; [c] depend on the background
- BD Accuri, BD Verse, and BD Astrios are currently being tested by the group of Dr. Paul Harrison. Preliminary results show that these instruments could detect PS beads down to about 200 nm.
- Raman microscpectroscopy is currently being tested by Edwin van der Pol (AMC). This method can gives information on chemical composition of MV [1].
- [7]: see references

IV. General comments from the contributors

Flow cytometry

- Minimum detection limit: The new generation of flow cytometers, e.g. Apogee A50-Micro, Stratedigm S1000Ex, BD Accuri, BD Verse, and BD Astrios, are able to detect PS beads between 100 nm and 200 nm by using light scatter. However, PS beads are not comparable to MV because the refractive index of PS beads is higher (~1.61) than that of MV (~1.38). Therefore, MV with sizes 100-200 nm will not be detected by these flow cytometers. To be noted, Apogee A50-Micro was developed for small (<1 μm) particle detection. Thus far, this flow cytometer is more sensitive in MV detection in comparison to other commercially available flow cytometers [2-4].

- Calibration using beads: The advantages of using synthetic particles such as PS and SiO_2 beads for calibrating flow cytometer are that these beads are reasonably monodisperse and their measurement can be made traceable. Mixtures of beads also could be formulated for size calibration purposes. The downside of PS bead is the refractive index, which is ~1.61, and thus much higher than the refractive index of MV (~1.38). The influence of the difference in refractive index between PS bead and MV can be eliminated by calibrating the flow cytometer using traceable PS beads and application of Mie theory [4]. To calibrate flow cytometry for the purpose of MV measurement, SiO_2 beads with a refractive index of 1.43-1.46, closer to the refractive index of MV, may be more appropriate than PS beads.

- Resolution: When trigged on SSC, we found for the BD FACScalibur an improved resolution compared to triggering on FSC, which for this type of flow cytometer is due to a more sensitive SSC detector (photomultiplier tube) compared to the less sensitive FSC detector (photodiode). We measured a mixture of NIST traceable PS beads with different sizes (125, 200, 400, 600, 800, and 1000 nm) triggered on both FSC (Figure 1A) and SSC (Figure 1B). We found that triggering on SSC improves the resolution of 400, 600, 800, and 1000 nm beads about 6-fold and allows the detection of 200 nm beads. However, the 125 nm-beads were under the detection limit of both settings (Figure 1). Dr. Paul Harrison suggests that triggering on fluorescence for each main type of vesicle and double labeling for e.g. phosphatidylserine or tissue factor and then back gating to the scatter plots may improve the resolution and lower limit of the detection using flow cytometry.

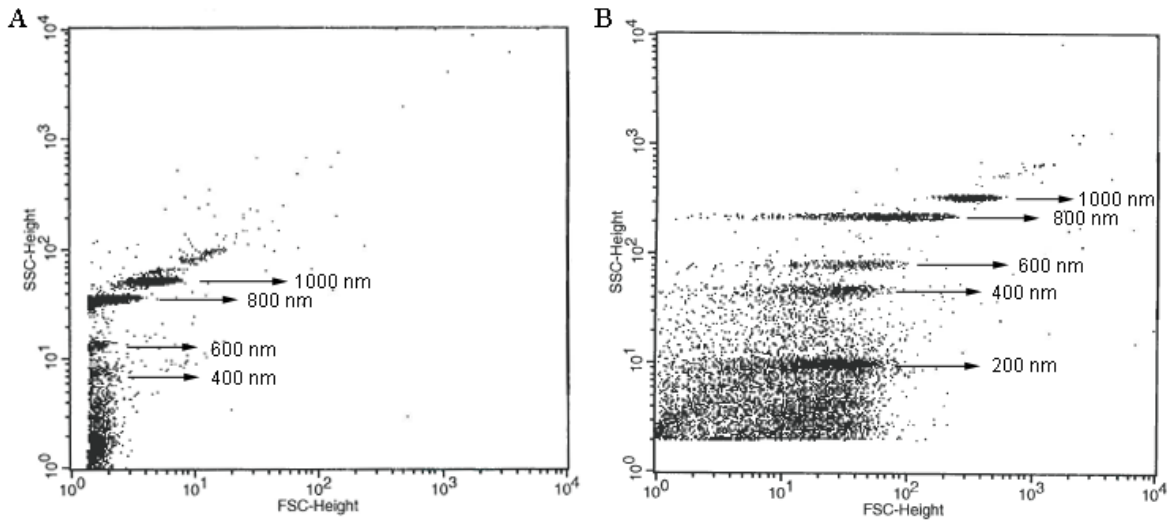

Figure 1. Scatter plots of a mixture of PS beads with different sizes (125, 200, 400, 600, 800, and 1000 nm) measured by BD FACScalibur. Events were collected by triggering on FSC (A) or SSC (B).

– Recent results from the AMC in testing Apogee A50-Micro are promising. This flow cytometer detects single 100 nm-PS beads (Figure 2A) and resolve a mixture of PS beads with sizes 248 and 315 nm or a mixture of SiO_2 beads with sizes 142 and 177 nm (Figure 2B, C). All beads are traceable and were provided by Dr. Felix Meli from Federal Office of Metrology METAS (Wabern, Switzerland). In addition, we measured a mixture of NIST traceable PS beads with sizes of 125, 200, 400, 600, 800, and 1000 nm and found that the intra- and inter-assay variations are less than 10%.

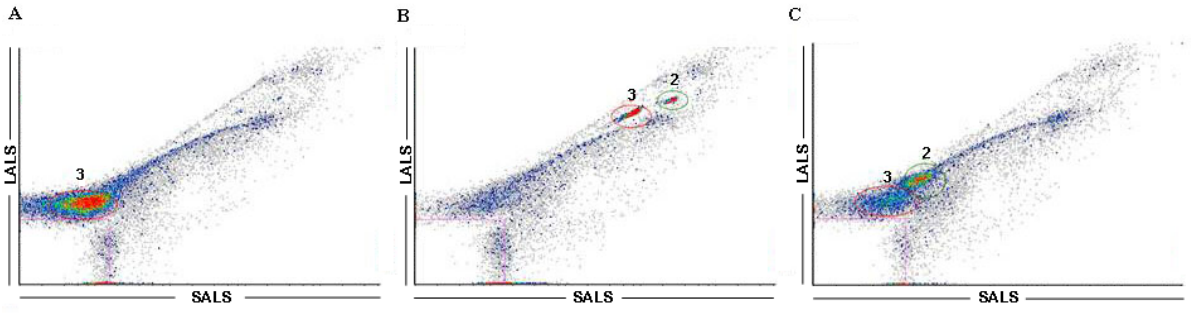

Figure 2. Measurement of PS and SiO_2 beads by Apogee A50-Micro. The 100 nm-PS beads are detected just above the noise of the instrument (A) and the bead population is indicated by the region 3. A mixture of PS beads with sizes 248 and 315 nm is detected (B). The bead populations are resolved as shown by the 248 nm-beads in region 3 and the 315 nm-beads in region 2. A mixture of SiO_2 beads with sizes 142 and 177 nm is detected (C). The bead populations are resolved as shown by the 142 nm-beads in region 3 and the 177 nm-beads in region 2.

– The group of Prof. Nolan has improved the light scattering sensitivity of their customized flow cytometer 2-fold by allowing the detection of vesicles as small as 35 nm in diameter. Instrument improvements will increase the number of different fluorophores measured from a factor of 2 to

more than a factor of 10. This also should improve the fluorescence detection limits from about 240 green fluorophores to less than about 100 fluorophores, with concomitant improvements in detection of fluorescent antibodies. However, this customized flow cytometer is not yet commercially available and only for research purposes.

Nanoparticle tracking analysis

- SiO_2 beads (100 nm) represent an improvement on PS beads for concentration calibration by NTA [5]. However, refractive index of silica is similar, but not identical, to that of MV. Clearly, a biological reference standard with traceable size and concentration is highly desirable for calibration of NTA and measurement of MV. Concentration measurement by NTA also remains intensity weighted, and thus particle size and refractive index weighted, while broadening of the size distribution occurs, particularly for polydisperse samples. Therefore, NTA is inaccurate in measurements of size distribution and concentration of vesicles.

- In the AMC we measured the same mixture of traceable PS and SiO_2 beads provided by Dr. Felix Meli and found that NTA using camera level 11 fails to resolve a mixture of traceable PS beads with sizes 248 and 315 nm (Figure 3A). Also NTA using camera level 14 fails to resolve a mixture of traceable SiO_2 beads with sizes 142 and 177 nm (Figure 3B). This indicates that the resolution of NTA for polydisperse samples is less than 22% (CV) and this will have an effect to the measurement of particle concentration.

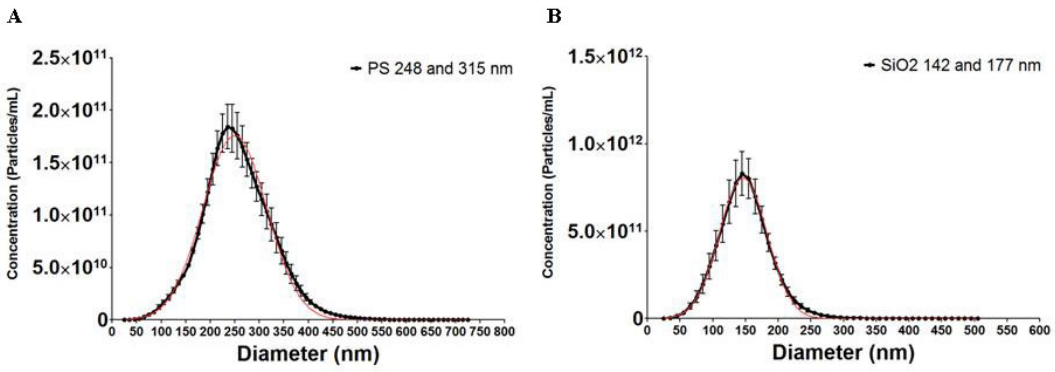

Figure 3. NTA measurement of a mixture of 248 and 315 nm-PS beads (A) and a mixture of 142 and 177 nm-SiO_2 beads. NTA fails to resolve the bead mixtures showing only one peak of bead population.

Resistive Pulse Sensing

- As RPS detection is not based on light scattering, this method is more accurate than NTA in the detection of particles/vesicles with different refractive index.

- The dynamic range of RPS is limited by the use of the non-conductive pores which are made by certain defined size range (NP100: 70-200 nm; NP200: 100-400 nm; and NP400: 200-800 nm). For a polydisperse sample, measurement using pores with different size range should be done in order to get the absolute concentration of particles/vesicles in the sample. Clogging caused by protein aggregates or clustering of vesicles is also a problem in measurement using RPS, particularly when measuring biological samples. The particle rate will change when clogging occurs, which will have an effect on the measurement of particle concentration. Also, clogging makes this system low-throughput.

- Besides all limitations of the RPS pores, two major concerns are that the concentration of calibration beads provided by the manufacturer of RPS is not traceable, and there is a pore-to-pore variation. The pore-to-pore variation causes variation in the minimum detectable particle size. Thus, the concentration and size measurements of MV by RPS are not traceable.

- In the AMC, we measured a mixture of traceable PS beads with sizes 248 and 315 nm as well as a mixture of traceable SiO_2 beads with sizes 142 and 177 nm. Our results show that RPS using NP200 could resolve the mixture of PS beads, but failed to resolve the mixture of SiO_2 beads (Figure 4).

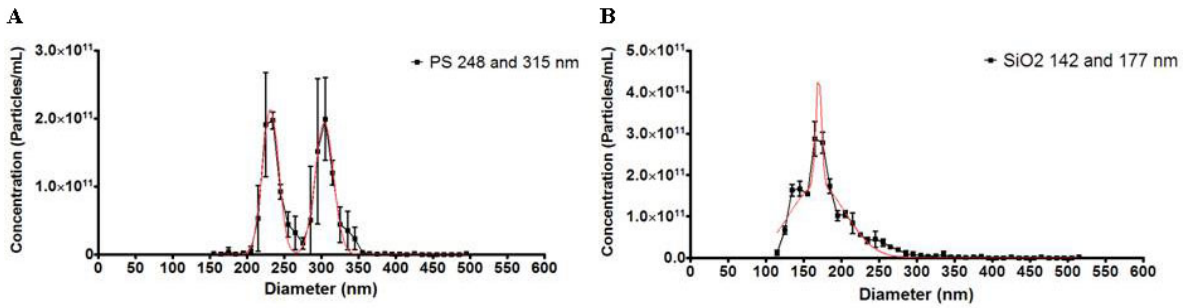

Figure 4. The mixture of 248 and 315 nm-PS beads (A) and of 142 and 177 nm-SiO_2 beads (B) are measured by RPS using NP200.

Transmission electron microscopy

- TEM is a sensitive method to detect MV (down to 10-20 nm). However, it also depends on the background of the sample, and particularly in the case of plasma samples, proteins and lipoproteins interfere with or even impair the detection of MV.

- The fixation and dehydration during preparation of MV for TEM measurement may cause shrinkage of the diameter of MV by approximately 12% [4]. This will influence the determination of size and shape of MV by TEM. TEM is also not suitable for quantitative analysis because of

several steps in the sample preparation (e.g. centrifugation and washing steps, adherence of MV on the grid, and blotting with filter paper). It has been observed that about 21% of the vesicles are recovered after centrifugation and binding to the formvar coating [4].

V. Required methods for dimensional characterization of microvesicles

When comparing results of MV measurements using different methods, we have to bear in mind that each method has a different minimum detection limit which may cause differences in the measurements of the particle size distribution and concentration. In the medical centres, flow cytometry, NTA, RPS, and TEM are used to detect MV. Thus far, flow cytometry is still the method of choice, due to high-throughput (minutes/sample), and the ability to detect subsets of MV using commercially available fluorescently labelled-antibodies in a small volume of sample (~50 µL for certain flow cytometers). The new generation of flow cytometers, such as Apogee, are relatively fast and allow multiple fluorescence detection, making them most applicable to clinical research. Apogee provides more sensitive detection of vesicles down to about 200 nm in comparison to conventional flow cytometers, which are only able to sensitively detect MV down to about 300 nm. In general, smaller MV, 100-200 nm, should be possible to be detected by flow cytometers dedicated to particle detection. Triggering on fluorescence for these smaller sizes MV may improve the sensitivity. For this purpose, specific fluorescently labelled-antibodies and bright fluorescent (lipid) dyes are needed.

All stakeholders agree that reliable measurements of MV can only be achieved by calibrating methods using appropriate reference standards. These reference standards should be from materials which at least reflect the characteristics of MV such as refractive index, biochemical composition, polydispersity, and surface charge. There are two types of reference materials tested for method calibration. One is synthetic reference materials such as PS and SiO_2 beads. These beads are reasonably monodisperse and they can be made polydisperse to mimic polydispersity of MV in solution by mixing beads with different sizes. Also, these synthetic reference particles can be made traceable. As the refractive index of PS bead is around 1.61, which is higher than that of MV (1.38), SiO_2 beads with refractive index of 1.43-1.46 may be more suitable to be used as a reference standard. However, PS and SiO_2 beads do not have the same biochemical composition as MV and probably also have a different surface charge.

Alternatively, reference material may come from biological materials such as liposomes, which are phospholipid vesicles with a refractive index of about 1.45. Liposomes can be produced in different sizes with certain concentrations and also loaded with different fluorescent dyes. When the sizes of liposomes and fluorescent-loaded liposomes are made traceable, they can be used to calibrate the

method based either on light scatters or fluorescent, and subsequently to calibrate the measurement of MV concentration.

Another important point in the measurement of MV concentration is that the stakeholders require reference standards with traceable particle concentration. This type of reference standard is not yet available and should be developed in the near future.

In the MetVes project, we have characterized the size distributions of an erythrocyte-derived MV sample by FF-TEM, NTA, RPS, SEC-DLS, and SAXS. As reported by Varga et al [6], all of the methods are capable of characterizing the mode diameter of the studied MV sample within small deviations, but traceability is not reached in this study. For traceable size determination of MV, the polydispersity of MV samples, for example due to the presence of (aggregates of) proteins and lipoproteins, is one of the key problems that need to be solved. In a more complex sample such as plasma, separation of vesicles with narrow size ranges and removal of (aggregates of) proteins and lipoproteins prior to measurement will be required. Techniques such as SEC and field-flow fractionation chromatography may be used to separate MV from proteins and lipoproteins.

VI. Conclusions

Based on the inputs of the stakeholders through the questionnaire, we conclude that for dimensional characterization of vesicles the instrument design needs to be improved for allowing measurement of MV in broader size range (e.g. 30 nm to 1 µm) and in much better resolution (e.g. 10 nm) with parallel improvements in detection of fluorescent antibodies, (lipid) dyes, or other ligands. Size and concentration calibration using traceable reference standards with comparable characteristics as MV will help to reliably determine the size and concentration of MV; and also to compare results of MV measurements in different medical centres. Traceable measurement of MV will be reached by acquiring less polydisperse MV samples and for this purpose, purification of MV from other contaminants such as proteins and lipoproteins is urgently required. Altogether, the most suitable method required for routine analysis of MV in medical centre in the near future is a method that can provide a high throughput traceable measurement of size, concentration, and biochemical composition (e.g. cholesterol, phospholipid composition, protein content) of MV based on single vesicles in suspension.

VII. References

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VII. Appendix A: Text of original questionnaire

No.	Method	Resolution (nm)	Detection limit (nm)	Measurement time (minutes)	Concentration[1]	Concentration calibration[2]	Size distribution[1]	Size calibration[2]	Shape[1]	Biochemical composition[1]

[1] please indicate with 'yes/no'; [2]if applicable, please specify your calibration procedure

Notification:

Satisfied with the method used Yes □ *No □ N.a. □

* Please explain the reasons and what need to be improved:

General comments: