EMRP JRP - HLT02 MetVes

Report on the needs, specifications and commercial sources of microvesicle reference materials

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This report is part of deliverable D4.1.1 in work package WP4

Contents

Abbreviations:

AFM	Atomic Force Microscopy
DLS	Dynamic Light Scattering
EQELS	Electrophoretic Quasi Elastic Light Scattering
EV	Extracellular Vesicle
FACS	Fluorescence-Activated Cell Sorting
FCM	Flow Cytometry
ISADE	Invitrox Surface Antigen Detection and Enumeration
MV	Microvesicles
NPP	Normal pooled plasma
NTA	Nanoparticle Tracking Analysis
PBS	Phosphate Buffered Saline
PFP	Platelet Free Plasma
PL	Platelet
PS	Polystyrene
RPS	Resistive Pulse Sensing
SAXS	Small Angle X-ray Scattering
SEB	Supplement Enzymatic Buffer
SEM	Scanning Electron Microscopy
SIOS	Scanning Ion Occlusion Sensing
SPT	Single Particle Tracking
SSL	Sterically Stabilized Liposomes
TEM	Transmission Electron Microscopy

1 Introduction

Within the European Metrology Research Programme (EMRP) the 3 year project “Metrological characterisation of microvesicles from body fluids as non-invasive diagnostic biomarkers” has started in June 2012. The aim of the project is to develop traceable measurement and characterisation techniques for the characterisation of microvesicles 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. In work package WP4 “Microvesicles reference materials” synthetic reference materials (task 4.1) and biological reference materials (task 4.2) will be developed and characterised.
Within task 4.1 “Development and distribution of synthetic MV reference materials“ METAS conducted a survey among JRP-Partners and stakeholder committee members on the used methods, needs, specifications and commercial sources of reference particles with physical properties related to MV. The here presented report contains the outcome of this survey.

A questionnaire collected existing information about:

- The methods intended to be used during the project by partners or stakeholders.
- Typical sample preparation and measurement conditions needed by these methods.
- Specific requirements of the mentioned methods with respect to reference particles.
- Known suppliers of commercial synthetic particle reference materials
- Experiences and recommendations with respect to reference materials already used.

The project attracted wide interest and 19 completed questionnaires were submitted.
The here presented report compiles the information obtained from the contributors. Some information given might be in contradiction to other given information or is valid only for specific situations.

The original questionnaire is attached in appendix A.

2 Contributors

The following persons and institutions completed the questionnaire and contributed with their profound experience to the results summarised here. Each institution might have even included several further contributing persons.

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3 Applied methods

3.1 General characterisations

The 19 contributors use in total nine basically different methods for micro vesicle investigations. These methods are briefly introduced here:

Flow Cytometry FCM: A laser beam is directed into a hydrodynamically-focused stream of liquid. The suspended particles pass through the beam and scatter the light. Many different detectors (Forward Scatter detector FSC, Side Scatter detectors SSC, and Fluorescence detectors) are aimed at the point where the stream passes through the light beam. The combination of the scattered and fluorescent light is picked up and analysed to derive physical and chemical information about the structure of each individual particle.

Dynamic Light Scattering DLS: Also known as photon correlation spectroscopy or quasi-elastic light scattering is a technique to determine the size distribution of particles in suspension. When coherent light is scattered by a suspension of particles in a liquid, variations or 'speckles' in the intensity of the scattered light are seen. These fluctuations are due to the fact that the particles are moved around by the Brownian motion of the surrounding molecules. The light fluctuations contain information about the time scale of the particle movement. The rate of particle movement is then related to the size of a sphere of equivalent hydrodynamic radius. This is a particle ensemble measurement.

Single Particle Tracking SPT (also Nanoparticle Tracking Analysis NTA): This method tracks directly the Brownian motion of individual particles in liquid suspension. An ultramicroscope and laser illumination is used to visualize the moving particles. The particle positions are captured over multiple frames to determine the random walk pattern of each individual particle. The rate of the particle movement is finally related to the size of a sphere of equivalent hydrodynamic radius.

Resistive Pulse Sensing RPS: The size of particles is determined by measuring the electrical resistance of a pore that connects two solution-filled reservoirs. A particle entering the pore displaces the conducting fluid, which leads to a transient change (or pulse) in the measured resistance. The magnitude and duration of the pulse are related to the size and position of the particles that caused it.

Transmission Electron Microscopy TEM: Microscopy technique in which electrons interact with an ultra-thin sample. An electron beam is focused and transmitted through a thin specimen. The interaction of the electrons transmitted through the specimen forms an image which is magnified and focused onto an screen (CCD sensor, photographic film or fluorescent screen). The basic principle is similar to a light microscope but it uses electrons and electromagnetic lenses instead of light and glass lenses, thereby obtaining sub-nm resolution. The specimens are observed in high vacuum.

Scanning Electron Microscopy SEM: Microscopy technique that produces an image by scanning a focused beam of electrons over the sample. The electrons interacting with the sample produce various signals which can be used for image formation. The specimens are observed in high vacuum.

Scanning Electron Microscopy in Transmission Mode TSEM: This Microscopy technique combines the overall versatility of a SEM with the high resolution of a TEM. A focused electron beam is scanned over and transmitted through a thin specimen. Brightfield and darkfield detectors can be used for image formation. The specimens are observed in high vacuum.

Small Angle X-ray Scattering SAXS: Technique based on the elastic interaction of photons with the electrons of the specimen. A monochromatic X-ray beam is sent through a sample from which some of the X-rays scatter. The scattered X-ray pattern is detected on a flat X-ray detector situated behind the sample. The small scattering angle requires a distance of several metres between sample and detector. The required intense monochromatic x-ray of low divergence is only available at synchrotron radiation facilities. The particles are directly observed in liquid suspension which is filled into a thin glass capillary. This is a particle ensemble measurement.

Atomic Force Microscope AFM: Microscope technique using an atomically sharp tip at the end of a cantilever in order to probe the specimen surface. Atomic forces (e.g. van der Waals) between tip and sample lead to a deflection of the cantilever. This deflection is measured using a laser beam reflected from the top side of the cantilever into photodiodes. A piezo actuator and a feedback loop maintain a constant tip-sample interaction force by adjusting the tip-sample distance. Raster scanning the tip across the sample surface leads to x, y, z coordinates which allow mapping the surface in 3D. AFMs can operate in air, liquid or vacuum. Particles must be deposited onto atomically flat cleaved mica surfaces.

3.2 Detailed Information

FCM

Used Instruments:
- Beckman-Coulter Cytomics FC 500 (2 laser setup: argon ion + solid state red laser)
- Beckman Coulter Galios
- Beckman Coulter NAVIOS
- Becton Dickinson LSR II, FACS Canto II
- Becton Dickinson FACS Calibur (488 nm, 633 nm, 4-color)
- Stratedigm S1000Ex (3-lasers: 405 nm, 488 nm, 640 nm, DI H_2O sheath)
- Apogee A50 (4 color flow, 2 lasers: blue and red)
- Fortessa
- Guava from Millipore

Comments:
- Using fluorescence staining, one can measure liposomes with diameters of 50 nm.
- The Apogee A50 can detect polystyrene microspheres down to approximately 100 nm in diameter; this is equal to a lipid based microvesicle about 200 nm in diameter, depending on the optical configuration.
- By testing particle size with various refractive indexes, important differences in the outcome of analysed particle sizes were observed.

Restrictions:
- PS beads result in underestimation of size of biological vesicles by flow cytometry. 100 nm diameter PS microspheres scatter the same amount of light as are equal to lipid based MV about 200 nm in diameter, depending on the optical configuration
- Since the volume of the focussed laser beam typically is ~50 pL, FCM may be detecting swarms of small MV as a single larger particle
- Reference particles above 3 microns (polystyrene) are incompletely counted. Sensitivity of detection based on scatter of polystyrene reference beads is 160 nm diameter.

Related methods:
- High Sensitivity Single Particle Fluorescence (= Custom designed fluorescence flow cytometry)
  Size range depends on the brightness resulting from intrinsic properties
- High Sensitive Flow Cytometry
  Size range: 0.25 - 80 µm (scatter threshold), 0.1 – 80 µm (fluorescent threshold)
  Sensitivity: 10 events / mm^3
  Fluorescent material preferred
- Fluorescence-Activated Cell Sorting (FACS)
  Apparatus: BD FACSCanto II
  Specimen measured: ejaculate (centrifugation of the material several

times before performing FACS) and follicular fluids, peripheral blood, bone marrow.
- Electrophoretic Quasi Elastic Light Scattering EQELS (Invitrox Inc.). Laser based technology that assesses cell surfaces as a function of activation, apoptosis or ligand binding.
  Particles smaller than 50 nm. Particles larger than 8 microns tend to settle with time and can induce errors.
- ISADE is a laser based light scattering device that detects, counts, and sizes particles. Phenotyping can be added.
  Particle size range is from 0.15 micron to 20 microns. Larger sizes are possible with different configuration of detectors.
  ISADE is not a dynamic light scattering technique, but a Mie scattering method.

SPT, NTA

Used Instruments:
- NanoSight NS500

Properties:
- Extracellular vesicles and other particles in the range of 50 nm to 1000 nm.
  Ability to distinguish two populations, as long as the diameter differs at least by a factor 2.
  Size range: ~ 50 nm - 500 nm, - 1µm
  Concentration range: 1E8 – 1E10; 1E6 -1E10/ml
  Reference materials with high refractive index are correctly sized.

Comments:

NTA measurement of extracellular vesicles in phosphate buffered saline, fluorescence NTA and zeta potential measurements by NTA. Measurements are generally made at ambient temperature but temperature may be controlled within a range of 10 - 37ºC. Possible to analyse many different man made nanoparticles.
- Refractive Index is important – must ideally be similar to biological vesicles. Very small biological vesicles (< 50 nm) may not be detectable by NTA. Upper limit is 1 micron.
- Aggregates and high background must be avoided.

Restrictions:
- Only isotonic saline is suitable for measurement of vesicles from biological sources. Other aqueous media may be used for non-biological particles, so long as the viscosity is known.
- Dilution into suitable buffer required into window of concentration optimal for measurement

TEM

Comments:
- Vacuum, e-beam energy: typical 100 kV

Restrictions:
- Dehydration and fixation in vacuum may affect the appearance.
- Polystyrene beads might shrink due to (e-beam) heating.

Related methods:

- Cryo-TEM
  Samples are observed as thin films of frozen-hydrated material
  Freezing by quick plunging a thin (100 – 500 nm thick) liquid film in liquid ethane, cooled down by liquid nitrogen.
  Detection of particles in the size range between 20 nm and 1 µm
  Resolution of 2 nm
  Concentration of MVs in PFP of healthy donors sufficient to be detected

- Transmission Scanning Electron Microscopy (TSEM)
  Energy range: 30keV
  Particles up to 200 nm (larger particles possible, but higher uncertainties)

- Freeze-Fracture combined TEM (FF-TEM)
  Energy range: 2 - 8 keV
  No size restriction
  Concentration: > 5 - 10 mg / ml (depending on the size)
  A few ul of a liquid sample is needed for freeze-fracture.

RPS

Used Instruments:

- Izon qNano

Comments:

- Sensitivity for small differences in size (5 nm)
- 5 % reproducibility for PS beads
- Ambient temperature or controlled temperature
- Particles suspended/diluted in aqueous electrolyte buffer
- Particle conductivity dependence?
- Size range: 40 nm - 50 nm to 10 µm, 70 nm to 800 nm
  Concentration range: 1E6/ml to 1E10/ml;
  10E5 to 10E12/ml depending on particles size
  Aqueous electrolyte (PBS, NaCl, KCl, cell culture media, urine, etc…)

Restrictions:

- Electrolyte required
- Filtration of the sample before analysis to avoid pore clogging.

Related methods:

- Scanning Ion Occlusion Sensing (SIOS)
  Apparatuses: qNano, qViro-X
  Size range: 50 nm - 10 µm
  Concentration range: 1E10 to1E5 /ml, depending on size.
  Izon Science calibration particles are NIST-certified carboxylated polystyrene beads, requiring 1/1000 dilution before use.
  Range is CPC70, 100, 200, 400, 500, 800, 1000, 2000 and 4000 to match corresponding nanopore membranes
  Requires electrolyte buffer (PBS, cell culture medium, Izon SEB)

SAXS

Comments:
- X-ray energies from 2 – 8 keV
- The liquid sample is filled into capillaries or vacuum compatible sample holder with membrane windows (for soft X-rays)

Related methods:
- Anomalous Small Angle X-ray Scattering (ASAXS)
  Uses tuneable X-ray energy near, but below, X-ray absorption edges of the elements comprising in the investigated sample. Thus element specific information, composition and density fluctuation can be obtained

Restrictions:
- Works best for monodisperse particle size distributions.
  Required continuous X-ray source (synchrotron radiation)

Metrology AFM

Used Instruments:
- Dimension 3500 (Bruker) metrology head
- Nanosurf InLens AFM
- Self-made metrology AFM

Properties:
- Size range of 4 nm to 2 µm.
- In air or liquid
- Deposition on atomically flat mica-substrates, with or without pre-treatment with Poly-L-Lysine.
- Deposition on atomically flat mica-substrates coated with antibodies against specific human antigens (e.g. anti-CD41 for vesicles derived from platelets).

Restrictions:
- Particles should be as spherical as possible, otherwise shape deviations can lead to systematic deviations.
- Extracellular vesicles attached/adhered to the substrate are flat. The height (z dimension) of the attached vesicle will only be around 10-20% of its x/y dimensions.
- Dilution might be necessary

Further applied characterization methods

Proteomics: Protein mass-spectrometry (Prof. Meyer, Ruhr-Universität Bochum, Germany)
Luminex: Multiplex spherical and planar affinity binder assays, monomolecular ELISAs and Western Blot.

Lipidomics: Mass-spectrometry (ESI-MS/MS, Triple quad GC/MS), High-content imaging, and other LipidomicNet consortium related enabling technologies (www.lipidomicnet.org .)

Transcriptomics (mRNA/miRNA): Microarrays (own institute), deep-sequencing (Prof. Meister, Institute of Biochemistry, University of Regensburg, Germany), Fluorescent dyes for EV-based miRNA/RNA-analysis.

3.3 Methods overview and comparison

Method	*Medium*	*Size*	*Concentration*	*Condition*	*Comment*
FCM	Aqueous	50 nm – 2 µm	1E6 – 1E8/ml	Ambient temperature and pressure	Intact MV analysis Fluorescent material Refractive index important
DLS	Aqueous	~50 nm - 20 µm		Ambient temperature and pressure	Equivalent hydrodynamic radius Ensemble method
SPT NTA	Aqueous	50 nm – 500 (1000) nm	1E6 – 2E9/ml	10 – 37 °C (usually ambient)	Determination of size and concentration directly in the medium Materials with high refractive index are correctly sized
TEM TSEM	Vacuum	2 nm - 1000 nm		100 keV e-beam 30 keV e-beam	Beads might shrink due to heating. Vesicles may change in size due to fixation and staining Diameters below 200 nm prefered Specific evaluation models required
RPS	Aqueous electrolyte buffer	70 nm – 800 nm	1E6 – 1E10/ml (size dependent)	Ambient temperature and pressure	Determination of size and concentration of particles in an electrolyte
SAXS	Aqueous	< 300 nm polydispersity <0.3	> 1 mg / ml	2-8 keV x-ray	Synchrotron radiation facility required Ensemble method
AFM	Air, aqueous and vacuum	4 nm – 2 µm		Particles deposited on mica	3D particle data More accurate for spherical particles

4 Commercial sources of MV reference materials

The following particle types and providers were mentioned by the contributors. This list is given without any recommendations and does not claim to be complete.

Manufacturer	**Product name**	**Sizes used**	**Product identification**	**Website**	**User review** ***(italics: from data sheets)***
Agar UK	PS Latex particles	88 nm	S130-1	www.agarscientific.com	*Tendency to coagulate* *Less ideal for AFM measurements*
Apogee UK	Calibration beadmix Polymer beads Green fluorescent beads	180, 240, 300, 590, 880, 1300 nm (η=1.42) 110, 500 nm (η=1.59) η=refractive index	1493	www.apogeeflow.com/	Use in FCM only. Various refractive indexes result in important differences in the outcome of analyzed particle sizes Concentration inaccurate (NTA)
Avanti Polar Lipids	Sterically stabilized liposomes (SSL)	80 – 90 nm (low polydispersity)	control liposome 300103	http://avantilipids.com/	Ideal reference system for MV form body fluids: similar main building blocks and structural features Fits to the requirements of SAXS (size, polydispersity and conc.) Store at 2°-8° C
Bang Laboratories, Fishers, Indiana, USA	Flow Cytometry Absolute Count Standard™	7-9 µm	580	http://www.bangslabs.com/	Only use in FCM to define the absolute concentration of vesicles in samples.
BioCytex	Megamix fluorescent beads	Mix of 0.1, 0.3, 0.5, 0.9, 3 µm	7801	www.biocytex.fr	*Fluorescent with specific 2:1 ratio between each pair of beads* *For FCM use only* *Low conc. 1000/mm^3*
Corpuscular Inc	Silica microspheres			www.microspheres-nanospheres.com	Size accurate Concentration highly inaccurate
Dako	CytoCount™	5.2 µm	S236630	www.dako.com	Concentration reference (counting) Fluorescent emission 520 nm - 700 nm
Fresenius Kabi	Intralipid 10%			http://www.fresenius-kabi.com/	Sterile fat emulsions containing soya oil, egg lecithin and glycerol
Invitrogen	FluoSpheres®	200 nm	F-8811	www.invitrogen.com	Yellow-Green fluorescent (505/515)
IRMM	Colloidal Silica in water	20 nm 40 nm	ERM- FD100 ERM- FD304	http://irmm.jrc.ec.europa.eu/	IRMM Certified narrow distribution
Izon Science NZ	Carboxylated PS beads (NIST certified)			www.Izon.com	*Own production* *Certified size and concentration* *Sonication before use and storage in a fridge* *Needs 1/1000 dilution in buffer* *Certification reports can be provided* *€80 per 0.5mL bottle*
Kisker D	Silica and PS beads			www.kisker-biotech.com/	Cheaper but lower quality than NIST Size differs 10 % from specifications Refractive index not well defined

					Silica beads > 1 µm may be porous
Microparticles GmbH	PS, PMMA, SiO2, Melamin	100 nm – 12 µm		www.microparticles.de/	High quality beads, also florescence and functionalized, coloured, magnetic and metal coated
NIST SRM	Au Nanoparticles PS spheres	10 nm 30 nm 60 nm 300 nm 100 nm 60 nm	SRM 8011 SRM 8012 SRM 8013 SRM 1691 SRM 1963a SRM 1964	www.nist.gov/srm	Well documented
Pelco	NanoXact und BioPure Au and Ag	5 nm - 100 nm	83420-175	www.tedpella.com	Tannic Acid Capped or Capped with a 10nm Silica Shell or Amine Terminated 10nm Thick Silica Shell
Polysciences	PS microspheres Nano- Microbead Silica microspheres	200 nm 400 nm 1 µm 2 µm 4 µm 0.15 µm 0.3 µm 1 µm 2 µm 4 µm	64013-15 64017-15 64030-15 64050-15 64070-15 24320 24321 24326 24328 24331	www.polysciences.com/	Excellent in term of size High refractive index (not good for light scattering methods) NIST traceable particles Better refractive index (closer to lipid based cellular MV) Size less accurate
RAS	Ag Nanoparticles			http://rasmaterials.com/products/silver-antimicrobial.html	High concentration, nice size distribution, suited for TSEM
SoftFlow Ltd HU	Custom made beads	300, 500, 900 nm	PE labelled	www.softflow.hu www.softflow.com	Bead agglomeration Problems with dim labelling
Ted Pella Inc.	Au calibration kit Au, Ag monodispersed colloids	5 -30 nm 2 nm – 250 nm	16200, 16205 AFM Kits	tedpella(1) tedpella(2)	Unconjugated Gold and Silver Sols No problems for deposition on mica for AFM measurements Trace amounts of citrate, tannic acid and potassium carbonate
Thermo Fischer Scientific (Duke Scientific)	NIST-traceable PS beads	20 nm -60 nm	Nanosphere Size Standards	www.thermoscientific.com www.dukescientific.com	Well-defined (diameter, conc., refractive index) Stand dev. 5 – 7 nm No problems for deposition nor for AFM measurements
UMR-CBMN-CNRS	Functionalized Au nanoparticles Liposomes	4 – 20 nm 40 nm by sonication 50 nm -800 nm by extrusion			Own production (not commercial) Annexin-5, antibodies Used for specific recognition of sub-populations of MVs broad size distribution of extruded liposomes

5 General comments from contributors

5.1 Aim

Translate pre-clinical analysis into accreditable/certifiable clinical methods and to generate evidence-based medicine data for novel surrogate biomarker evaluation that are reimbursed by health insurance companies. Characterizing platelet or red blood cell extracellular vesicles in transfusion medicine to find biomarkers for the early diagnosis of arterial/venous thrombosis risk in vascular and metabolic disease, acute vs. chronic inflammation and neurodegenerative diseases.
Accreditation of the above mentioned cell type specific EV-analysis needs refraction index-specific and stable more narrow PL-EV size discrimination for routine applications and as validation of haemapheresis product preparations for transfusion.

5.2 Vesicles

To be measured preferably in physiological solution or directly in the body fluid in order to avoid artefacts (loss of populations, clumping, changes in composition)

Size range of vesicles is estimated between 30 nm and 1 µm with concentration of 1E10/ml.

5.3 Liposomes

Liposomes are widely used as model membrane systems and have been characterized by numerous analytical methods. Liposomes are difficult to produce in uniform diameters above 200 nm. Extrusion through nucleopore filters produces reasonably uniform distributions in the range of 50-200 nm.

Preparation of liposomes:

- sonication for the small ones (40 nm)
- extrusion through nucleopore filters from 50 nm to 800 nm. Large size distribution for extruded liposomes (larger than 100 nm).

Sterically stabilized liposomes (SSL) would be an ideal reference system for microvesicles form body fluids, since their main building blocks (phospholipids, cholesterol) and their structural features are the same.

Dox-NP™ (doxorubicin HCl liposomes for injection) is doxorubicin hydrochloride (HCl) encapsulated in long-circulating, pegylated, nano-liposomes. Doxorubicin is a cytotoxic anthracycline antibiotic isolated from Streptomyces peucetius var. caesius. (Avanti Polar Lipids)

5.4 Reference particles

Reference particles should be stable in time, do not clump or fragment during experiments, have neither batch-to-batch variations nor toxic contaminants, widely and easily available, cheap.

Reference particles should have the same range of size, density (=concentration) and refringency (=refractive index) as the analysed particles.

There is a need to develop standard reference materials within the size range from 20-30 nm to 1000 nm, with a density approaching that of human cellular exosomes

According to literature, the range of vesicles is usually estimated between 30/50 nm and 1 µm. So, ideally, size range of beads should be between 30/50 nm and 1 µm.

The concentration depends on use. When reference particles are used for setting size thresholds/gates, then concentration irrelevant. However, when used for quantification purposes, then concentration should be known. Because concentration of vesicles often estimated to be larger than 1E10/ml, the concentration of reference particles should be sufficient to allow reliable calculations of such concentrations.

Maybe it will be possible to generate standards of biological vesicles including exosomes, microvesicles and apoptotic bodies which will be stable by lyophilisation?

The refractive index of polystyrene beads is much higher than for biological vesicles (approximately 1.38-1.39). So, perhaps (the much higher refractive index of polystyrene beads?), polystyrene beads of e.g. 200 nm when analysed by flow cytometry can be used to set the 1 µm gate/threshold for light scattering?

Cooperation with the flow cytometer company ApoGee in UK for the development of non-/fluorescent multisize silica/latex beads (Range 50 nm-2 µm). A first product is available on the market since May 1st 2012. Also standard calibration beads (Megamix) covering the size range of interest from Biocytex. Additional products with different refractive indices (RI/η) and fluorescence coatings are under development. Development of a stackertube filtration/centrifugation consumable to simplify and standardize pre-analytical procedures for flow cytometry and NTA of EVs in body fluids and culture media.

5.5 Polymer beads

Polymer beads are extensively used as size reference materials and also as calibrated intensity standards.

The Nanosphere products from Polysciences were found to be excellent in terms of size, although the relatively high refractive index means that they are a poor calibration material for concentration measurements of biological vesicles by light scattering methods.

Polystyrene beads result in underestimation of size of biological vesicles by flow cytometry.

5.6 Silica beads

Silica and polystyrene microspheres from Polysciences (www.polysciences.com) have been used. They provide size certified beads with a narrow range of variation. Worked well in calibrating the flow cytometer, size versus light scatter (Chandler et al., J Thromb Haemost 2011;9:1216). Silica microspheres appear to have a refractive index closer to lipid based cellular microvesicles.

5.7 Calibration

For instrument setting, day to day variation and serial control, reference size particles ranging from 50 nm - 2 µm (ApoGee, UK) are in use for platelets (PL), red blood cells (RBC), granulocytes (PMN, polymorphonuclear leukocytes), monocytes/macrophages (Mo/Mac), adipocytes (AT), hepatocytes (HEP) and endothelial cells (EC) and their released *extracellular vesicles* PL-EVs, RBC-EVs, PMN-EVs, Mo/Mac-EVs, AT-EVs, HEP-EVs, EC-EVs.

5.8 Preparation of samples

Preparative methods include density-gradient ultracentrifugation, cell sorter, chromatographic methods, preparative free flow electrophoresis (e.g. Isotachopheresis).

Particles are not usually stable when diluted.

Centrifuging samples to remove platelets also removes up to 80 % of larger MV, in the 300 to 1000 nm range.

Plasma samples from fasted patients preferred to avoid presence of quantities of lipoproteins

Often larger bead populations may be contaminated with ‘bead dust’, typically small particles not filtered out or sometimes due to bacterial contamination. These generally show up in the 500 nm particle range, based on polystyrene reference.

Polystyrene beads may literally ‘dissolve’ and break-down into smaller pieces due to long-time sonification.

5.9 FCM

With the realization that flow cytometers may be detecting swarms of small microvesicles which appear as a single larger particle in the cytometer detector (Van Der Pol, J Thromb Haemost 2012;10:919), we are evaluating the effect of coincidence on particle detection and apparent false positive double labelling of microvesicles.

5.10 NTA

For NTA, we try to normalize acquisition. We have heard suggestions to use silica beads as reference particles to set up and fix the settings of the machine for acquisition. But in our hands it is difficult to set up the conditions of acquisition on one sample containing silica beads, and then load another sample in the chamber and perform the analysis without changing any conditions, including set up of the camera etc. So if possible, I would suggest trying to generate reference particles which could be spiked in every sample, and which could be quantified in the same chamber as the sample, and thus used to normalize both the acquisition and the analysis conditions. That would require for instance fluorescent silica beads if the experimental samples

are not fluorescent, which could be distinguished from the analysed samples thanks to their fluorescence.

NTA to quantify exosomes: A reference sample should have the same range of size, but also density, as the analysed particles. For NTA, it should also have the same type of refringency, but do we know the refrigency of every single type of vesicles / particles? It probably changes with the composition of both the membrane and the inside of the vesicles.

NTA agrees with AFM on the sizing and concentrations of vesicles in biological fluids e.g. plasma or urine.

5.11 Cryo-TEM

The best starting material would be PFP sample, frozen at -20°C.

6 Appendix A: Text of original questionnaire

Questionnaire on particle reference materials and methods (WP4)

With this questionnaire we would like to collect existing information about:

- The methods intended to be used during the MetVes project by your institution or by the stakeholders you know.
- Typical sample preparation and measurement conditions needed by these methods.
- Specific requirements of the mentioned methods with respect to reference particles.
- Known suppliers of commercial synthetic particle reference materials
- Your experiences and recommendations with respect to reference materials already used.

Just add as much text as you like into the boxes

Name and Institution:
Nanoparticle measurement methods used for this Project (e.g. SPM, SEM, TEM, DLS, DMA, NTA, RPS, SAXS, etc.) Include important measurement conditions like aqueous, air, vacuum, pressure, humidity, electron or x-ray energies, heating (temperature), etc.
Known specific requirements and restrictions of the mentioned methods with respect to reference particles e.g. particle size range, particle concentration, particle material parameters, etc.
Known manufacturers or vendors of synthetic nanoparticles in the size range of 1 nm to 1000 nm made of any material. Include the product identification and web-site, if possible.
Additional knowledge on specific reference particle products you have worked with like quality, homogeneity, size deviation, size distribution, agglomeration, additives, impurities, concentration, price, availability etc. (e.g. previous reports and relevant publications)

General comments: