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1 Executive Summary

Introduction

Diagnosis requires reliable information, which in part comes from analysis of body fluids. Body fluids contain cell fragments (microvesicles; MV) that differ in common diseases such as cancer and cardiovascular disease, and thus provide novel biomarkers. Unfortunately, prior to the MetVes project the results of MV measurements were incomparable between instruments and hospital laboratories. Therefore the MetVes project developed (i) a procedure that is now being used worldwide to isolate MV, (ii) reference materials to improve standardisation of MV measurements, (iii) discovered a method to measure a hitherto unknown but important physical property of MV required for standardisation, and (iv) improved the comparability of MV concentration measurements in 30 hospital laboratories worldwide; supported by the International Society on Thrombosis and Haemostasis.

The Problem

The costs of health care are increasing rapidly in the EU due to ageing of the population. One instrument to reduce health care costs is early diagnosis of disease, which improves the efficacy of medical treatment. However, making a medical decision requires reliable information, which comes from routine analysis of body fluids. Predominantly such information comes from the routine analysis of body fluids such as blood and urine, as these fluids contain cells and biomarkers that reflect health or disease. During the last decade, human body fluids were shown to contain not only cells and soluble biomarkers, but also numerous and extremely small cell-derived MV. MV are membrane-surrounded cell fragments, which can trigger blood clotting, thrombosis and inflammation, promote tumour growth and metastasis and may cause diseases such as preeclampsia, etc. Numerous studies have shown that the concentration, cellular origin and composition of MV markedly differ between healthy and disease states. However, because the measurement results for MV were hitherto incomparable between different instruments and laboratories, the clinical usefulness of MV as biomarkers for disease could not be explored, prior to the MetVes project.

The Solution

The MetVes project produced:

- Methods for the dimensional characterisation (measuring size and size distribution) of MV, including Atomic Force Microscopy, Small Angle X-ray Scattering and Transmission Scanning Electron Microscopy.
- MV reference standards for downstream analysis and software to correct for differences in the refractive index between selected reference materials and MV, and to correct for differences in the optical configuration of instruments used in hospital laboratories.
- Reliable MV sample procedures, including controlled and standardised collection, handling, and storage of body fluids for MV and for the isolation and purification of MV from these body fluids.
- Methods for measuring the concentration, morphology and (bio)chemical composition of MV, and to distinguish MV from other biomaterials in body fluids (lipoproteins, viruses). As well as a method to determine the refractive index (RI) of single nanoparticles – including MV - in suspension.

The project's reference materials, biological samples and software have been distributed, measured and tested in more than 30 hospital laboratories worldwide.

Impact

Since the start of the MetVes project in 2011, it has had an important impact in the biomedical sciences community as it was the first project involved in the standardisation of MV measurement. The project has introduced the term "metrology" and corresponding terminology to a large and global (bio)medical audience and provided a sound metrology structure for MV measurement that will be further developed and explored within a newly initiated working group on the standardisation of vesicle measurements by flow cytometry. This new working group is a collaboration between the International Society for Extracellular Vesicles (ISEV), ISTH and the International Society for Advancement of Cytometry (ISAC) (www.evflowcytometry.org). The MetVes project coordinator (who is the Chair of the Scientific and Standardisation Committee on Vascular Biology of the ISTH) has been asked to participate in this international working group and so far has given 2 invited presentations to this working group.

The MetVes project also developed (i) MV reference standards and SOPs for the collection, handling and storage of blood, urine and saliva for MV analysis, (ii) a method for the isolation of MV that is being used in 170 (mainly clinical) laboratories worldwide, and (iii) reliable procedures for the dimensional characterisation of MV and for measuring the concentration, morphology and (bio)chemical composition of MV.

The sample procedures developed by the project, to standardise the collection, isolation and purification of MV from body fluids are being widely used by investigators in the MV field, including researchers in hospital laboratories, not only in Europe but also worldwide, e.g. investigators of the National Institute of Health (Washington DC, USA).

Finally, the project developed a novel and label-free method for distinguishing MV from other biomaterials in body fluids using the RI of MV that offers clinical laboratories the opportunity to analyse body fluids containing MV more efficiently and economically.

All these results have enabled the comparison of MV measurement results between different instruments and different medical institutions, which means that for the very first time it will become possible to perform multi-centre MV trials. The results have also provided a sound basis for the standardisation of MV measurements and contributed to the further establishment of MV as biomarkers of disease, an important step towards earlier diagnosis of many common and rare diseases and thus more efficient medical treatment.

2 Project context, rationale and objectives

2.1 Context

The costs of health care are increasing rapidly due to ageing of the population throughout Europe. One of ways of reducing health care costs is the early diagnosis of disease, which improves the efficacy of medical treatment. However, making a medical decision requires reliable information. Predominantly such information comes from the routine analysis of body fluids such as blood and urine, as these fluids contain cells and biomarkers that reflect health or disease. During the last decade, human body fluids were shown to contain not only cells and soluble biomarkers, but also numerous and extremely small cell-derived fragments, microvesicles (MV). MV are surrounded by a membrane, and are capable of triggering blood clotting, thrombosis and inflammation, promoting tumour growth and metastasis, and may even lead to diseases such as preeclampsia. Taken together, it is not surprising that a huge clinical and scientific interest arose in detection and characterisation of MV.

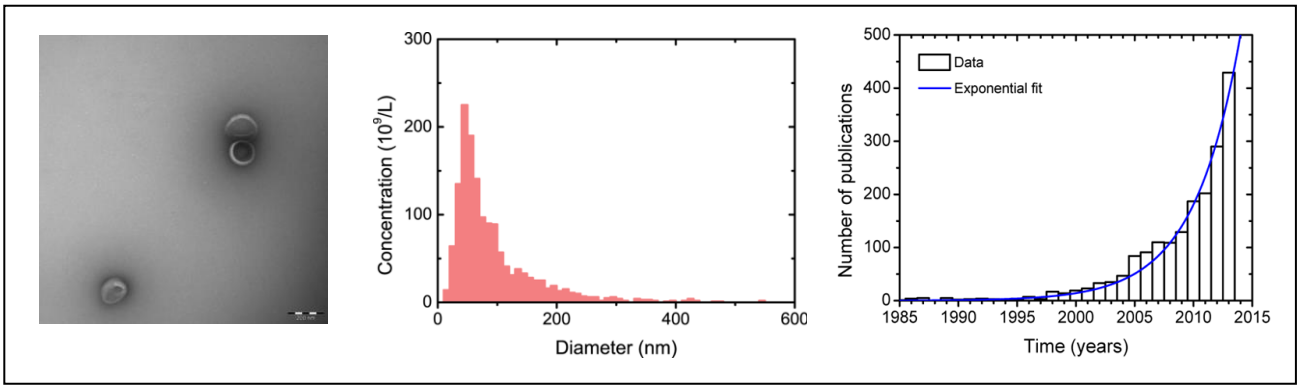


***Figure 1**. Left: Microvesicles (MV) in the urine of a normal healthy subject visualised by electron microscopy (scale bar: 200 nm). Centre: Estimated size distribution based on analysis of 5,000 MV by electron microscopy in human urine. Right: Number of publications on MV per year.*

2.2 Rationale

Although there is consensus that the concentration, cellular origin, composition and function of MV differ in most if not all diseases the measurement results for MV are incomparable between methods and laboratories. For example, even recently (2010-2013) reported concentrations of MV in plasma of healthy humans differ 10^8-fold between methods and laboratories. Therefore, without standardisation, it will be impossible to determine concentrations of MV in body fluids of healthy 'normal' human subjects, and without

knowing what is “normal” it will be impossible to know when there is a deviation from this i.e. what is “abnormal” or a disease state.

Furthermore, most of the available methods that are used to detect MV lack sensitivity, and there is consensus that only “the tip of the MV iceberg” is detected, although it remains unclear how large the iceberg and its tip actually are. Therefore, in order to exploit the full potential of MV as novel clinical biomarkers, improved understanding of MV measurements and the standardisation of measurement results are required.

In hospital laboratories two methods are commonly used to measure single MV. Both methods measure the amount of light that is scattered by a single particle or MV in suspension. However, the problem is that the amount of scattered light depends on the size (diameter) and composition of the particle or MV. For example, a plastic (polystyrene) particle scatters more light than a MV having the same diameter. This property is called the refractive index (RI), and in order to derive the diameter of a MV from the amount of scattered light, it is essential to know the RI. Unfortunately, a method to determine the RI of nanometre-sized particles did not exist. But once the RI can be determined, the diameter of MV in suspension can be derived, which then allows estimation of the detection limit of applied methods, which should improve the comparison of measurement results between instruments and institutes.

Methods measuring light scattering also lack specificity as they do not provide information on the type of particle that is scattering light. This lack of specificity causes problems especially when studying MV in human plasma, because plasma contains not only MV but also lipoprotein particles which overlap in size (diameter) with MV. Therefore, to solve this problem, information on the (bio)chemical composition or other properties, such as morphology are needed to distinguish MV from other biomaterials in body fluids.

Previous studies showed that MV in body fluids are dependent on pre-analytical variables, such as blood collection conditions. Therefore the comparison of MV measurements results also requires standardisation of pre-analytical variables. However such standard operating procedures for sample procedures for the collection, handling, and storage of body fluids for MV did not exist prior to the MetVes project. In addition, MV research commonly requires isolation/purification of MV for analysis and although laboratories apply centrifugation-based protocols, numerous erroneous artefacts have been published in MV analysis. Therefore, there was a need for an isolation and purification method for MV from body fluids.

2.3 Objectives

Based on the above clinical needs, the following scientific and technical objectives were formulated for the MetVes project:

1. To develop methodologies for the dimensional characterisation (measuring size and size distribution) of MV.
2. To develop MV reference standards. Standards should be stable, provide repeatable measurements, and should have properties similar to MV (size, morphology, refractive index (RI)).
3. To develop reliable MV sample procedures, including controlled and standardised collection, handling, and storage of body fluids for MV and for the isolation and purification of MV from these body fluids.
4. To develop methodologies for measuring the concentration, morphology and (bio)chemical composition of MV, and to distinguish MV from other biomaterials in body fluids (lipoproteins, viruses).

3 Research results

3.1 To develop methodologies for traceable dimensional characterisation (measuring size and size distribution) of MV

3.1.1 Introduction

The determination of the size distribution of MV is important from the point of MV classification and is important for choosing the best method to accurately enumerate MV. Size characterisation of MV is challenging due to their small size and heterogeneity. The diameter of vesicles typically ranges from 1 µm down to 30 nm, which is below the detection range of common devices such as conventional flow cytometers. In addition, many new techniques have not been validated yet. Consequently, there is a need for traceable size determination of MV. “Traceable size determination” means that the measurement result can be related to the SI unit ‘meter’ through an unbroken chain of comparisons with known uncertainties. Traceable measurements of MV can then be used to develop reference materials with similar properties to MV and to calibrate other detection methods, thereby facilitating standardisation.

A number of methods were proposed and used in this project to measure the size distribution of MV. These included the microscopic methods dry atomic force microscopy (AFM), wet AFM, transmission electron microscopy (TEM) and transmission scanning electron microscopy (TSEM), which require the MV to be bound to a surface, and with the exception of wet AFM, also require drying of the sample, which can be detrimental to naturally wet soft matter, since in their natural state MVs are freely suspended in liquid. Therefore, several methods to measure the size distribution of MV free in suspension were also performed by the project. These included nanoparticle tracking analysis (NTA), resistive pulse sensing (RPS), flow cytometry (FCM) using the newest highly sensitive flow cytometers, and small-angle X-ray scattering (SAXS). Unlike microscopic methods, measurement methods in suspension can only provide information about the size and size distribution of MV, and not information on the morphology of (single) MV. In the case of SAXS, an ensemble averaged size distribution can be obtained from the data by model fitting. Whereas, in case of NTA, RPS and FCM, single particles can be detected and the size distribution can be obtained as a histogram of single events. FCM is also widely available in hospitals and clinical centres, albeit usually with conventional, that is less sensitive FCM devices. In contrast, Freeze-fracture TEM (FF-TEM) sits in-between these categories, as in FF-TEM, the sample is snap-frozen, broken and examined using microscopy. However the assumption with FF-TEM is that the freezing does not disturb the sample and the resulting micrograph gives an impression of the freely suspended MV.

The project used highly concentrated and purified erythrocyte-derived MV, isolated from outdated erythrocyte concentrates for measurements with all the above mentioned methods. Additionally, MV samples derived from platelets, erythrocytes, plasma, urine and saliva were prepared by means of centrifugation only. Whilst these latter samples were closer to real ‘clinical’ samples i.e. those collected from patients, the MV concentration was several orders of magnitude lower than the erythrocyte-derived MV samples and they included impurities due to proteins and other components. Because of this, these ‘clinical’ MV samples from platelets, erythrocytes, plasma, urine and saliva by centrifugation only, were much more challenging to measure than the highly concentrated and purified erythrocyte-derived MV samples.

3.1.2 Methods in suspension

Nanoparticle Tracking Analysis (NTA)

NTA is widely used in MV research for the characterisation of the size distribution and concentration of MV. With NTA, the Brownian motion of individual particles in solution is tracked based on their light scattering. Variables include the camera level, detection threshold, viscosity, temperature, and the dilution of the sample. An NS500 (NanoSight Limited, London, UK) was used by the project for MV measurements, equipped with an electron multiplying charge coupled device camera (Andor Technology, Tokyo, Japan) and a 405 nm laser.

Resistive Pulse Sensing (RPS)

RPS is capable of determining the size and concentration of MV based on the Coulter principle. An RPS instrument (qNano, Izon Science Ltd., Christchurch, New Zealand), equipped with an NP100A type membrane (particle detection range: 70-200 nm), was used by the project to measure the size distribution and concentration of MV in suspension.

Freeze-Fracture Transmission Electron Microscopy (FF-TEM)

In case of FF-TEM, the sample is frozen within a few milliseconds, which inhibits crystal formation in aqueous samples, thus preserving the original morphology of the MV. The frozen sample is then broken, and a replica of the surface is made and examined using TEM. FF-TEM does not require fixation and negative staining, which are procedures that are most likely to affect the MV sample. Therefore, FF-TEM can provide a more realistic picture of the structure of MVs in suspension than TEM.

Small-Angle X-ray Scattering (SAXS)

SAXS can provide structural information on nanomaterials in the 1 nm to 200 nm size range. SAXS is based on the elastic scattering of X-ray photons on the electrons of the sample at low angles. The scattering intensity is measured as a function of the momentum transfer q, which is related to the half of the scattering angle θ between the direction of the incident beam and the scattered light according to $q = 4\pi/\lambda \sin(\theta)$, where λ denotes the wavelength of the incident X-ray beam. The structural features of the sample with a size d are indirectly represented in the scattering curve by the intensity at $q=2\pi/d$, and can be extracted by fitting theoretical models to the measured data. SAXS has already been used to describe different vesicle systems from synthetic and natural origins, prior to the MetVes project, however it had not been used for the characterisation of MVs.

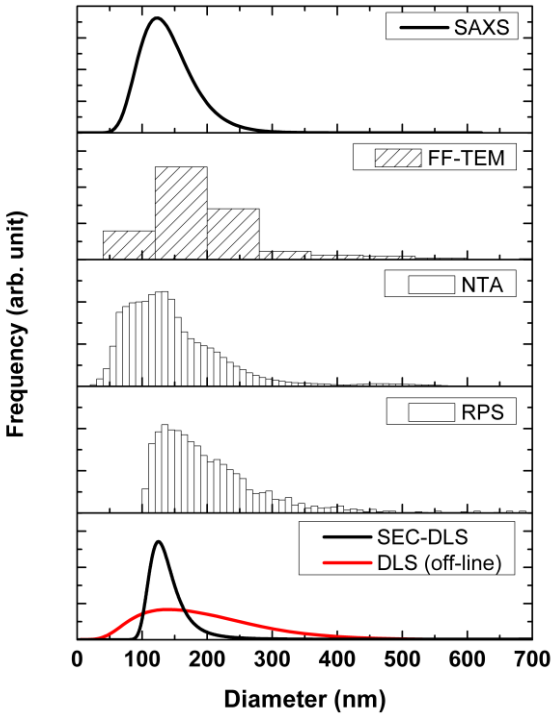


Figure 1. *Comparison of size distributions obtained in suspension on a concentrated erythrocyte vesicle sample*

Figure 1 compares the size distributions obtained by SAXS, FF-TEM, NTA and RPS on a concentrated MV sample derived from erythrocytes. The first noteworthy observation is that SAXS, FF-TEM and NTA also detected vesicles with a diameter below 100 nm, despite the fact that the applied gravitational force during sample preparation was believed to separate out from the suspension (i.e. as sediment) vesicles larger than 100 nm. RPS is an exception, because the pore size of the used membranes was insufficient for sizing vesicles with diameter less than 100 nm. All methods, except FF-TEM, resulted in a distribution with a mode value between approximately 120 nm and 140 nm, but there were pronounced variations in the width of the

distributions amongst the different techniques. For example, with FF-TEM, most of the vesicles belong to the 120 nm to 200 nm size range.

For a traceable measurement of the MV size distribution, the length scale of the method has to be traceable to the SI unit 'meter' and the sample has to fulfil certain requirements. Full traceability could not be obtained using either SAXS, FF-TEM, NTA or RPS for a variety of reason. For example, upon purification of erythrocyte MV, formation of “rouleaux” (i.e. aggregations of red blood cells) occurred, thus producing a polydisperse suspension and impairing the measurement of size distribution. Further details of these results can be found in a published article by the MetVes project[1].

3.1.3 Microscopic methods

In contrast to the methods in suspension, which provided mainly statistical information about the ensemble of particles such as MV in suspension, microscopic methods are based on the analysis of a micrograph. Therefore, these methods can provide additional insight in for example morphology or classification of a MV and the size distribution can be obtained by analysing many particles/MVs.

Atomic force microscopy (AFM)

AFM is a method which evaluates the forces acting on an ultra-sharp tip scanning the sample surface. A two-dimensional height map of the surface is generated, and the size of particles adhered to the surface can be evaluated from the height and width of the deflections on the height map. In addition to dry surfaces surfaces immersed into liquid can also be measured.

Transmission scanning electron microscopy (TSEM)

TEM is a method, where the sample is imaged onto a two-dimensional detector using an electron beam and magnetic lenses. TEM was performed in the MetVes project using the microscopes Philipps CM-10 and FEI Tecnai-12. Instead of imaging using lenses, TSEM applies a single focused electron beam scanning through the sample to build up the image. The TSEM measurements were performed in the project on the same grids using a Zeiss Supra 35VP scanning electron microscope equipped with a transmission detector.

Figure 2 shows the images obtained using different microscopic methods. Platelet and erythrocyte derived MV samples were measured by AFM, although quantitative evaluation of the size was only possible for the erythrocyte derived MV samples due to the higher concentration and more regular shape of the MV (Figure 2d). Under both wet and dry conditions, MV in the size range from 20 nm up to 160 nm could be found. On the TEM and TSEM pictures, a number of other objects were visible, out of which only doughnut-shaped objects were counted. Both TEM and TSEM find objects in a size range from 100 nm to 300 nm, however, the number of objects found on these images was too small to create meaningful histograms.

Flow cytometry (FCM)

FCM is a method which guides particles through a laser beam in a hydrodynamically focused fluid stream. The scattering from individual particles is detected under a fixed angle. The size of the particles can be estimated from the intensity but only if the refractive index of the particles is known. The absolute concentration of particles can be estimated from the flow rate. FCM is of special interest to the MetVes project, because it is widely available at medical centres and used on a regular (daily) basis to count cells in patient samples.

FCM measurements were performed on an A50-Micro (Apogee Flow Systems Ltd., Hemel Hempstead, United Kingdom) in the project. Scattering of light with a wavelength of 405 nm was measured using two photomultiplier detectors and in the forward scattering direction, the small angle light scatter (SALS) detector and in the side scattering direction the large angle light scatter (LALS) detector were used. The fluorescence channels were not considered throughout analysis.

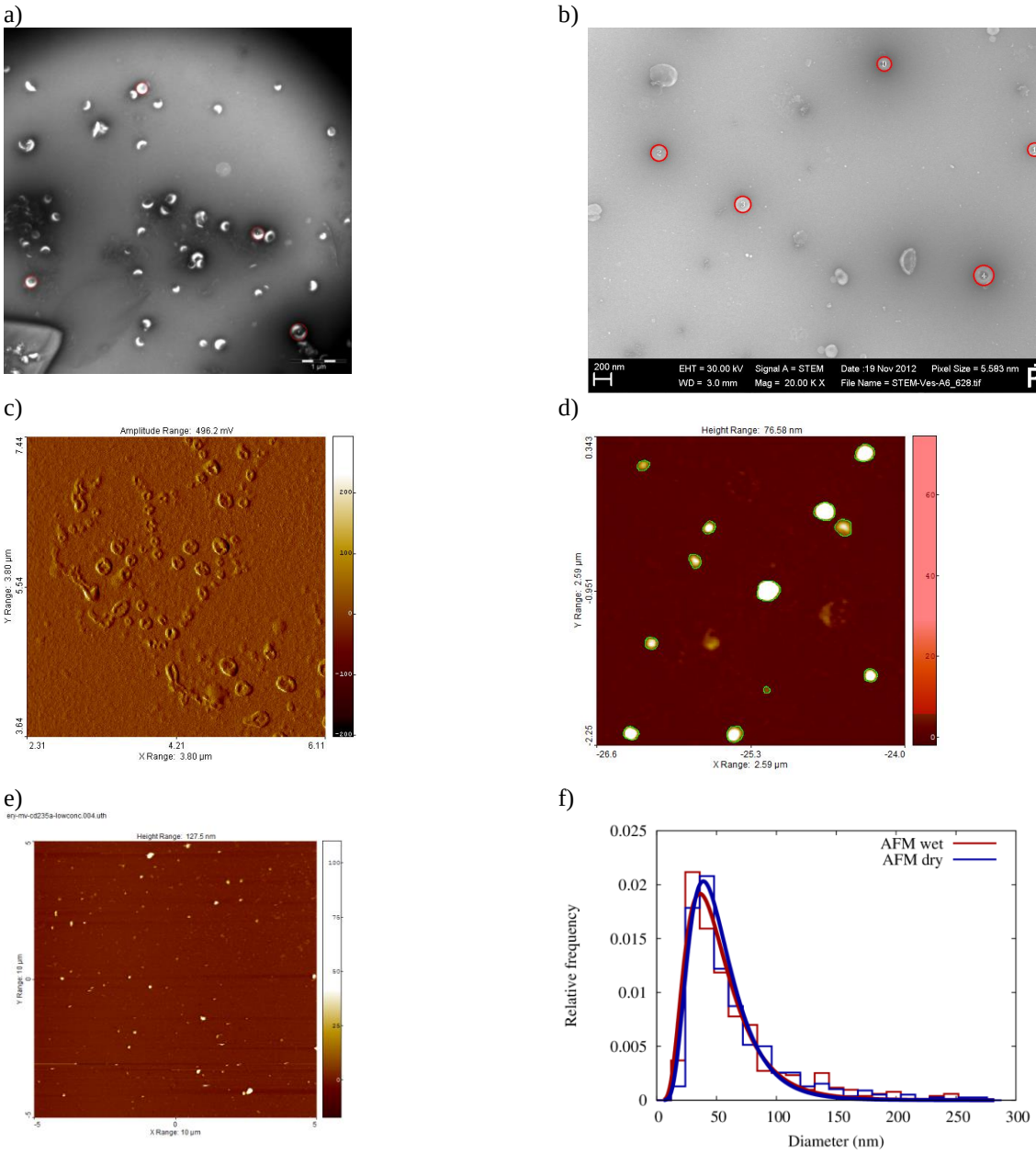
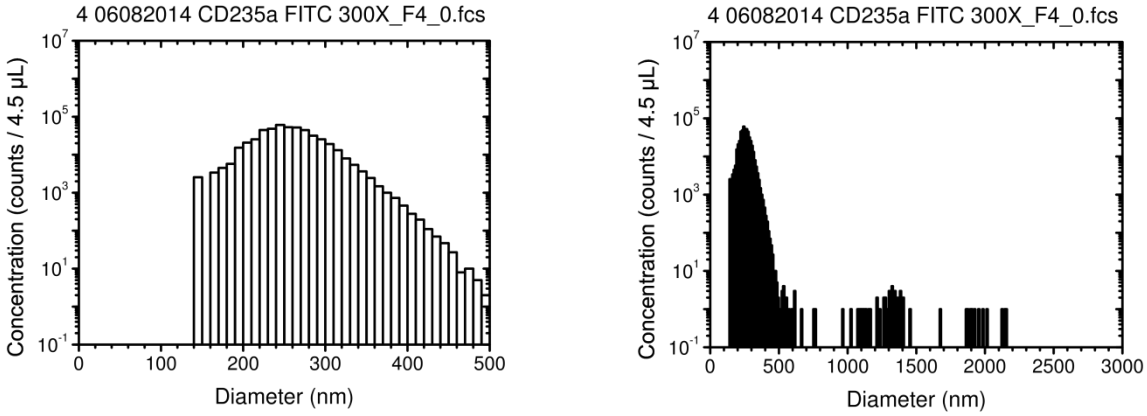


Figure 2. *Images of MV obtained by microscopic methods. Erythrocyte vesicles were measured by TEM (a), TSEM (b), dry AFM (d) and wet AFM (e). Platelet vesicles imaged using dry AFM are shown in (c). The size distribution from dry and wet AFM is shown in (f)*

Figure 3 shows the size distribution obtained by FCM for concentrated erythrocyte derived MV samples. A major peak of the size distribution at around 230 nm was obtained and a minor peak at 1100 nm – 1500 nm. The mode of the major peak could be extracted with good precision using a lognormal fit. However, this peak was close to the lower cut-off, where probably the sensitivity of the flow cytometer was decreased, therefore the real mode of the particles should be at lower diameters, near or below the cut-off size. The minor peak at larger diameters could be caused by empty erythrocytes, which can be still present in the sample, even after centrifugation, due to their low density.



a) b)

Figure 3. *Size distribution as obtained by FCM for particles below 500 nm (a) and in the full range (b) Already above the sharp cut-off at 150 nm, the sensitivity of the device is decreased. The size distribution has a bin size of 10 nm*

3.1.4 **Conclusions**

In summary, the project developed a range of methodologies for the dimensional characterisation (measuring size and size distribution) of MV, including SAXS, FF-TEM, NTA, RPS, AFM, TSEM and FCM. Most methods could be used to measure a sample of concentrated erythrocyte derived MVs. However, a traceable size measurement for MV in 'clinical' samples (from platelets, erythrocytes, plasma, urine and saliva by centrifugation only) remains difficult to measure as methods like SAXS, electron microscopy or AFM which can deliver traceable results for artificial nanoparticles and MV, require a higher concentration and purity of MV than the 'clinical' samples available. In contrast the methods which can cope better with these limitations in concentration and purity for 'clinical' samples, like RPS and NTA, have difficulties in achieving traceability in their measurements. Further to this, FCM, the method of choice for clinical laboratories for measuring larger particles at a high throughput, struggle with the small size of the majority of MV. To overcome these difficulties, clinical laboratory methods (like FCM, NTA and RPS) need to be developed further to become more sensitive to size. If this can be done, then engineered MV reference materials (such as those developed as part of objective 2 see Section 3.2), which can be characterised using traceable methods, could then be used for the calibration of clinical laboratory methods.

3.2 MV reference standards. Standards should be stable, provide repeatable measurements, and should have properties similar to MV (size, morphology, refractive index (RI))

3.2.1 Introduction

As concluded in section 3.1.4, the clinical laboratory used techniques NTA, RPS and FCM required MV reference standards for traceable calibrations and validations. These reference standards/materials should preferably be certified beads with properties comparable to MV, for example, the RI and size should be similar, as most of the clinical methods are based on light scattering. In addition, these reference materials should be stable and able to provide repeatable measurements.

Two types of MV reference materials, synthetic and biological, were developed and characterised by the MetVes project.

3.2.2 3.3.1 Synthetic MV reference materials

A survey amongst clinicians, by the MetVes project, gathered information from 40 stakeholders and end-users on the available commercial sources of reference particles with physical properties related to MV and on the measurement methods, needs and further requirements. From the results of the survey, 14 different particles made of either Silicon dioxide (Silica; SiO_2) or Polystyrene (PS) having nominal diameters ranging from 50 nm to 315 nm (i.e. matching the size range of MV and the detection range of the methods to be used) were characterized using the AFM, in order to find the most suitable synthetic MV reference materials candidates. The samples were characterised with respect to the shape of the particles, their size, the width of the size distribution, the RI and their purity. The best candidates were then used in an inter-laboratory comparisons in Section 3.2.4). Figure 4 shows 3 candidates of different qualities for the synthetic MV reference materials.

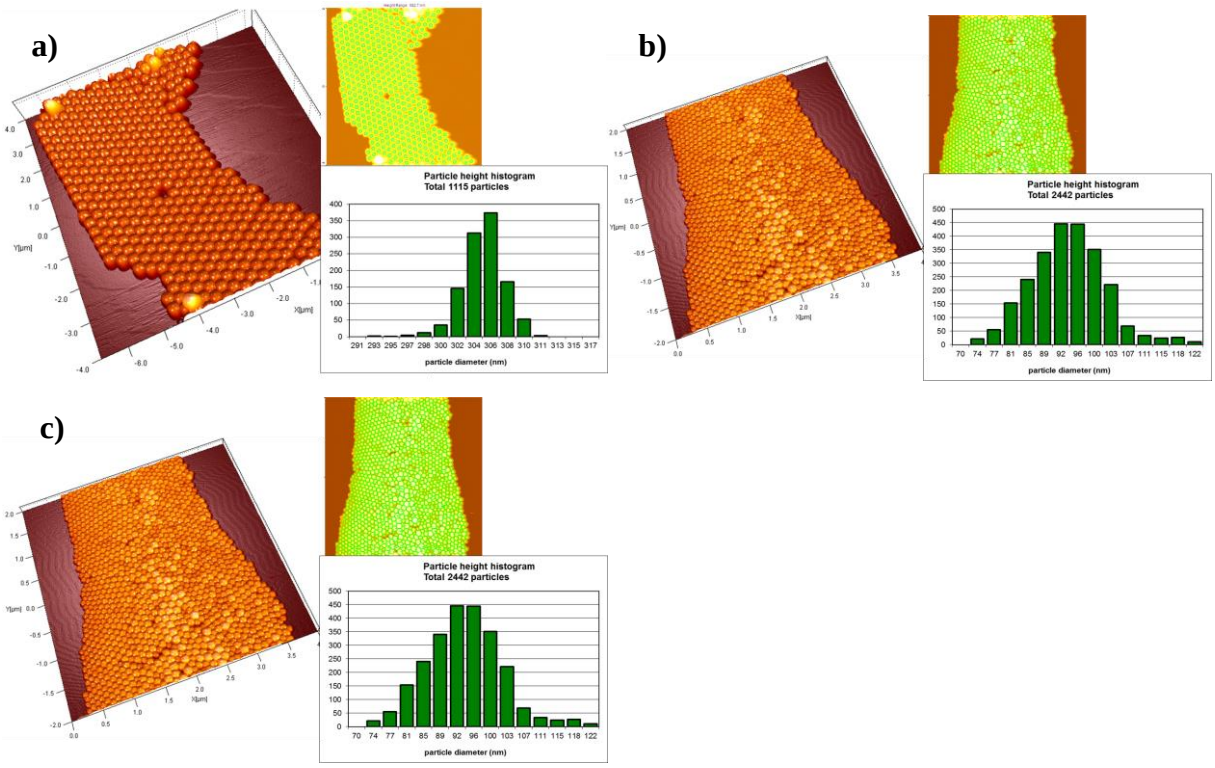


Figure 4: *Characterization of synthetic MV reference materials. a) high quality particles b) medium quality particles c) low quality particles.*

3.2.3 Biological MV reference materials

The synthetic MV reference materials were monodisperse, stable and spherical. However their structural composition and RI was still somewhat different from 'real' MV. Therefore biological MV reference samples were also required for reliable measurements of MV. These biological MV reference materials should also prove stability, low polydispersity and good sphericity. Different candidates such as intralipids, liposomes, lipoproteins and purified erythrocytes MV were considered by the project. However, their characterisation was much more complicated than for the synthetic MV reference samples. The main issues were the requirements of the different analytical methods e.g. NTA and RPS require particles bigger than 50 nm and SAXS requires highly concentrated samples. Furthermore, with the microscopy methods (AFM and TSEM), a complex background due to impurities in the solution and unstable samples made the evaluation difficult.

The intralipid and liposome candidates were both polydisperse and imaging proved difficult. In contrast, the lipoproteins, in spite of their relatively good monodispersity, were too small for the clinical methods. The erythrocytes were stable but polydisperse and included too many undefined impurities. Therefore, unfortunately none of the tested candidates for the biological MV reference materials fulfilled all the requirements needed and the project was unable to identify a successful biological MV reference standard. Table 1 show the suitability of the proposed biological MV reference materials across different the measurement methods.

Laboratory	Intralipids	Liposomes	Lipoproteins	Erythrocytes MVs
AMC – RPS + NTA	✗	✓ ✗	✗	✗
PTB – SAXS	✗	✓	✓	✗
PTB - TSEM	✗	✗	NA	NA
SMD – AFM	NA	✗	✗	✗
VSL – AFM	✗	NA	✗	✓
METAS - AFM	✓	✗	✗	✗
General difficulties	Polydispersity, unstable	Polydispersity	Size, costs	Polydispersity

Table1. *The suitability of the proposed biological MV reference materials across different measurement methods*

Although the project's biological MV reference sample evaluation was not successful, candidates were proposed for future investigations such as naturally occurring particles (e.g. coccoid bacteria, picoplankton or viral particles) or particles from purified biochemical components or membranes (nanoerythrosomes, oil droplets, etc.). As well as purified erythrocytes, if using an improved purification process.

3.2.4 Inter-laboratory comparison of the synthetic MV reference materials

A set of five monodisperse samples of high quality synthetic MV reference material particles was selected in section 3.3.1 for an inter-laboratory comparison. The synthetic MV reference material particles were made of either SiO_2 or PS with nominal diameters ranging from 50 nm to 315 nm. Two bimodal samples were also prepared for size resolution measurements and to estimate the relative size dependent count rate of the methods. The particles were distributed to the project partners AMC, PTB, SMD, VSL and METAS and the size and size distribution of the synthetic MV reference materials were evaluated using three independent traceable measuring methods (AFM, SAXS and TSEM) and the two clinically available methods (NTA and RPS). The traceable methods use different physical principles; thus method independent reference values and uncertainties were obtained and the measurements with NTA and RPS illustrated the current capabilities within clinical laboratories. For each evaluated sample, absolute reference values and uncertainties were calculated and these values showed good agreement with the traceable methods (AFM, TSEM and SAXS). An example evaluation for a monodisperse SiO_2 sample is shown in Figure 5.

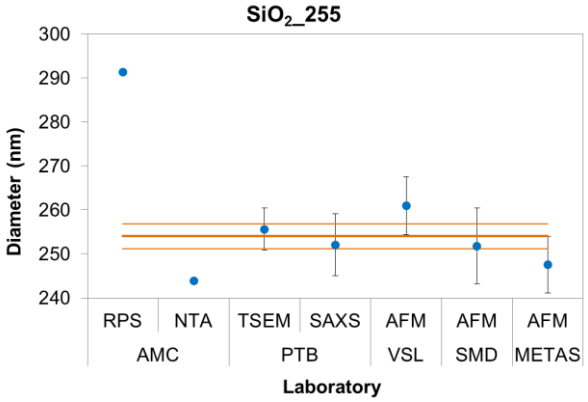


Figure 5 *Monodisperse particle mean size value with expanded uncertainty (k=2) of a nominal 255 nm SiO_2 sample. The thick straight line is the reference value, and the two thin lines indicate the uncertainty range (k=2).*

For the bimodal synthetic MV samples prepared by mixing high quality monodisperse particle solutions, the traceable methods (AFM, SAXS and TSEM) agreed within the specified size uncertainties of the subpopulations (Figure 6). However, due to size selective sample preparation and a lack of an uncertainty contribution for the relative number of particles in each size fraction, (possibly due to the size dependent sensitivities and different size detection limits of the methods), the particle size comparisons for the bimodal MV samples were more difficult compared to the monodisperse synthetic MV samples. Therefore, further systematic comparisons of bimodal synthetic MV samples are needed, so that they may be used to bridge the gap between traceable measurements of monodisperse synthetic MV samples and hitherto untraceable measurements of MV or other samples with broad size distribution.

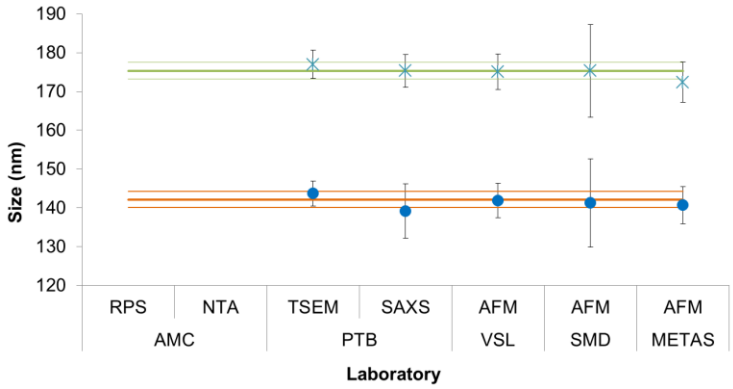


Figure 6. *Particle mean size values with expanded uncertainty (k=2) and reference values for the size fractions of the silica bimodal sample. The thick straight lines are the reference values, and the two corresponding thin lines indicate the expanded uncertainties (k=2).*

Unlike the traceable methods (AFM, SAXS and TSEM), the clinical methods (NTA and RPS) have no calculable uncertainty and are often calibrated using monodisperse PS beads provided by manufacturers. Therefore to determine the size accuracy of the methods, their detected mean sizes for the bimodal synthetic MV samples were compared to the mean size of the traceable methods, and the deviation from the traceable reference values for the monodisperse PS beads was smaller than 7 nm. However, for the SiO_2 (silica) beads, the deviation was as high as 37 nm for RPS. For RPS, which derives the diameter from the electrical resistance of the particle, this high deviation may have been caused by the difference in resistance between SiO_2 and PS beads. Furthermore, both RPS and NTA overestimated the mean size of the 48 nm SiO_2 beads by up to 88%. However, this may most likely, be due to the fact that the 48 nm beads were below the detection limit and aggregates were measured instead, but, in this case, RPS and NTA should have provided a warning instead of an incorrect result. The size resolution of the two clinical methods (RPS and NTA) was also studied and showed the current limitations of the methods as illustrated in Figure 7 with a PS sample with a bimodal distribution. Although the two size fractions were clearly apart from each other (250 nm and 315 nm), NTA was not able to distinguish the two size populations.

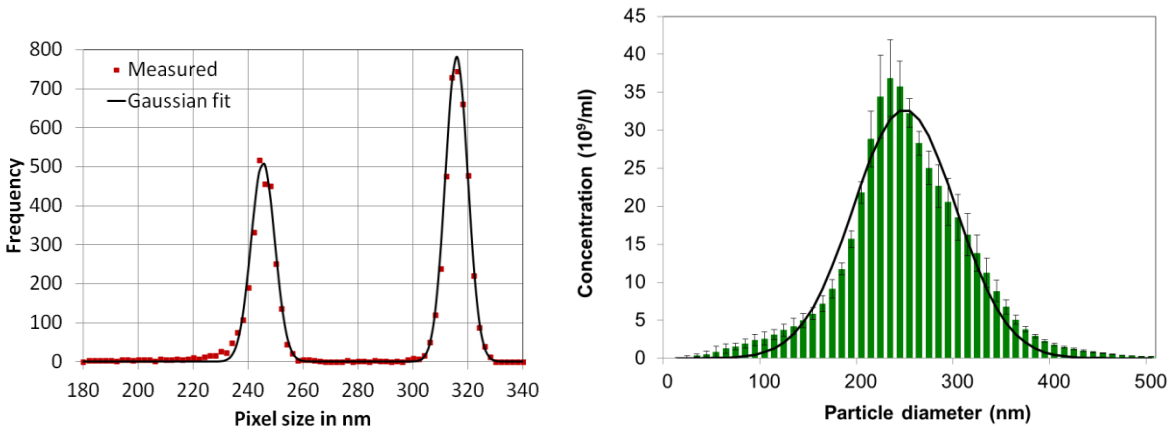


Figure 7. *Size distribution for the bimodal polystyrene sample measured with TSEM (left) and NTA (right) shown with Gaussian fits. In this sample, the used particle fractions had a clear size difference, but NTA was not able to distinguish them.*

Traceable long-term stabilities of the synthetic monodisperse MV reference materials were also performed by the project, in order to verify the minimum stability times and hence the expected useful shelf-life. No indication of instability was found over an evaluation period of 18 months for the synthetic MV reference materials (Figure 8). Upper limits for particle size changes, including the measurement uncertainties, were in the order of 2.5 % /year for the monodisperse samples.

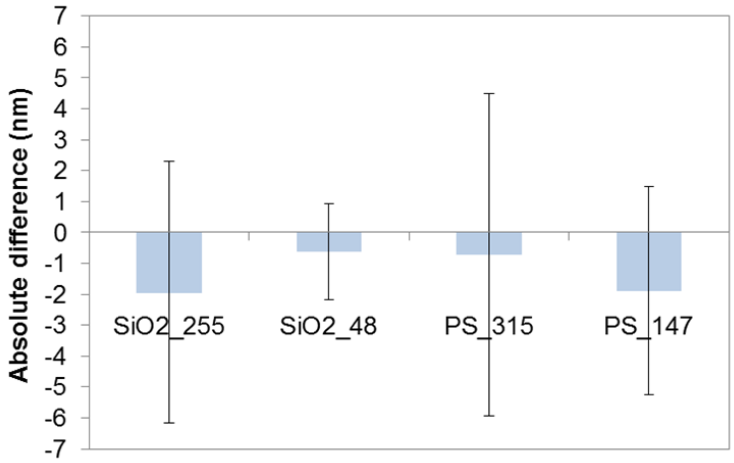


Figure 8. *Mean diameter changes during the time span of the long-term stability measurements with respect to the initial measurement given with the combined uncertainty (k=2) for the monodisperse samples.*

3.2.5 **3.3.4 Clinical inter-comparison of MV using FCM**

Based on the results of the previous sections, the project produced a set of synthetic MV reference materials, made out of polystyrene and silica reference particles and biological MV samples, and distributed them to 33 clinical laboratories with over 45 different laboratory FCM instruments/devices. The participating laboratories are shown on Figure 9. The most important results of the study showed that 1 in 3 of the studied flow cytometers was unable to detect MV in the synthetic MV reference materials in the largest MV size range 1,200 to 3,000 nm.

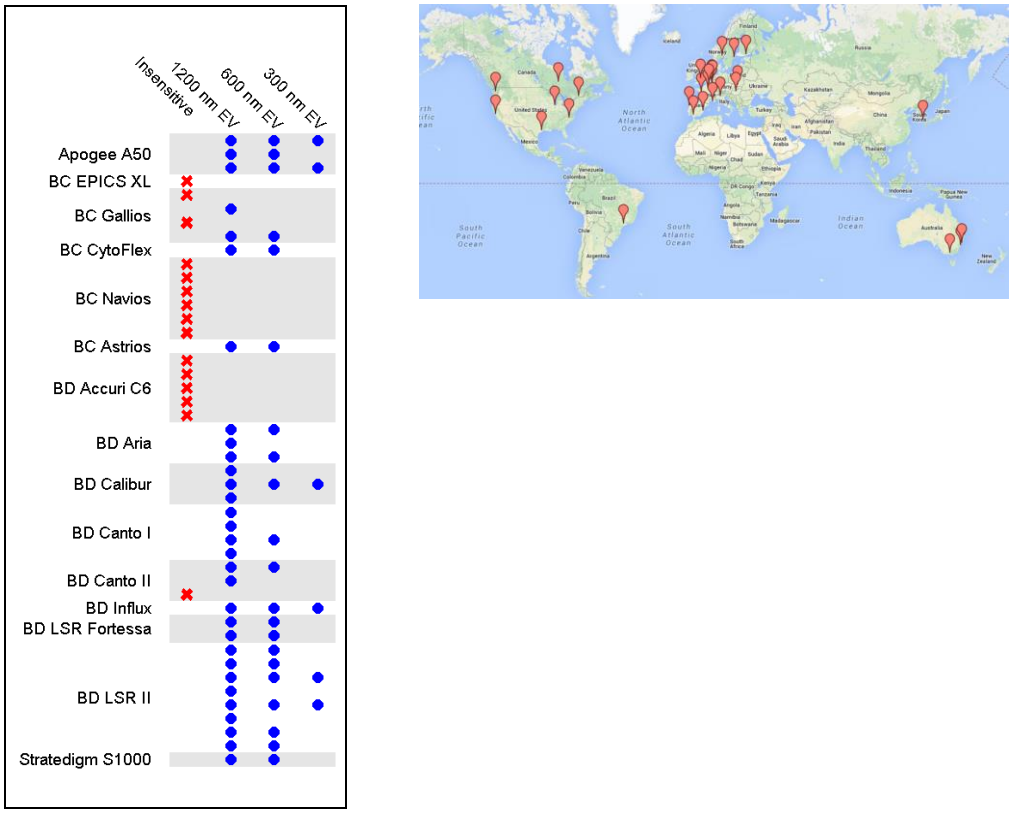


***Figure 9**. Left. Participating institutes in the clinical inter-comparison study of measuring MV on FCM. Right. Assessment of instrument sensitivity. Open blue markers indicate whether a system was capable of detecting the signal of a 1200, 600 and/or 300 nm vesicles above the threshold level. For a number of systems this could not be determined, because their scatter was too insensitive to detect 400 nm fluorescent polystyrene beads. A 400 nm fluorescent bead corresponds to 1200-1900 nm on different side scatter optical configurations and to 700-900 nm on different forward scatter configurations.*

Because all the participants in the clinical inter-comparison had a track record of MV analysis by FCM, the obtained results provide important information about the (lack of) sensitivity of commercially available flow cytometers, and should be taken into account by end-users when referring to previously published scientific work in which insensitive flow cytometers have been used. The results of this work was supported by Exometry B.V, a company who developed software to correct for differences in RI between synthetic MV reference materials and biological MV, and to correct for differences in the optical configurations of the involved flow cytometers. Support was also provided to the project by the International Society on Thrombosis and Haemostasis (ISTH) in distributing the reference materials and biological samples.

3.2.6 **Conclusion**

To improve the standardisation of MV measurements between instruments and laboratories, reliable and well characterised reference materials must be available. Based on the results of a survey, the MetVes project selected 14 commercially available synthetic reference materials made out of silica and polystyrene with a particle diameter between 30 nm to 315 nm. After measuring the size distribution of the particles in the reference materials by AFM, 5 synthetic monodisperse particle samples were selected for their high quality monodispersity (i.e. their particle uniformity). In addition, 2 synthetic bimodal reference particle standards were also chosen to mimick polydispersity as in most clinical samples MV have a polydisperse size distribution. The size distributions and stability of the 5 monodisperse and 2 bimodal synthetic reference materials were measured over 18 months by a variety of traceable and clinically used methods including AFM, SAXS and TSEM and NTA and RPS (repsectively). A comparison of the results showed that (i) RPS and NTA overestimate the mean size of silica particles (of 48 nm) by up to 88 % as these beads are below

the detection limits for the methods and therefore the method often measures aggregates of the particles instead, and (ii) NTA is incapable of measuring the size distributon of a bimodal MV reference materials.

The project also performed inter-comparsions of the MV reference materials, including studing the ability of flow cytometers to detect MV in 33 clinical laboratories worldwide. The results showed that 1 in 3 of the flow cytometers was unable to detect MV in the largest MV size range 1,200 to 3,000 nm, and provide important information about the (lack of) sensitivity of commercially available flow cytometers, which should be taken into account by end-users when referring to previously published scientific work in which insensitive flow cytometers have been used.

3.3 Development of reliable MV sample procedures, including controlled and standardised collection, handling, and storage of body fluids for MV and for the isolation and purification of MV from these body fluids

3.3.1 Introduction

Prior to the start of the MetVes project, published recommendations for the standardisation of pre-analytical variables for blood collection, handling and storage for MV analysis (e.g. Thrombosis Research 2011; 127(4): 370-377) relied on flow cytometry for MV detection, and were therefore based on measuring only 1-2% of the MV present (the lack of sensitivity of flow cytometry is described in objective 1). In addition, no MV sample procedures existed for urine and saliva. Therefore the project developed standard operation procedures (SOPs) for the collection, handling and storage of blood, salvia and urine for MV analysis. To do this the project determined the effects of centrifugation, single freeze-thaw cycle, time between collection and handling, storage temperature and storage duration on blood, urine and saliva, using not only FCM but also RPS, NTA and TEM.

3.3.2 Isolation of MV from single cell types

The size, concentration, and composition of MV in human blood are not only disease (state) dependent but also depend on pre-analytical conditions[1]. For example, most MV in human blood originate from platelets, which can easily become activated during blood collection and handling. This activation results in the release of MV, and therefore variations in blood collection and handling procedures hamper the reproducibility of MV measurements. Although attempts have been made to standardise such pre-analytical variables of blood collection to minimise the release of MV during collection and handling of blood samples, these attempts have been unsuccessful so far because of the lack of sufficiently sensitive techniques to detect MV[2].

To tackle this problem, the size, size distribution and concentration of MV (using the methods from objectives 1 & 2; Sections 3.1 and 3.2) were studied during variations in the collection and handling of blood. Based on these results, SOPs were developed that minimise the sources of pre-analytical variation of MV measurements, but at the same time can be applied in a clinical routine environment. The developed SOP was then applied to collect and process blood samples from patients (see section 3.3.4).

The initial goal was to isolate MV from platelets, red blood cells (erythrocytes) and single cell types such as cell cultures. With regard to the latter, the obtained concentrations were too low and samples proved to be insufficiently pure for downstream analysis by methods such as AFM and SAXS. Therefore, it was decided to focus on the 2 types of MV that occur in highest concentrations in human blood i.e. platelets and erythrocytes.

3.3.3 Isolation of MV from human blood

Blood contains cells which are present in a fluid called plasma. Plasma also contains MV that originate mostly from platelets and erythrocytes. Because plasma is by far the most studied body fluid with regard to biomarker analysis, the behaviour of both platelet MV and erythrocyte MV was studied independently. Both platelet MV and erythrocyte MV were isolated and their behaviour was studied over a variety of conditions; during (after); centrifugation (18,890 x g and 100,000 x g; both tested at 0.5 and 2.0 hours); a single-freeze-thaw cycle; effect of time delay between blood collection and plasma preparation (5 minutes and 1 hour); stability during storage (6 months and one year); at different temperatures (-20°C, -80°C, and -196°C) and measurement by RPS, NTA, FCM and TSEM.

The 2 main conclusions from these studies were that:

1. the effects of centrifugation are dependent on the cellular origin of the studied type of MV (Figure 10). Platelet MV clump during or after centrifugation. This clumping has practical and clinical consequences: whereas sensitive detection methods such as RPS detect lower concentrations after centrifugation, insensitive detection methods such as FCM will detect an increase; in contrast. Eerythrocyte-derived MV do not clump and are too small to be detected as single (individual) MV by FCM. Because centrifugation

[1] *J. Thromb. Haemost.* 2004; **2**: 1842-1851
[2] *Biochem. Biophys. Res. Commun.* 2010; **399**: 465-469

also resulted in clumping of plasma MV, centrifugation was no longer used in subsequent studies in order to circument artefacts;

2. the effects of a single freeze-thaw cycle, time delay between blood collection and plasma preparation and storage time were insignificant on platelet MV and erythrocyte MV under the tested conditions.

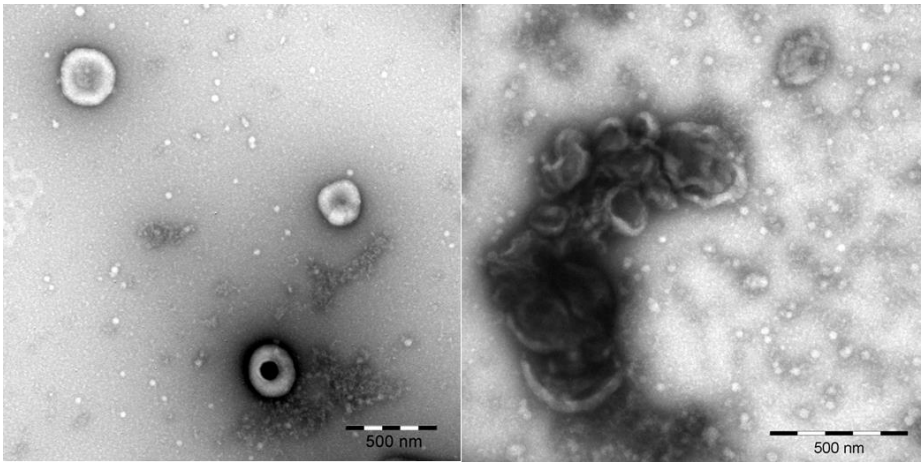


***Figure 10**. The effects of centrifugation. MV originating from erythrocytes (left) do not form aggregates during or after centrifugation, whereas MV of platelet origin (right) will clump together. Because both types of MV are present in human plasma samples, the effects of centrifugation on concentration and size distribution depends on the cellular origin of the MV.*

As part of this work an important but unforeseen development was the rediscovery of size exclusion chromatography (SEC) for isolation of MV from human blood and other body fluids. For downstream analysis, for example measuring size distribution of MV by SAXS or AFM, highly purified fractions of MV are essential, but thus far available protocols which are all based on centrifugation all produce fractions of MV that are insufficiently pure for such detailed downstream analysis. Similarly, for more clinically-oriented applications such as proteomics or genomics, the existing isolation methods are of insufficient quality to produce reliable measurement results.

However, because isolation of MV by SEC does not damage MV and allows almost complete removal (>95%) of soluble components such as proteins and most lipoproteins. In fact this method, as championed by the MetVes project has already become the new method of choice by more than 170 laboratories worldwide for MV isolation (>170 laboratories in 2015; Figure 11). This can then followed by further downstream analysis, such as electron microscopy (Figure 12) or proteomics.

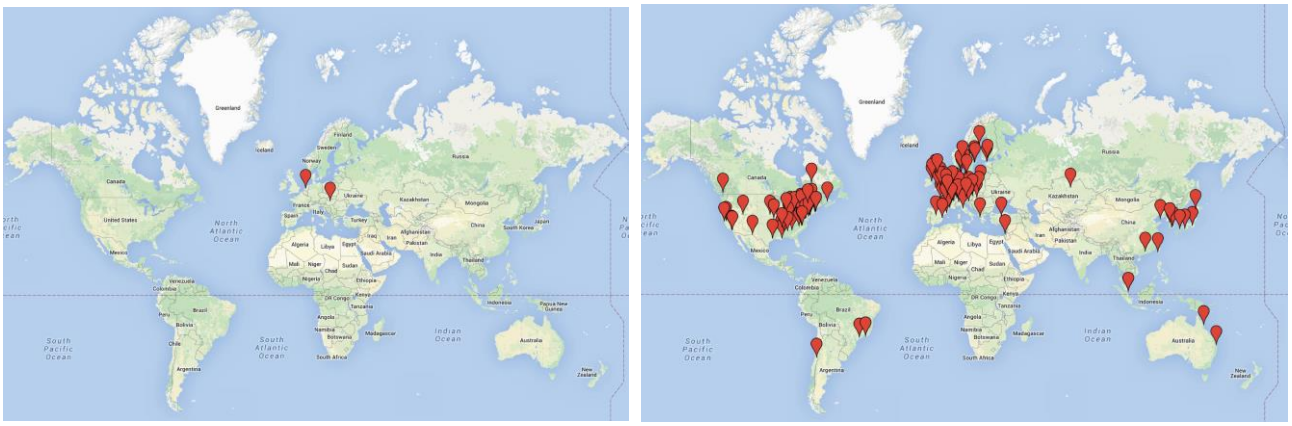


***Figure 11**. Use of size exclusion chromatography (SEC) to isolate MV from human body fluids. Left: exploration of SEC and publication of the first in 2014. Right: use of the MetVes-introduced MV isolation procedure in May 2015 (>170 laboratories).*

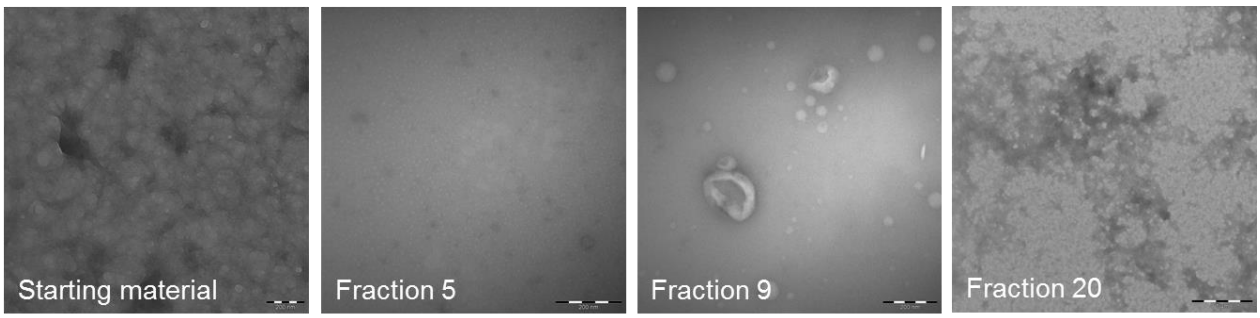


***Figure 12**. Single step isolation of MV from human plasma using size exclusion chromatography. From left to right: the starting material is plasma, which contains not only MV but also a high concentration of soluble proteins making electron microscopy of MV impossible; fraction 5 contains buffer, fraction 9 contains MV, and fraction 20 contains the soluble proteins.*

A demonstration movie of SEC is available on the project website and the results have been published (J Extracellular Vesicles 2014). The SEC method developed by the project has been commercialised by project partner AMC in collaboration with iZon (www.izon.com; http://www.izon.com/assets/PublicationFiles/qEV-Brochure-April-15.pdf).

3.3.4 **Application of the developed SOPs on clinical samples**

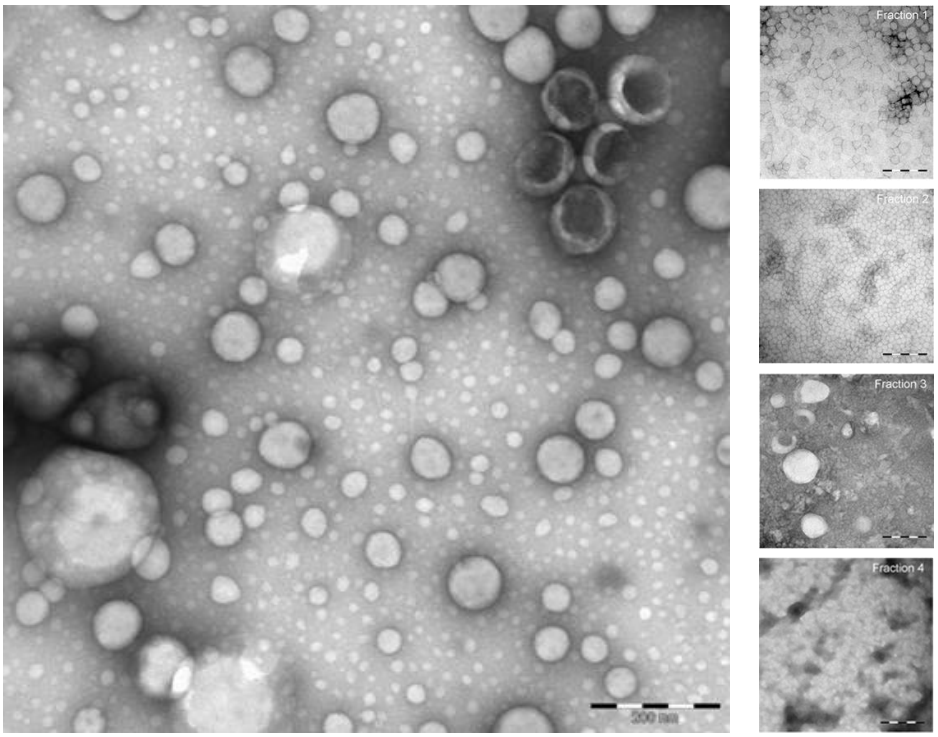


***Figure 13**. Plasma contains MV and lipoprotein particles. Left: plasma contains not only MV (5 MV visuable with their characteristic donut shape in the upper right) but also numerous lipoprotein particles and chylomicrons. Right. Four fractions prepared from plasma by potassium bromide density gradient centrifugation, a procedure commonly used in hospitals to isolate different fractions of lipoproteins for downstream analysis. From top to bottom, Fraction 1 contains particles with a density (d) < 1.006 g/ml, Fraction 2 contains particles with 1.006 g/ml < d < 1.063 g/ml, Fraction 3 contains particles with 1.063 g/ml < d < 1.21 g/ml, and Fraction 4 contains particles with d > 1.21 g/ml. Fraction 3 is the fraction containing the HDL particles, high density lipoprotein particles, but, importantly, also MV. Thus, fraction 3 does not contain pure HDL.*

To test and validate the developed blood collection and handling SOP from section 3.3.2 in daily clinical practice, blood was collected from patients with ovarian cancer, preeclampsia, and from patients suffering from diabetes mellitus type II. The MV isolated from the samples from these patinets were then studied by FCM, RPS and NTA. The MV measurement results confirmed the findings obtained from the blood of healthy/control subjects, thus confirming the clinical applicability of the developed SOP.

However, because plasma also contains lipoproteins, particles consisting of lipids and proteins, which overlap in size range en density with MV, lipoproteins were isolated from plasma by density gradient ultracentrifugation, which is a method commonly applied to isolate and purify lipoproteins from human plasma. The main finding of this was that high-density lipoproteins (HDL) and MV overlap in density and are co-isolated in a fraction (fraction 3 in Figure 13). The direct and practical consequence of this finding was that density gradient ultracentrifugation should be avoided in plasma biomarker studies and that previously published results should be interpreted with extreme caution (J Extracellular Vesicles 2014; 3: 23262

3.3.5 SOPs for MV isolation from blood and other body fluids

SOPs for the collection and handling of other body fluids such as urine and saliva (i.e. other than blood) were not available prior to the MetVes project, therefore SOPs were developed for the collection and handing of both urine and saliva. Similar to blood, analysis of MV in urine and saliva requires the removal of cells as a first step. However, because urine and saliva do not contain very small cells such as platelets, the removal of mainly epithelial cells and thus the preparation of cell-free but MV-containing urine and saliva is easier for these fluids than it is for blood, and the small cells were removed using routine, existing clinical guidelines.

Similar to plasma, MV in cell-free human urine and saliva was studied over a variety of conditions; during (after) centrifugation (18,890 x g and 100,000 x g; both tested at 0.5 and 2.0 hours); a single-freeze-thaw cycle; effect of time delay between blood collection and plasma preparation (5 minutes and 1 hour);stability during storage (6 months and one year); at different temperatures (-20°C, -80°C, and -196°C) and measurement by RPS, NTA, FCM and TSEM. These studies have resulted in the first ever SOPs for successfully isolating MV in urine and saliva and the results have been published (J Extracelllular Vesicles 2015; 4 29260) and are available on the project's website.

3.3.6 Conclusion

The project developed SOPs for the collection, handling and storage of blood, salvia and urine for MV analysis. To do this the project determined the effects of centrifugation, single freeze-thaw cycle, time between collection and handling, storage temperature and storage duration on blood, urine and saliva, using not only flow cytometry but also RPS, NTA and TEM. One of the most important findings was the fact that centrifugation of samples, even at relatively low g forces, results in losses of MV sub populations. The SOPs are available for end users to download from the project website, and so far 226 unique end-users have downloaded the SOPs (Dec 2014 – Aug 2015 website statistics).

In terms of the isolation and purification of MV from body fluids; the MetVes project has pioneered the use of an old, but unused isolation method for extracellular vesicles, for use with MV isolation. The method, size exclusion chromatography (SEC) allows fast isolation of MV from complex body fluids such as plasma in a single step, and is sufficiently reliable to be used in medical clinics. Once MV have been isolated by SEC, 'downstream' MV analysis becomes possible e.g. by proteomics, lipidomics and mRNA analysis, which allows end-users to study the biochemical composition of MV in detail; this was hitherto impossible. The biochemical composition of MV is important as this can be used for the identification of unique proteins, lipids and mRNAs, which can be used as biomarkers for the diagnosis of disease. The SEC method developed by the project has been commercialised by partner AMC in collaboration with iZon and within the space of a year the use of the method has increased to 170 laboratories worldwide.

3.4 Development of methodologies for measuring the concentration, morphology and (bio)chemical composition of MV, and to distinguish MV from other biomaterials in body fluids (lipoproteins, viruses)

3.4.1 Introduction

For the application of MV as biomarkers for clinical diagnostics, not only their size, size distribution or size range in a certain sample is relevant, but also the type (i.e. morphology and (bio)chemical composition) of MV and the concentration is important. When parameters such as the type of MV and their concentration can be reliably extracted from a 'clincal' sample, reference values for MV in bodily fluids can be establised. These reference values then be used tro indicate the range of the number of a certain type of MV for a healthy person, which could be compared to reference ranges for cells, a widely used diagnostic tool in, for example, Clinical Chemistry, Haematology and Pathology. Within the MetVes project, various methodologies were tested to analyse the concentration, morphology and (bio)chemical composition of MV, including anomalous small-angle X-ray scattering (ASAXS), attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), X-ray fluorescence (XRF), AFM using functionalised probes and TEM.

3.4.2 (Bio)chemical composition of MV

Anomalous small-angle X-ray scattering (ASAXS)

ASAXS is based on the energy dependence of the atomic scattering factors of the elements. In an ASAXS measurement, scattering experiments are performed at different X-ray energies close to the absorption edge of one of the elements of the sample under investigation. When the energy of the X-ray beam is close to the edge, absorption processes also occur, resulting in specific changes in the measured intensity curves. From these changes, information about the distribution of the resonant atoms in the sample can be obtained. ASAXS measurements were performed by the project on the erythrocyte derived MV samples at X-ray energies close to the iron edge at 7112 eV.

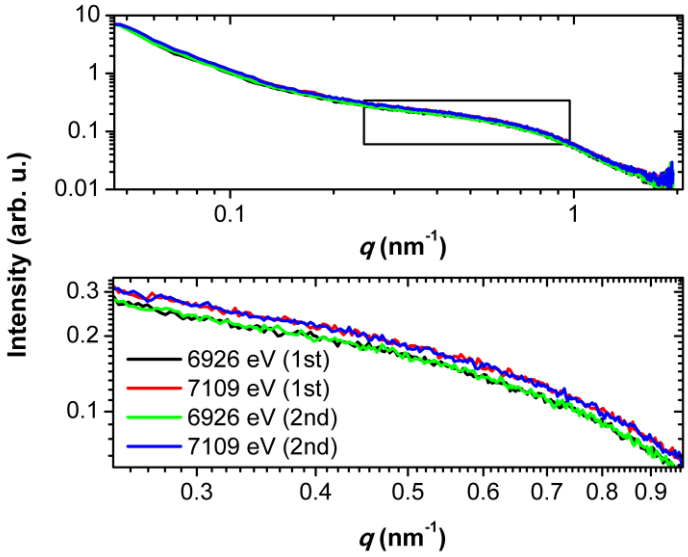


***Figure 14**. ASAXS curves of the erythrocyte-derived MV sample measured at X-ray energies close to (E=7109 eV) and far (E=6926 eV) from the absorption edge of iron. Subsequent measurements at the same energies indicate no radiation damage, but there is a constant shift towards higher intensities in case of the measurements at E=7109 eV (close to the absorption edge)*

The aim of these ASAXS measurements was to reveal if iron containing haemoglobin was present within the erythrocyte derived MV samples. Figure 14 shows the ASAXS curves of the erythrocyte derived MVsample measured at X-ray energies of 6926 eV and 7109 eV, in two consecutive measurement cycles. The ASAXS curves were normalised to the primary beam intensity, and to the transmission values of the MV sample at the different energies. The erythrocyte derived MV sample was not degraded during the measurements as

the subsequent measurements at the same energy did not differ. However, there was a consequent shift in intensity towards larger values in the case of the curves measured at 7109 eV. This trend can be explained by assuming that the resonant atoms are distributed in the matrix, i.e. if there is no co-localization of MVs and haemoglobin. In summary, ASAXS indicates that haemoglobin, also present in the MV preparation, is not associated to the erythrocyte derived MV samples.

X-ray fluorescence (XRF)

During XRF analysis the electrons of the sample are excited with X-ray photons, which results the emission of fluorescent photons with energy characteristic to the constituent elements of the investigated sample. XRF is widely used in materials science, geochemistry and archaeology. Erythrocyte-derived MV samples were studied by XRF using the excitation energy of 3.5 keV. The use of a sample cell with SiN windows was needed due to the low penetration length of X-ray photons at this energy. Figure 15 shows the XRF spectra of two erythrocyte-derived MV samples. Sulphur and phosphorus can be assigned to MVs, however, the phosphorus from the buffer also contributes to the signal. The chlorine peak originates also from the buffer, while the silicon, aluminium, and nickel peaks can be attributed to the sample holder.

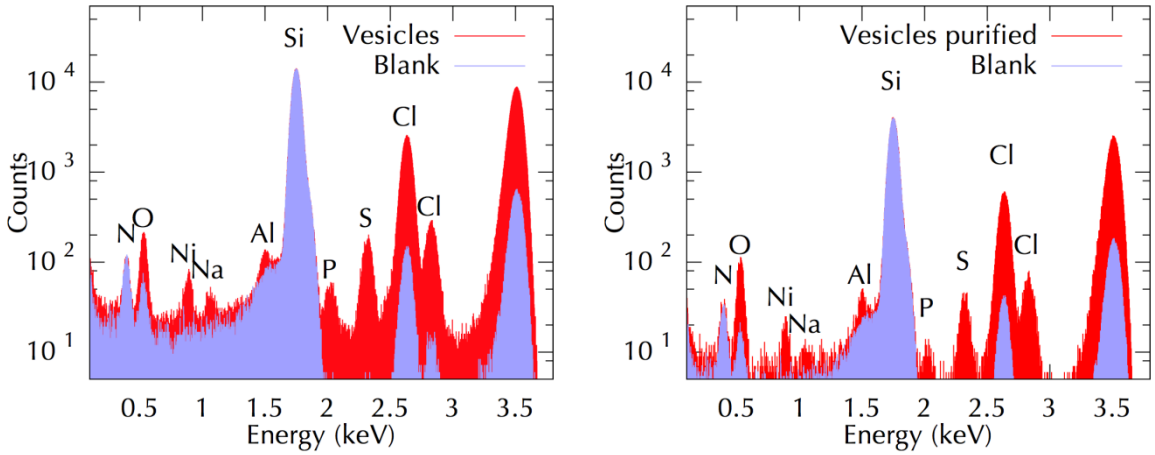


Figure 15. *XRF spectra of erytrocyte-derived MV isolated at 18,890 x g (left), and at 100,000 x g followed by purification by size exclusion chromatography. The lines characteristic to the different elements are denoted by their symbols. Elements form the MVs (P and S), from the buffer (P and Cl), and from the sample holder (Si, Al, Ni) can be detected by XRF.*

3.4.3 Distinguishing MV from other biomaterials in body fluids (lipoproteins, viruses)

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR spectroscopy based protein quantification is already commercialised and provides a solution for many problems associated with colorimetric assays and UV-detection used in protein research. However, despite its advantages, ATR-FTIR spectroscopy had not previously been used to characterise MV samples. ATR-FTIR spectra were measured by the project using a Varian 2000 FTIR Scimitar Series (Varian, Inc, USA) spectrometer equipped with a liquid nitrogen cooled mercury-cadmium telluride detector and with a 'Golden Gate' single reflection diamond ATR accessory (Specac Ltd, UK). 128 scans were co-added at a nominal resolution of 2 cm^{-1}. The measurements were performed at room temperature, immediately after drying the sample on the top of the diamond ATR element. ATR correction was applied after each data collection. Spectral analysis was performed using GRAMS/32 software package and for the interpretation of the MV spectra, a high density lipoprotein (HDL) sample (Merck Millipore, Damstadt, Germany) was also measured by ATR-FTIR.

The amount of plasma proteins and an estimate for the protein-to-lipid ratio were deduced from the ATR-FTIR measurements. Figure 16 shows the IR-spectra of two investigated erythrocyte derived MV samples, together with the spectrum of a HDL sample and characteristic vibrational bands are highlighted. To describe the amount of proteins in each sample, the Amide I bond at 1690 to 1600 cm^{-1} was used, while the C=O band (at 1765 to 1710 cm^{-1}) characteristic to lipid moieties was used for the phospholipids. The areas under the indicated bands were calculated for the two erythrocyte derived MV samples and for HDL, and the known

protein-to-lipid weight ratio of the latter was used to the estimate the ratio for the erythrocyte derived MVs. The composition of the HDL according to the manufacturer was 45 to 55 w/w% protein, and 45 to 55 w/w% lipid. Using the average of these values, and taking into account that the 3 to 5 w/w% free cholesterol does not contribute to the lipid C=O vibration, we used 1.17 as the average value of the HDL sample. Using this value we obtained 5.385 for the protein-to-lipid ratio of the sample centrifuged at 18,890 x g, and 4.025 for the 100,000 x g sample. This indicates that the protein content of the erythrocyte derived MV samples is around 15 and 20 w/w% for the samples isolated at 18,890 x g and 100,000 x g, respectively. Considering the fact, that CL-2B filtration removed the majority of plasma proteins in the case of the 100,000 x g sample, the amount of proteins which are not associated to the vesicles is estimated to be 5 w/w%.

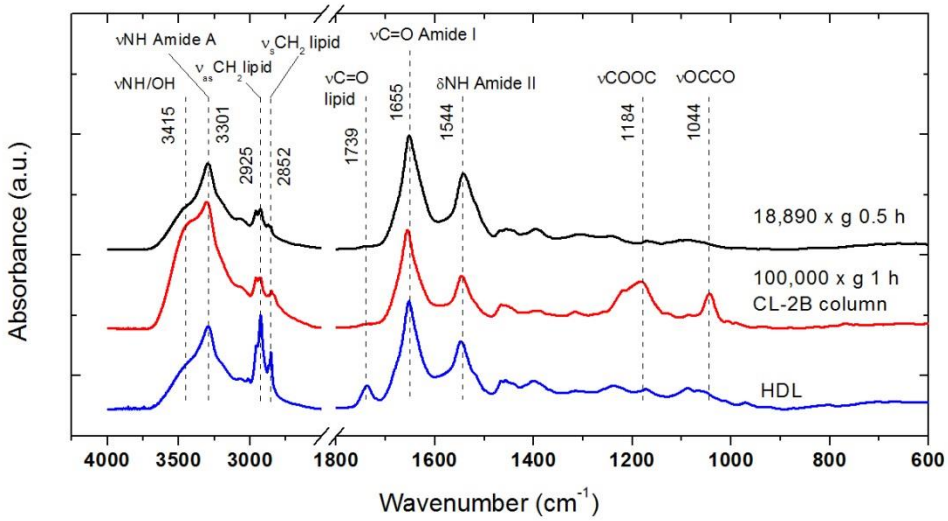


Figure 16. *ATR-FTIR spectra of the investigated MV samples together with the spectrum of HDL. The assignments of the main bands are also shown in the figure.*

AFM with functionalised tip probes

AFM probes can be transformed into sensitive, chemically selective biosensors by attaching ligand molecules to the AFM tip and by probing samples for receptors on the surface. The ligand can be an antibody, substrate, inhibitor, etc. The receptor can be a protein immobilised on various substrates such as mica, or it can be a protein exposed on MV. The probing of the sample is performed by force spectroscopy in which, Force-Distance curves are performed: i.e. the functionalized AFM tip is approached until it interacts with the sample and then is retracted to its resting position. During this approach, a receptor–ligand complex can be formed based on the specific ligand–receptor recognition. Following subsequent retraction of the tip from the surface, an increasing force is exerted on the ligand–receptor connection until the interaction bond breaks at a specific and critical unbinding force. These force spectroscopy experiments can provide valuable information about the structure and dynamic of molecular unbinding events at the molecule level and in molecular recognition AFM, the binding of ligand molecules to the AFM tip or to the surface is a critical step. Therefore, this requires careful AFM tip sensor design such that the attached ligands should have enough mobility to interact with the complementary molecule. To allow this, a long and flexible poly ethylene glycol (PEG) chain is generally inserted between the tip and the probe molecule. Here, an antibody directed against human glycophorine A (CD235a) is linked to the AFM tip using a bifunctional commercial PEG linker. The specific interactions of the antibody and its target antigen are then detected by force spectroscopy in a liquid environment.

For the recognition measurements in the project, the MV were deposited on a mica substrate, after which a regular AFM image of the surface was obtained. Subsequently, at the position of interest the interaction force was measured and recorded as in Figure 17, which shows the typical ligand-receptor unbinding between a CD235a-coated AFM probe and an erythrocyte-derived MV.

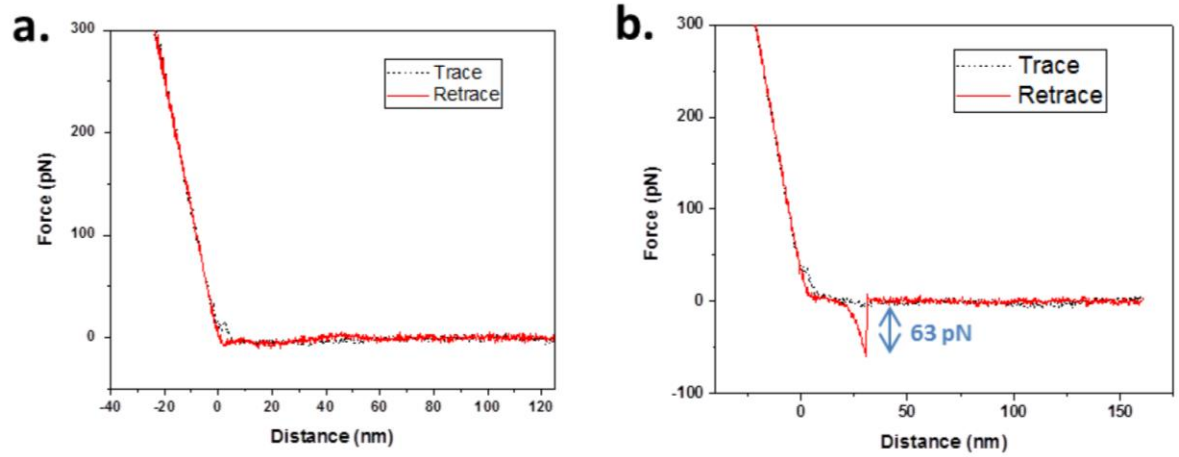


Figure 17. *Force measurements of interaction between cd235a antibody-tethered tip and erythrocyte derived MV captured on mica substrate. An example of different force curves observed are presented: (a) an event free curve and (b) a specific recognition event.*

Although the results for the AFM with functionalised tip probes measurements of MV were very promising, it was not been possible to probe isolated MV, but only agglomerates, as these were easier to identify. In addition, although the magnitude of the unbinding force (61 +/- 27 pN) was in the expected range, the number of ligand-receptor complexes that contributed to the total measured force was unknown, meaning that in order to positively identify a certain MV, the number of ligands tethered to the AFM tip needs to be quantified, which will require further research.

3.4.4 **3.2.3 Morphological analysis of MV**

In order to measure the shape/morphology, of MV microscopy techniques such as TEM and liquid-AFM were used. Figure 18 shows a typical measurement for TEM. However, when a similar erythrocyte derived MV sample was measured using AFM, the picture is completely different, and only two types of morphologies are observed: round shapes and “doughnuts”, as can be seen in Figure 19.

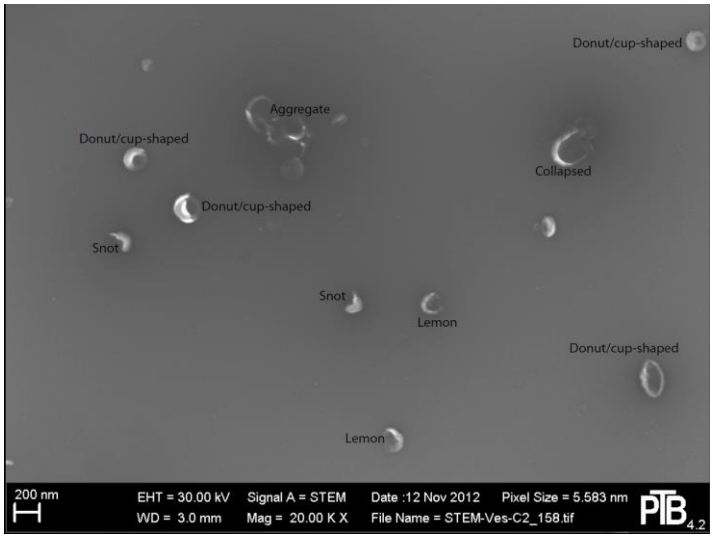


Figure 18. *TEM image of MV showing a large variety of morphologies.*

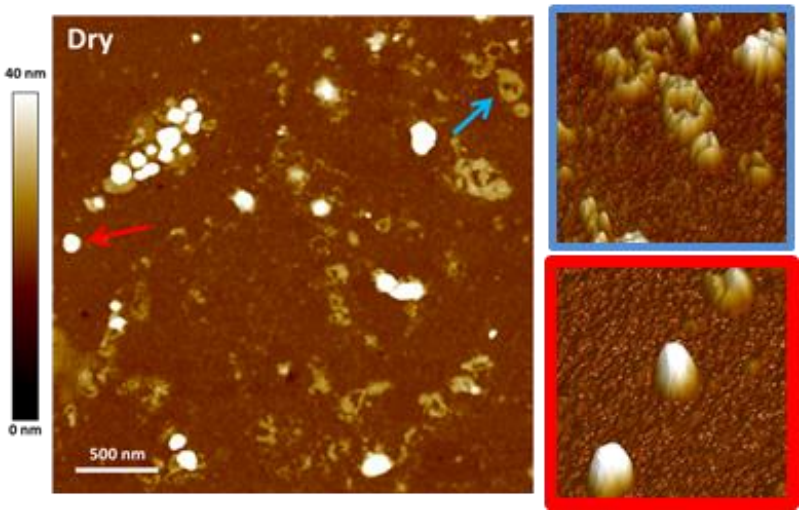


***Figure 19**. AFM image of erythrocyte derived MV measured under dry conditions and 3D views of collapsed MV (blue) and an uncollapsed MV (red)*

When measured in a liquid media, the doughnut shaped MV are very rare. From this, we deduced that the doughnut shapes as observed in AFM under dry conditions are collapsed MV. This also suggests that the different morphologies of MV as observed with TEM are due to the specific sample preparation and do not reflect any intrinsic properties of MV.

Compared to TEM the sample preparation of liquid-AFM is much less invasive as the MV are continually kept in a buffer media. Therefore, if we take a closer look at the round shapes in the AFM images, we find that the 3D-shape is ellipsoidal, with the height much smaller than the in-plane diameter[3], see Figure 10 (a). It can also be assumed that in suspension the morphology of MV is spherical, rather than ellipsoidal, as this would be the energetically most favourable shape. These results imply 2 things; firstly, that the interaction of MV with their substrate is significant and secondly, that liquid-AFM does not provide an accurate view of true, intrinsic MV morphology. However, AFM can still be used to deduce the size distribution of MV (as per objective 1 and Section 3.1.3). Figure 20 (b) shows the size distribution of erythrocyte derived MV as measured with liquid AFM.

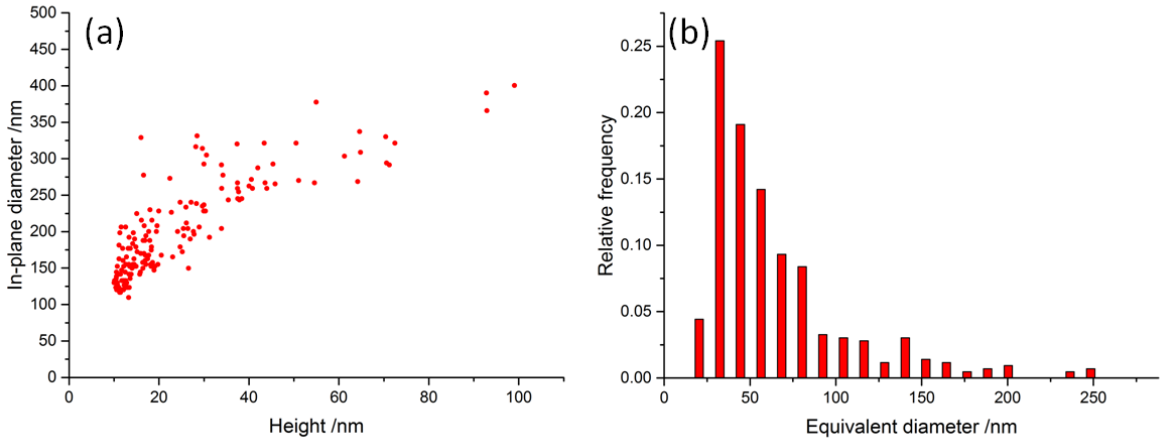


***Figure 20**. **(a)** The height of erythrocyte derived MV plotted versus the in-plane diameter as measured with liquid-AFM. **(b)** Distribution of the equivalent diameters of erythrocyte derived MV as measured with liquid-AFM.*

The results have also shed new light on the validity of the dimensional metrology of MV using electron microscopy (e.g. SEM, TEM, and TSEM). As these techniques can only provide the 2D in-plane dimensions

[3] The in-plane diameter provided is the diameter of a circle with the same area as the measured area of the shape after the AFM image has been corrected for tip-sample convolution.

of MV and cannot be used to extract the equivalent diameter, unless the MV remain spherical after deposition on the substrate, which is highly unlikely. Therefore, to gain an accurate measurements of the size and true morphology of MV arguably the best technique would be FF-TEM, where the intrinsic dimensional properties are preserved by rapid freezing of the sample.

3.4.5 **3.2.4 MV concentration analysis**

Although microscopy methods such as AFM and TEM can be used to count MV after deposition on a substrate, they cannot be used to directly measure the concentration of a MV sample, because the relationship between the surface coverage and the number of particles/MV in the applied suspension is in most cased unknown. Therefore, in this respect methods that measure directly in suspension such as NTA and RPS (see Section 3.1.2) are in principle better suited to determine the concentration of MV samples. Two relatively new techniques that are currently used in clinical laboratories to determine the (size-dependent) concentration of MV are NTA and RPS and these techniques were evaluated for their applicability to the enumeration and concentration of MV. The results showed that both NTA and RPS are able to measure the size-dependent concentration of MV in clinical samples. But because both methods are laborious, require monodisperse suspensions and are unable to distinguish MV from similar-sized particles, both NTA and RPS have only limited value for the analysis of MV in clinical samples.

3.4.6 **Conclusions**

The MetVes project has shown that differences in MV size distribution and concentration measurements (of the same MV sample) are primarily due to differences in the minimum detectable MV size of different methods, i.e. 70–90 nm for NTA and 70–100 nm for RPS. This knowledge can now be used to enable end-users to compare MV concentration measurements from different methods, once the minimum detectable MV size is known.

For the (bio)chemical composition and morphology of MV, the project developed a method using AFM with “functionalised tips” to study the cellular origin of single MV. The functionalised tips are coated with antibodies against a protein present on only a single type of MV. This method allows end-users to establish the cellular origin of MV by AFM, and can also be used to distinguish MV from other biomaterials in body fluids.

4 Actual and potential impact

Actual impact

Since the start of the MetVes project in 2011, it has had an important impact in the biomedical sciences community as it was the first project involved in the standardisation of MV measurement. The project has introduced the term “metrology” and corresponding terminology to a large and global (bio)medical audience and provided a sound metrology structure for MV measurement that will be further developed and explored within a newly initiated working group on the standardisation of vesicle measurements by flow cytometry. This new working group is a collaboration between the International Society for Extracellular Vesicles (ISEV), ISTH and the International Society for Advancement of Cytometry (ISAC) (www.evflowcytometry.org). The MetVes project coordinator (who is the Chair of the Scientific and Standardisation Committee on Vascular Biology of the ISTH) has been asked to participate in this international working group and so far has given 2 invited presentations to this working group.

The project has received ongoing interest from a large group of renowned (medical) institutes and universities such as Harvard Medical School and the Mayo Clinic in the US and Oxford University in the UK, as well as support from relevant international societies such as the ISAC, ISEV and ISTH. For example, the ISTH invited the MetVes project to present a State of the Art Lecture on “Innovation in detection of microparticles and exosomes” at the XXIV ISTH Congress meeting in Amsterdam in July 2013 and to contribute with an invited review in the ISTH’s associated state of the art book which was published as an additional volume of the Journal on Thrombosis and Haemostasis.

In addition, to ensure the uptake and dissemination of the MetVes project’s results to stakeholders and end-users, a wide range of activities were undertaken.

These activities included:

- the production of the MetVes website (www.metves.eu) visited by 2065 unique visitors in 2014, and by 2314 unique visitors in Jan 1 – August 17, 2015. The project’s results/reports are also available on the website for end-users to download. For example the project’s report on ‘MV reference materials’ has been downloaded 1,085 times by unique visitors (- August 17, 2015) and the project’s report on the ‘Co-isolation of extracellular vesicles and high-density lipoproteins using density gradient ultracentrifugation’ has been downloaded by 734 unique visitors (August 17, 2015),
- 8 peer-reviewed papers mainly in health journals and 4 more papers are in preparation/have been submitted
- 46 presentations on the MetVes project at international scientific (mainly biomedical) conferences and a further 8 presentations at seminars/workshops in the UK, US, Ireland, Finland and the Netherlands.
- the training of 19 graduate and PhD students from 7 different European countries in the MetVes project’s developed protocols and methods on the isolation and detection of MV , as part of the students internship with the project partner AMC.
- the production of instructional (YouTube) movies for end-users. The movies are also available on the project’s website since March 2015 and include:
    - Collection and preparation of human blood samples for MV measurements (226 unique visitors)
    - Isolation of MV from human blood samples by SEC (271 unique visitors)
    - MV size and concentration using NTA (187 unique visitors)
    - MV size and concentration using TRPS (117 unique visitors)
- two articles/media interviews on MV research and the MetVes project in leading Dutch newspapers (e.g. Trouw), as well as 4 other press releases in Germany and the Netherlands.
- a METVES workshop attended by over 50 metrologists and health care professionals. The workshop was used to disseminate the most important results from the project such as the MV reference materials and MV measurement methods. The workshop program included live demonstrations of MV sample preparation and measurements, as well as lectures and discussion and networking sessions.

The MetVes project also developed (i) MV reference standards and SOPs for the collection, handling and storage of blood, urine and saliva for MV analysis, (ii) a method for the isolation of MV by SEC that is being

used in 170 (mainly clinical) laboratories worldwide, and (iii) reliable procedures for the dimensional characterisation of MV and for measuring the concentration, morphology and (bio)chemical composition of MV.

The SOPs developed by the project, to standardise the pre-analytical phase and the development of a method for isolation and purification of MV from body fluids are being widely used by investigators in the MV field, including researchers in hospital laboratories, not only in Europe but also worldwide. The method to isolate MV from body fluids using SEC has also been widely accepted, and is being used by many research organisations and hospital laboratories, worldwide. For example, investigators of the National Institute of Health (Washington DC, USA) are using SEC to isolate MV for downstream analysis. Although SEC is not a novel method as such, the application of SEC in within the field of MV is novel, and therefore it was commercialised by the project, in order to facilitate standardisation.

Finally, the project developed a novel and label-free method for distinguishing MV from other biomaterials in body fluids using the RI of MV that offers clinical laboratories the opportunity to analyse body fluids containing MV more efficiently and economically.

All these results have enabled the comparison of MV measurement results between different instruments and different medical institutions, which means that for the very first time it will become possible to perform multi-centre MV trials. The results have also provided a sound basis for the standardisation of MV measurements and contributed to the further establishment of MV as biomarkers of disease, an important step towards earlier diagnosis of many common and rare diseases and thus more efficient medical treatment.

5 Website address and contact details

Project website www.metves.eu

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6 List of publications

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