FINAL PUBLISHABLE REPORT

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RMG: -		

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1 Overview

Extracellular vesicles (EVs) are cell-derived particles present in body fluids, which have excellent potential as biomarkers for the diagnosis of diseases as cancer and thrombosis. This project aims to tap into the clinical potential of EVs by developing traceable measurements of number concentration, size distribution, refractive index (RI) and fluorescence intensity of EVs in human blood and urine. The METVES II project has developed synthetic reference materials with physical properties resembling EVs, ready-to-use biological test samples, and instrumentation and procedures to standardise EV measurements in clinical laboratories, which were evaluated in an inter-laboratory comparison study across standard flow cytometers in clinical laboratories.

2 Need

European healthcare costs are estimated to increase by five to six percent annually for the next decade, and healthcare costs are projected to become unsustainable between 2040 and 2050. A dramatic reduction of treatment costs can be achieved by early diagnosis of disease, because the costs of early-stage treatment are a fraction of late-stage treatment. Moreover, early-stage treatment improves the clinical outcome and the quality of life of patients, and hence a healthier society. However, early diagnosis requires real time diagnostic information from easily accessible samples. Body fluids are so well suited for this purpose that they are often called “liquid biopsies”. Current liquid biopsies are mainly based on the analyses of (macro)molecules, cell-free DNA or cells, however EVs are rapidly gaining interest as a new category of liquid biopsy biomarkers.

The exploitation of EVs as biomarkers requires reliable measurements, however this is currently very difficult as most EVs are smaller than 200 nm. At present, flow cytometry is one of the most appropriate techniques for EV analysis in clinical samples, because flow cytometers are present already in clinical laboratories and can measure EVs at high throughput.

A flow cytometer measures light scattering and fluorescence intensity of single EVs in a flow. However, due to technical variations between different flow cytometer models, measurements of EV concentrations are currently incomparable. Therefore, EV reference materials and methods are urgently needed to calibrate flow rate, light scatter intensity and fluorescence intensity in the sub-micrometre size range. The ideal reference material should contain particles with a traceable number concentration to calibrate flow rate, a traceable size and RI to calibrate scatter intensity, and a traceable fluorescence intensity. Applications of such dedicated reference materials also require testing and validation using biological test samples in clinical laboratories.

3 Objectives

The overall goal of this project is to enable the standardisation of concentration measurements of cell- specific EVs in human body fluids by developing reference materials and related reference measurement methods. The specific objectives are:

1. To develop clinically relevant synthetic reference materials that contain stable spherical particles with (1) concentrations in the range of 10^9 to 10^{12} particles mL^{-1}, (2) discrete diameters between 50 nm and 1 000 nm, (3) a refractive index (RI) in the range of 1.37 1.42 and (4) a visible fluorescence intensity between 100 and 100 000 molecules of equivalent soluble fluorochromes (MESF).
2. To develop traceable measurement methods for the number concentration, size distribution, fluorescence intensity and RI of the reference materials from Objective 1. The uncertainty for each method will be determined.
3. To develop traceable methods to characterise the number concentration, size distribution, RI, and fluorescence intensity of biological test samples containing EVs from human body fluids. The uncertainty for each method will be determined.
4. To evaluate and validate the performance of the clinically relevant synthetic reference materials from Objective 1 via an inter-laboratory comparison with an adequate number of clinical end users. This should include an assessment of the reproducibility of measurements of the concentration of EV from the biological test samples from Objective 3, across a range ($\geq$ 20) of standard flow cytometers in clinical labs.
5. To facilitate the take up of the technology and measurement infrastructure developed in the project by the measurement supply chain (accredited laboratories, instrumentation manufacturers), standards developing organisations and end-users (medical practitioners, clinical and academic laboratories).

4 Results

4.1 Objective 1: To develop clinically relevant synthetic reference materials that contain stable spherical particles with (1) concentrations in the range of 10^9 to 10^{12} particles mL^{-1}, (2) discrete diameters between 50 nm and 1 000 nm, (3) a refractive index (RI) in the range of 1.37 1.42 and (4) a visible fluorescence intensity between 100 and 100 000 molecules of equivalent soluble fluorochromes (MESF).

By definition, reference materials need to be homogeneous and stable with respect to one or more properties and established for a given measurement. Since flow cytometry is based on light scattering and fluorescence from single particles, an established reference material for EVs should resemble the optical properties of EVs. Light scattering depends on the size, RI, shape and structure of the particle, the RI of the medium, and the optical configuration of the flow cytometer. EVs are membrane-bound biological nano- and microparticles released by cells into the extracellular environment. EVs are heterogeneous but share several common characteristics.

Namely, EVs are bounded by a phospholipid bilayer, which surrounds an aqueous core containing soluble proteins and nucleic acids. The thickness of a phospholipid bilayer is typically in the range of 3.5 nm to 5 nm. On the other hand, in the case of biological membranes that also contain membrane proteins, the overall thickness ranges between 5 nm to 10 nm. The RI of the phospholipid bilayer ranges between 1.42 to 1.45. The lumen of EVs can be considered as protein solutions, the RI of which only slightly exceeds the RI of water at physiologically relevant concentrations. The size distribution of EVs is heterogeneous but generally follows a power-law function, with most of the particles being in the sub-200 nm range. Therefore, ideal reference materials for flow cytometry regarding light scattering should be stable, monodisperse, concentric core-shell particles with a high-RI shell and a low-RI core with the nominal values described above.

Detection of EVs by fluorescence in flow cytometry is based on their labelling either with a membrane dye or a fluorochrome-conjugated antibody specific to one of the specific membrane proteins of EVs. Using the estimation of the number of lipid molecules and specific membrane proteins, ideal reference materials for the fluorescence detection of EVs by flow cytometry should be labelled with commonly used fluorochromes in the 100 to 1000 MESF range.

In order to ascertain and meet the needs of potential end users, an online survey was carried out at the beginning of the project on the most desired properties of reference materials to standardise EV measurements. The survey prepared by partners TTK and AMC, was sent to the members of the Stakeholder Committee of the METVES II project and to the members of the EV Flow Cytometry Working Group which is established by flow cytometry experts from the International Society for Extracellular Vesicles (ISEV), International Society for the Advancement of Cytometry (ISAC), and International Society on Thrombosis and Haemostasis (ISTH). The findings of the survey confirmed the targeted ranges for the physical properties of the EV reference materials matched those formulated by the project in Objective 1, namely, the concentration of the candidate reference materials should fall in the 10^9 - 10^{12} particles mL^{-1} range, which corresponds to 10^{12} to 10^{15} particles kg^{-1}, with diameters between 50 nm and 1000 nm, a RI in the range of 1.37 - 1.42, and a visible fluorescence intensity between 100 and 100000 MESF.

It was deemed highly unlikely that one particle type could satisfy all of the necessary reference material criteria, therefore three types of reference materials were considered in METVES II: hollow organosilica beads (HOBs) prepared at TTK; liposomes prepared at BD and TTK; and low-RI particles prepared at BAM and PolyAn. Initial characterisation of the candidate materials was performed using small- and wide-angle X-ray scattering (SAXS and WAXS) at PTB and using flow cytometry performed by AMC and evaluated by Exometry.

Development of hollow organosilica beads (HOBs)

Hollow silica particles (HSPs) with porous shells have recently attracted considerable attention, mainly due to their applicability as drug delivery vehicles. The low RI of porous silica materials and the possibility of synthesising well-defined hollow shell structures with tuneable shell thickness also makes these kinds of particles ideal candidates for reference materials in the flow cytometry analysis of EVs. The general method for creating HSPs is known as the template-assisted method, which can be divided into two categories: (i) soft templating and (ii) hard templating. In the soft templating method, templates such as emulsion droplets, vesicles, and micelles are used as soft organic cores for the growth of porous shells, which are then converted into hollow structures without the need for additional chemical treatment. These soft templates may be

thermodynamically unstable and can be affected by temperature, pH, solvent polarity, and ionic strength, leading to the production of non-uniform structures.

In contrast, the hard templating method involves using solid cores, such as inorganic particles, as templates to create core-shell particles, followed by etching the inner core under alkaline conditions to create uniform hollow structures. Silica nanoparticles produced using the Stöber method are often used as hard templates for HSPs because of their easy synthesis and ability to be precisely sized. Additionally, hydrofluoric acid or alkaline solutions can easily dissolve silica templates. The synthesis of an organosilica shell on the surface of a silica template to form a core-shell particle, followed by the selective removal of the inner core to create a uniform hollow particle, requires careful optimisation of the process parameters. Several methods can be used for selective etching of the core. The most common is using 1,2-bis(triethoxysilyl) ethane (BTEE) as a precursor to form an organosilica shell, which is more resistant to basic hydrolysis than the silica core, which thus enables the selective etching.

In the METVES II project, seed particles from commercial sources (Alpha Nanotech Inc, Kisker GmbH), as well as in-house synthesised solid silica nanoparticles (SNPs) by the Stöber method, were used.

Two different kinds of HSPs were prepared by partner TTK. The so-called surfactant-free approach was used to prepare HOBs with a shell thickness of approximately 10 nm. In contrast, porous hollow organosilica particles (pHOPs) were synthesised by using hexadecyltrimethylammonium bromide (CTAB) as a structure-directing agent for the formation of mesoporous organosilica shell. The details of the synthesis of pHOP and their characterisation with Single Particle Inductively Coupled Plasma Mass Spectrometry (spICP-MS) by LGC were described in *Nanomaterials* 2022, *12*, 1172.

Commercial silica particles, including PSI-0.2, PSI-0.4 and PSI-06 particles from Kisker GmbH (Steinfurt, Germany) and colloidal Silica Nanoparticles with 200 nm, 300 nm, and 400 nm nominal diameter from Alpha Nanotech Inc. (Vancouver, Canada) were used. In-house prepared SNPs were synthesised with the Stöber method, which is based on the controlled hydrolysis and condensation of alkoxysilanes ($Si(OR)_4$, where R = CH_3, C_2H_5, C_3H_7) in water-ethanol solution using ammonia as a base catalyst. Organosilica core-shell particles were prepared using the basic amino acid catalysis route for the hydrolysis and condensation of BTEE to the surface of SNPs. In the last step of the synthesis, the silica core is dissolved in NaOH solution at pH 12.75.

The prepared HOB samples were suitable for traceable size measurement using SAXS at PTB and for concentration measurements using spICP-MS by LGC and LNE. Their diameter ranged from approx. 200 nm to 400 nm, and they had a shell thickness of approx. 10 nm, and therefore fulfil the criteria set in Objective 1 for a reference material for the light scattering calibration of the flow cytometry measurement of EVs. Figure 1 shows representative TEM images of the various seed, HOB and pHOP particles prepared in the project.

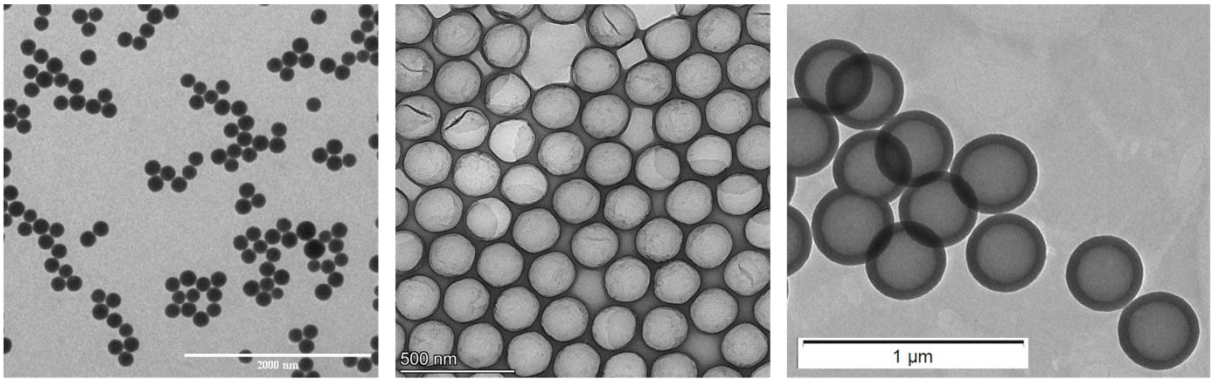


Figure 1. Representative TEM images of solid silica nanoparticles (SNP088, left), hollow organosilica beads (HOB-AN200, middle) and porous hollow organosilica particles (pHOP-280-213).

Two main challenges were encountered during the preparation of HOBs. The first was the variation in the quality of the commercial SNPs used as seeds, which resulted in unreproducible preparations. Developing our own seed particles solved this problem. However, another issue that compromised reproducibility was that scaling up the synthesis caused changes in the quality of the final HOBs in terms of size distribution and homogeneous shell thickness. The latter (homogeneous shell thickness) is because mixing plays a major role in both the formation of the organosilica layer and in the etching of the inner core. Re-optimisation of the

geometry of the reaction vessel partially solved this issue. But in cases where this did not provide a solution, the number of preparations was increased by the project partners.

Including the surfactant CTAB during the synthesis of the organosilica core-shell particles resulted in the formation of a mesoporous layer. The shell thickness of this layer was larger than in the case of the surfactant-free approach, but the etching of the inner core is faster and results in a more homogeneous core removal. The use of CTAB produces in an increased shell thickness, from approx. 10 nm for HOBs prepared by the surfactant-free method to approx. 35 nm for pHOP particles. According to flow cytometry measurements, this also caused the increase of the scattering cross-section of the particles, which made them stronger scatterers than most of the EVs. In this regard, HOBs resemble the light scattering properties of EVs better than pHOPs. However, the porous shell of pHOPs is more beneficial for surface modification and fluorescent labelling of the particles.

Surface functionalisation of HOBs

To attach fluorochromes to the surface of HOBs, the silanol groups on their surface must first be converted into reactive groups (e.g., primary amines), which can then be reacted with the fluorochrome. The following 3 covalent surface modification strategies were used in the METVES II project:

Strategy 1. Amino-surface modification with amino-functionalised organosilanes, such as 3- aminopropyl(diethoxy)methylsilane (APDEMS). The resulting amino-silica particles could directly react with amino-reactive fluorescent dyes, such as fluorescein isothiocyanate (FITC) or NHS-esters of fluorescent molecules.

Strategy 2. Direct biotinylation of the surface of HOBs was carried out using biotin-PEG-silane (Creative PEG Works, USA). Biotin-PEG-Silane is a chemical compound that consists of a biotin group, a PEG (polyethylene glycol) spacer, and a silane group. It is a linear, heterobifunctional reagent that can be used for crosslinking or bioconjugation purposes. The biotin group binds specifically and with high affinity to avidin and streptavidin, while the silane group can react with glass, silica, or other hydroxylated particle surfaces. Biotin-PEG-Silane can be used to functionalise silica particles by attaching the silane group to the surface of the particles.

Strategy 3. Indirect biotinylation of the surface of HOBs using the amino-HOBs prepared according to the procedure described in the first modification strategy (above) and then reaction of the primary amino-groups with biotin-NHS (biotin N-hydroxysuccinimide) as per the second modification strategy (above).

pHOP particles were amino-functionalised and labelled with FITC according to method 1. The effect of the reaction parameters of the surface modification were described in *Materials* **2022**, *15*(7), 2696. The success of the fluorescent labelling of pHOPs with FITC was confirmed by fluorescence spectroscopy and flow cytometry.

The amino-surface modification according to strategy 1 was also used to label HOBs prepared with the surfactant-free approach. Due to the less porous and thinner shell of HOBs, the procedure resulted in a lower concentration of primary amines on the surface; and consequently, a lower number of FITC molecules were attached. Although fluorescent labelling was confirmed by measuring the bulk fluorescent spectrum of the sample, the fluorescence intensity of individual particles measured using flow cytometry was at or below the detection limit according to measurements performed by AMC. Increasing the number of amino groups on the surface of the particles was attempted to try and increase the fluorescent labelling, but due to the charge reversal of the particles upon amino modification, this caused colloidal instability. Therefore, this sample proved inadequate as reference material for the fluorescent detection of EVs using flow cytometry.

Strategies 2 & 3 were also used to fluorescent label HOBs i.e. biotinylation of the surface of the particles and attaching avidin-conjugated fluorochromes to biotin groups. Either performing the biotinylation directly with biotin-PEG-silane (strategy 2) or indirectly (strategy 3) using amino-modified HOBs and biotin-NHS, the success of the labelling with bulk fluorescent analysis was confirmed however, the MESF of the particles was below the detection limit of flow cytometry.

Development of liposomes

Phospholipid bilayers are the basic building block of cell membranes and EVs. Purified or synthetic phospholipids form self-assembled bilayers when dispersed in water due to the amphipathic character of these molecules. The shape of the most common membrane-forming lipids is not perfectly cylindrical; hence, the curvature of the formed bilayers results in the formation of spherical liposomes. Therefore, their use as reference materials for EVs should be straightforward. Liposomes can have higher homogeneity in size and composition (compared to EVs) and are usually composed of a single phospholipid bilayer, whereas EVs can have a more complex structure. Regarding light scattering, liposomes resemble the optical properties of EVs, but due to the contribution of proteins to the RI of EVs, their RI is smaller compared to that of phospholipids.

There are a variety of preparation methods for producing liposomes with different size distributions and homogeneity. But the extrusion method results in the narrowest size distribution amongst these procedures.

Fluorescent labelling of liposomes is also straightforward because synthetic phospholipids conjugated with different fluorophores are already commercially available. Biotin-conjugated phospholipids are also available; therefore, avidin-conjugated fluorescent proteins can also be easily attached to the surface of liposomes.

Reference materials need to be homogeneous and stable with respect to one or more properties. The monodispersity of liposomes depends on the pore-size distribution of the membrane filters used for extrusion and the tendency to form thermodynamically stable liposomes that are smaller than the pore size of the used filter, which depends on the lipid composition. Using common lipid compositions (which include phosphatidylcholine as the main lipid and cholesterol), the practical upper limit of monodisperse liposomes was found to be approx. 100 nm to 200 nm in diameter.

But another challenge in developing liposomal EV reference materials is that the traceable size and concentration determination of liposomes is much more difficult than that of solid inorganic nanoparticles. Due to the polydispersity of the liposomes and the internal structure of the phospholipid bilayer, the size determination by SAXS is subject to high uncertainty. As for the concentration determination by spICP-MS, the phosphorus atom of the lipid molecules could be used, but the low number of phosphorus atoms in single liposomes makes their detection by spICP-MS challenging.

In the METVES II project, liposomal candidate reference materials were prepared by BD and TTK. BD used its proprietary method to prepare liposomes with different compositions and sizes using extrusion with a centrifugation device. In addition, monodisperse liposome fractions were prepared by sorting a heterogeneous liposome sample using flow cytometry. Unfortunately, the concentrations of the as-prepared liposome samples were insufficient for traceable determination of their size, concentration and fluorescence properties.

The classical thin-film hydration and extrusion method was used for the liposomes prepared by TTK. To overcome the challenge of the traceable characterisation of classical liposomes, a new approach was introduced to determine the concentration of liposomes based on the determination of the exact lipid amount within a single liposome.

Liposomes containing phosphatidylcholine and cholesterol as main lipids and a biotin-PEG-lipid conjugate were prepared and fluorescently labelled with avidin-conjugated Allophycocyanin (APC) and Phycoerythrin (PE) at three different fluorophore concentrations. Testing of these liposomes with flow cytometry was performed at AMC. Due to the use of a membrane filter with a pore size of 200 nm during the extrusion, the final diameter of the liposomes was in the range 150 nm to 200 nm measured by Dynamic Light Scattering (DLS). According to the project's flow cytometry results, the MESF values were in the range 29 to 568 for FITC-labelled liposomes, below the detection limit for PE-labelled liposomes, and in the range 65 to 123 for APC-labelled liposomes. Traceable determination of the size, concentration and fluorescence properties of these liposomes was not feasible due to (i) the broad size distribution, (ii) the low concentration of MS-detectable element, and (iii) the low concentration of the fluorophore and small volume.

The project used a novel method to enable the traceable size determination of liposomal EV reference materials. Liposomes containing phospholipids with saturated hydrocarbon chains were prepared using 1,2 dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycerol-3-phosphoglycerol (DPPG) fluorescently labelled with 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-(carboxyfluorescein) (PE-CF). DPPC/DPPG liposomes were in the so-called gel phase at 25 °C, which implied that the lipid molecules were in an ordered sub-lattice within the phospholipid bilayer. The repeat distance of this sublattice can then be determined with WAXS, and form this the area-per-lipid within the liposomes can be calculated. SAXS can also be used to provide the overall size of the liposomes. Using the size and area-per-lipid data, the number of lipid molecules in a single liposome can be calculated, which can be used then to determine the

number concentration of the liposomes by using the molar concentration of the phospholipids. Using the fluorescent PE-CF lipid in a trace amount (maximum 1 mol % of the total phospholipids), sufficient fluorescent labelling was able to be achieved to enable traceable quantum yield measurements.

However, the drawback of using this kind of liposomes is that their phospholipid bilayer is rigid; therefore, a monodisperse sample can only be prepared by using a 100 nm pore-size membrane during the extrusion. Consequently, the average size of the liposomes is around 80 nm in diameter, which is below the detection limit of most of the currently used (can commercially available) flow cytometers. It should be noted that the majority of EVs are in the sub-100 nm size range, and newer developments in flow cytometers are working towards reaching this size range. Overall, 5 different samples were prepared with different PE-CF concentrations ranging from 0 % to 1 mol % of PE-CF by TTK. SAXS and WAXS characterisation was carried out at PTB, while flow cytometry based on fluorescence detection was measured at AMC and evaluated by Exometry.

The requirement for traceable determination of the fluorescent quantum yield is that the absorption at the excitation wavelength (at the vibronic shoulder of the absorption band or at maximum in the longest wavelength absorption band) is around 10 % with a minimum of at least 2 %. This criterion was successfully achieved by the DPPC/DPPG/PE-CF liposomes, which enabled traceable fluorescent quantum yield measurements by partner BAM.

Development of low-RI solid particles

The third group of candidate reference materials developed in the METVES II project were low-RI solid particles. The core concept was to reach an effective RI of the particles below 1.42 by using a mesoporous silica shell with tuneable shell thickness and a polymer core with a sufficiently small size. The consortium expected that these low-RI solid particles would have four advantages (i) core particles with high monodispersity are commercially available, (ii) the thickness of the mesoporous silica shell is tuneable, (iii) fluorescent staining of the polymer core is established, and (iv) the mesoporous shell protects the fluorescently labelled polymer core.

The first experiments used commercially available polystyrene (PS) particles, followed by CTAB- or DTAB-templated silica shell growth. TEM showed that although the core particles were monodisperse, non-uniform shell growth and strong homo-nucleation (formation of small silica particle byproducts) were observed, probably due to poor surface wetting of the silica precursor. Moreover, instability (melting) of the polymer core was observed in the presence of the surfactant used to prepare the mesoporous silica layer. Next, poly(methyl methacrylate) (PMMA) particles from PolyAn were used instead of PS. Despite achieving partial success using 500 nm PMMA particles, the homo-nucleation of the silica precursor and the instability of the core particles made it impossible to prepare homogeneous low-RI particles with this method.

Since the RI of PMMA is lower (RI = 1.50) than that of PS (RI = 1.61), PMMA particles of approx. 100 nm, 200 nm, and 400 nm diameters labelled with either FITC, R-PE, or APC at different dye concentrations were prepared. This was done in order to obtain PMMA particles of different fluorescence intensities as candidate reference materials for fluorescence calibration in flow cytometry. However, it was found, based on preliminary flow cytometry results, that the PMMA particles are unpredictably sticky, i.e. they have a tendency to stick to the tubing of the flow cytometer, and their fluorescence intensity is at the detection limit of the flow cytometer used, rendering these particles unsuitable as EV reference materials.

Conclusions

Table 1. summarises the 3 types of candidate EV reference materials prepared in the METVES II project.

HOBs resemble the light-scattering properties (i.e. RI distribution) of EVs and can be reproducibly prepared in small volumes. Upscaling the preparation procedures was found to be problematic due to the complex colloid chemical processes taking place during the synthesis. The fluorescent labelling of HOBs with classical methods, such as amination and attachment of amino-reactive fluorophores to the surface, is feasible, as confirmed by bulk fluorescence measurements. However, the production of HOBs with a number of fluorophores reaching the lower detection limit of flow cytometry measurements has been hampered. Therefore, HOBs were only traceably characterised for size and concentration and can be used as candidate EV reference materials for these properties.

The preparation of liposomes using established protocols was straightforward, and their fluorescent labelling was easily carried out using either fluorophore-conjugated lipids or functionalised lipids (e.g. biotin-lipid conjugates) to bind protein-based fluorophores. But the use of liposomes as EV reference materials suffers from two challenges. Firstly, the traceable size and concentration determination of these organic soft-colloidal particles is difficult. Secondly, the width of the size distribution of liposomes prepared by the extrusion method increases with increasing pore sizes. Therefore, liposomes were only traceably characterised for fluorescence properties and can be used as candidate EV reference material for this property.

The polymer core-based low-RI particles were found to be unsuitable as candidate EV reference materials for the scattering and fluorescence detection of EVs using flow cytometry. The difficulties encountered with the preparation of these low-RI particles included non-homogeneous layer formation and the melting of the polymer core during the synthesis.

Table 1. The candidate EV reference materials used for traceable measurements in METVES II

	Sample name	Type	Reference property
1	SNP073	solid silica particle	concentration
2	SNP082	solid silica particle	concentration
3	SNP083	solid silica particle	concentration
4	SNP088	solid silica particle	concentration
5	02-HOB-AN200	hollow organosilica bead	size and concentration
6	04-HOB-SNP082	hollow organosilica bead	size and concentration
7	09-HOB-K400-05	hollow organosilica bead	size and concentration
8	11-HOB-AN400	hollow organosilica bead	size and concentration
9	Liposome APC A2	liposome	fluorescence
10	Liposome unstained	liposome	fluorescence
11	DPPC/DPPG 0 PE-CF	liposome	fluorescence
12	DPPC/DPPG 0.25 PE-CF	liposome	fluorescence
13	DPPC/DPPG 0.5 PE-CF	liposome	fluorescence
14	DPPC/DPPG 0.75 PE-CF	liposome	fluorescence
15	DPPC/DPPG 1 PE-CF	liposome	fluorescence
16	PolyAn2000	PMMA particle	RI
17	PolyAn1300	PMMA particle	RI
18	PMMA-F-0.8 Lot PMMA-F-L1057	PMMA particle	RI
19	PMMA-F-0.6 Lot PMMA-F-KM28	PMMA particle	RI

4.2 *Objective 2: To develop traceable measurement methods for the number concentration, size distribution, fluorescence intensity RI of the reference materials from Objective 1. The uncertainty for each method will be determined.*

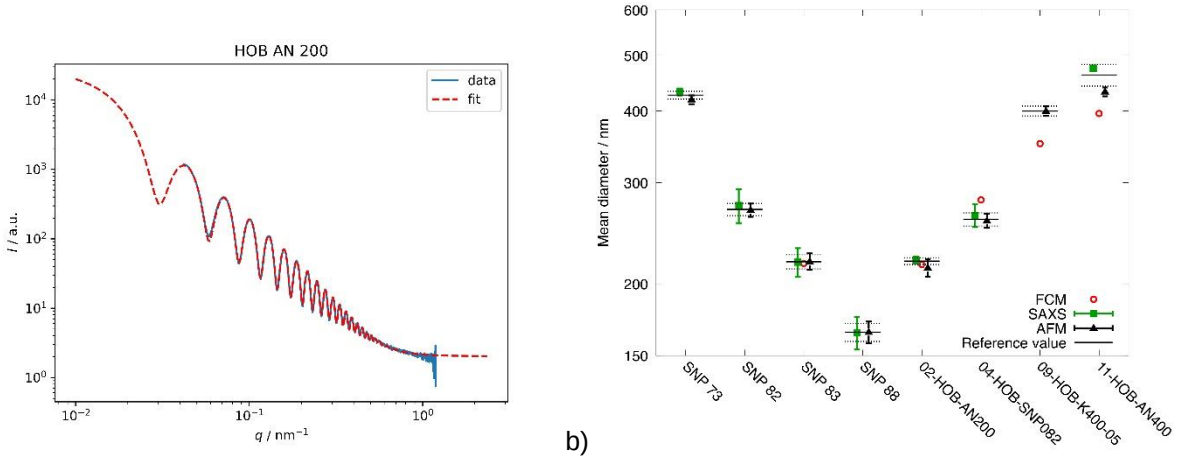
Many metrological methods have been developed in recent years for the characterisation of nanoparticles and other particles with a diameter below 1 µm. In particular, many well established methods including best practices codified in ISO standards are available for the measurement of the particle size. Methods also exist for number concentration, fluorescence intensity and RI, but are generally less developed and less widely available. A common problem is that accurate metrological methods have difficulties coping with real world EV samples which can be polydisperse, heterogeneous, impure and of low concentration. Whilst other (non-metrological) measurement methods can analyse such real-world and more difficult samples, this generally results in lower accuracy and precision. In Objective 2, different measurement techniques were applied to the most promising candidate reference materials developed in Objective 1, and the best results were used to guide the selection of the reference materials and combined into the final reference values.

Size distribution

The size distribution of the reference particles was established using atomic force microscopy (AFM) and SAXS. AFM was performed by VTT on a Jupiter XR device AFM in tapping mode and using standard silicon tips for the measurements. The z-scale of the AFM was calibrated using step height standards calibrated by VTT's metrological AFM (MAFM) which is directly traceable to the metre via online interferometric position measurement. The particles were deposited on a MICA surface treated with poly-L-lysine and the particle height was measured.

SAXS measurements were carried out by PTB at the four-crystal monochromator-beamline, which is owned and operated by PTB and located at the BESSY II synchrotron radiation facility in Berlin-Adlershof. Each colloidal solution was filled into separate round capillaries made of borosilicate glass and sealed with a welding torch before measurement. The filled capillaries were then loaded into the vacuum chamber of the SAXS facility and irradiated with a pencil beam of synchrotron radiation with an energy of 8 keV and a cross-sectional area of 150 µm × 400 µm at the position of the capillaries. The radiation scattered by the samples was then recorded by a vacuum-compatible Pilatus 1 M detector, at a distance between 3 m to 5 m behind the sample. For the background correction, the pure suspension liquid (usually water) was measured in addition to the samples.

Size distribution measurements are well established for both AFM and SAXS methods and were carried out according to best practices for both HOBs and SNPs (Objective 1) used as templates in the production of the HOBs. While mostly standard methodologies could be applied, a new numerical model for the SAXS data analysis of the HOBs was devised comprising independent size distributions for the core size and the shell thickness. Figure 2a) demonstrates the exceptionally good fit of this model to the measured data.



a) b)

Figure 2. Representative scattering curve for HOBs (a, blue line) together with a fit of the new SAXS model (red dashed line) and comparison of the size measurements on all particles using different techniques (b) together with the reference values.

Figure 2b) also shows a comparison of the SAXS and AFM size measurements with those determined by flow cytometry, which was performed at AMC on a calibrated flow cytometer (Apogee Flow Systems, Hemel Hempstead, UK) and evaluated by Exometry. Flow cytometry is not currently a metrological method and relies on assumptions on the RI of the particles, the suspending liquid and the detection geometry of the device. Therefore, no uncertainties could be determined. However, the results are in general agreement with the metrologically determined reference values, although they fall outside the confidence band of the reference values for the larger particles.

Number concentration

The number concentration of the HOBs and the SNPs (from Objective 1) was determined traceably by spICP-MS carried out at LGC and LNE, and by SAXS performed at PTB in combination with freeze-drying at TTK. Additionally, particle tracking analysis (PTA) was performed by LGC.

Previously, number concentration has been traceably determined for solid spherical gold nanoparticles by spICP-MS and SAXS. However, a metrologically sound measurement for silica particles poses much greater challenges for both methods. In the case of spICP-MS, silicon is much more difficult to measure at low levels than (e.g. gold) due to the higher contribution of procedural blanks and the poor ionisation efficiency of the element. The project mitigated this challenge at LNE by replacing (silicon containing) glass parts of the sample introduction system with sapphire and at LGC by using instrumentation equipped with fast, microsecond detection. LNE and LGC also approached the transport efficiency determination (a prerequisite in accurate quantification of particle number concentration) in different ways. LGC used the DMF method, which relies on weighing the mass of introduced sample and the mass of the sample being nebulised. Whereas, LNE followed the size method, which is a calibration strategy using gold particles of known diameters.

For SAXS, the key point is the determination of the effective electron density of the particles, which is required to convert the scattering intensity to number concentration. While the density is a known constant for gold particles, it can vary for silica particles depending on their synthesis. To overcome this issue, the SAXS data was complemented by measurements of the dry mass of the samples using freeze drying performed at TTK. The SAXS intensity and the dry mass content could then be jointly evaluated in order to determine both the density and the number concentration. As this approach requires a relatively large amount of material (~3 mL concentrated suspension), which is lost after the measurement, only the SNPs were analysed.

Further to this, PTA measurements were performed as a confirmatory method, to support data obtained with the other methods. PTA, is a popular laboratory method, but is not directly traceable to the SI.

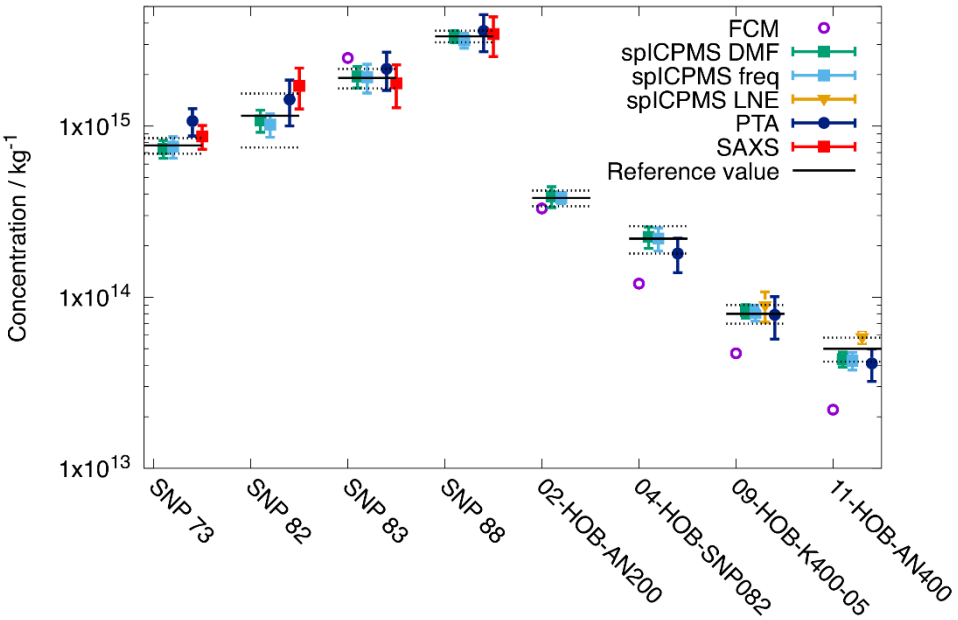


Figure 3. Comparison of the number concentration measurements using different techniques and determined reference values

The final reference values were obtained by combining the spICP-MS and the SAXS measurements. Again, the values measured by flow cytometry, shown without uncertainties in Figure 3, generally agree with the metrologically determined values, but fall outside the confidence bounds.

Fluorescence intensity

From the EV reference materials developed in Objective 1, only the DPPC/DPPG PE-CF liposomes were fluorescent. The fluorescence was measured at BAM using an integrating sphere setup (Quantaurus, Hamamatsu Photonics) using standard protocols for the spectral correction of the emission spectra and the reabsorption correction. From the measured emission and absorption spectra (Figure 4a), the quantum yield and the brightness (Figure 4b) were determined. The brightness equaled the product of the absorbed light flux and the quantum yield assuming equal liposome concentrations. The concentrations were adjusted by dilution until equal scattering contributions were reached. Due to the strong scattering background the quantum yield determination was only possible by using an integrating sphere. Unfortunately, relative quantum yield determinations failed in scattering media.

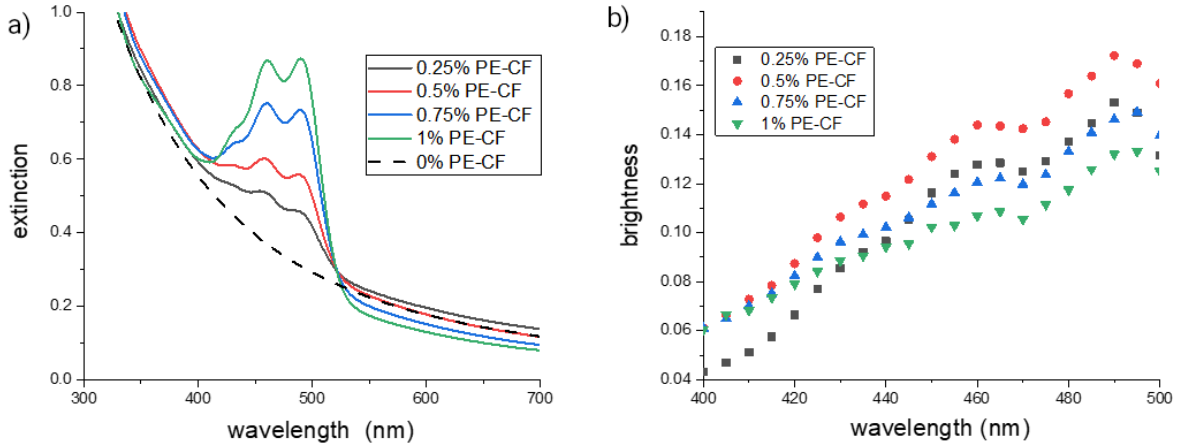


Figure 4: Extinction spectra (a) and brightness (b) of the fluorescent reference particles

Refractive index (RI)

The RI is one of the key properties for single particle detection using flow cytometry. Since the signal depends on the ratio of the RI between the particle and the suspension medium, both must be determined. The RI of relevant liquids (PBS buffer and diluted pooled plasma) was determined using a minimum deviation angle setup at VSL with an expanded (k=2) uncertainty better than 2×10^{-6}, which is a new record for the determination of the RI of fluids. This was achieved by a custom-built setup of the minimum deviation angle method on top of a precision goniometer.

The RI of solid PMMA particles (Objective 1) in suspension was determined at PTB using spectrally resolved collimated transmission measurements with a custom-built setup. In this setup, the collimated transmittance of a sample suspension in a cuvette was measured using low-divergence white-light illumination and a detection with a very low aperture. This allowed the collection of light only in the forward direction and suppressed light scattered at small angles into the spectrometer's aperture. Thus, it was possible to remove unwanted contributions to the directed transmittance when analysing the measurements, which is not possible in standard spectrophotometers.

The extinction spectra of microparticles typically exhibit oscillating behaviour – so-called Mie resonances. Their presence allows the simultaneous determination of particle RI, size distribution and concentration from the spectra without other independent measurements. For smaller particles, these resonances shift to shorter wavelengths. But if they move out of the accessible spectral range the spectra become less informative about the particles. Furthermore, the extinction and scattering signal of a given amount of suspended particle material per volume decreases with particle size. Hence, particles below a certain size require higher concentrations for acceptable signal-to-noise ratio, which is ultimately limited by the occurrence of multiple scattering. Both

these effects limit the particle size range that can be measured with spectrally resolved collimated transmission measurements.

Based on the analysis of the corresponding ensemble with an averaged extinction cross section with Lorenz-Mie Theory, the RI and the size distribution of PMMA particles in a size range from 2 µm down to 600 nm was determined. As shown in Figure 5, the RI is close to the expected values from measurements on bulk material.

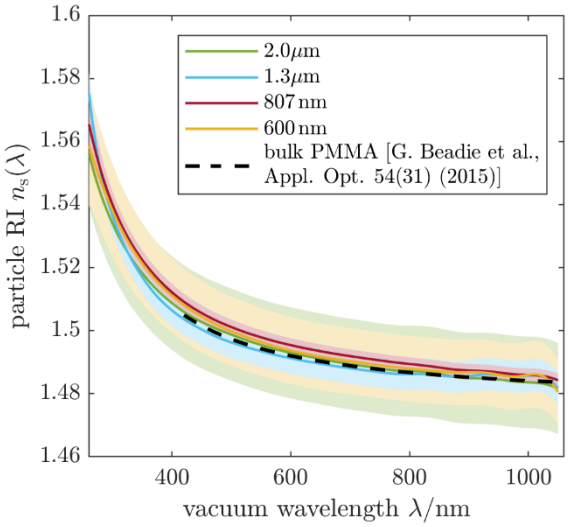


Figure 5: RI of the PMMA reference particles in comparison to the RI of bulk PMMA

Conclusions

The reference values determined for the EV reference materials developed under Objective 1 are shown in Tables 2 and 3.

For each of the relevant properties, the reference values were determined with sufficient accuracy. As explained in Objective 1, different reference materials had to be selected for these different properties.

Table 2. Reference values determined for the size and concentration

Sample	Mean diameter / nm	Size distribution width / nm	Concentration / 1e15 kg-1
11-HOB-AN400	462 ± 20	28	0.050 ± 0.008
09-HOB-K400-05	400 ± 8	45	0.08 ± 0.01
04-HOB-SNP082	259 ± 7	10	0.22 ± 0.04
02-HOB-AN200	216 ± 6	4	0.38 ± 0.04
SNP_073	426 ± 5	12	0.77 ± 0.08
SNP_082	269 ± 7	7	1.15 ± 0.40
SNP_083	219 ± 7	6	1.91 ± 0.25
SNP_088	165 ± 6	11	3.35 ± 0.26

For the size characterisation of the SNPs and HOBs, multiple methods were shown to agree, which provides reliability of the results.

The concentration could also be determined by two independent methods for the SNPs, whereas the results for the HOBs are derived from a single primary method (spICP-MS). The reference values for both are in

agreement with the (indirectly) traceable method PTA, which for the first time allows the establishment of the number concentration of silica-based particle suspensions.

For the RI and the fluorescence intensity of particles, only a single method was available. However, the results obtained agree with expected values and this can be accepted as reference values. In addition, the RI of commonly used liquids could be determined with an uncertainty below 2×10^{-6} by an improved setup, which was used to complement the data.

Hence, reference values were established for the number concentration, size distribution, fluorescence intensity RI of the reference materials from Objective 1.

Table 3. Reference values determined for the fluorescence intensity (Brightness) and RI

Sample	Nominal mean diameter / nm	RI @ 488 nm	Brightness@ 485 nm
DPPC/DPPG 0.25 PE-CF	< 100	—	0.145± 0.025
DPPC/DPPG 0.5 PE-CF	< 100	—	0.164 ± 0.028
DPPC/DPPG 0.75 PE-CF	< 100	—	0.14 ± 0.024
DPPC/DPPG 1 PE-CF	< 100	—	0.125 ± 0.021
PolyAn2000	2000	1.500 ± 0.015	—
PolyAn1300	1300	1.4970 ± 0.0049	—
PMMA-F-0.8	807	1.5020 ± 0.0024	—
PMMA-F-0.6	600	1.501 ± 0.012	—

4.3 Objective 3: To develop traceable methods to characterise the number concentration, size distribution, RI, and fluorescence intensity of biological test samples containing EVs from human body fluids. The uncertainty for each method will be determined.

Concentration measurements of EVs in body fluids are potential disease biomarkers. Most laboratories use flow cytometers to measure EV concentrations, but EV concentrations are currently incomparable between flow cytometers and institutes. To improve comparability the METVES II project aimed to develop (1) reference materials (Objective 1) and methods (Objective 2) to standardise/calibrate all aspects of a flow cytometry (flow rate, light scattering and fluorescence). An inter- laboratory comparison study (Objective 4) was organised in which stable, well-characterised and ready- to-use biological test samples containing pre-labelled EVs (Objective 3) were used to validate the developed reference materials from Objective 1.

In the previous inter-laboratory comparison studies, organised by the preceding EMRP project HLT02 METVES, frozen human plasma samples were distributed between the participating laboratories. Before measuring the EVs present in the provided plasma samples, the plasma was thawed, and EVs were labelled with fluorochromes-labelled antibodies, and diluted before measuring the concentration of labelled EVs by flow cytometry. This sample distribution and handling likely contributed to the observed variation in the measured EV concentration. For example, several participants reported visible clumps in the plasma after thawing. Such clumps are likely to be due to small blood clots, which contain fibrin and fibrinogen, and which are known as scavengers of EVs from platelets and possibly other cells.

To try and avoid this issue, in Objective 3 a procedure was developed to produce stable biological test samples containing EVs from human plasma and/or urine. The biological test samples should be (i) stable, (ii) compatible with flow cytometry, (iii) ready-to-use to avoid interlaboratory variation due to sample preparation, and (iv) traceably characterised. In addition, the EVs present in the test sample should be characterised for number concentration, size distribution, RI, and fluorescence intensity.

Development of biological test samples

AMC and UH developed a procedure to prepare biological test samples from human body fluids. AMC developed a biological test sample based on human blood plasma, which is (i) stable, (ii) compatible with flow cytometry, (iii) ready-to-use to avoid interlaboratory variation due to sample preparation. The biological test sample was developed based on the requirements of the EV community and stakeholders needs. The project's developed plasma-derived EV test sample was called PEVTES.

In parallel, UH developed a biological test sample based on human urine. The urine derived EV test sample showed a large variation in the EV concentration between the prepared aliquots of the same batch. Therefore, UH together with AMC decided that the developed urine test sample was unsuitable as a biological test sample in the upcoming inter-laboratory comparison study (Objective 4).

The concentration of pre-labelled and stabilised EVs present in the PEVTES proved stable for at least one year and was therefore used as EV-containing biological test sample in the scheduled inter-laboratory comparison study (Objective 4). AMC produced a new batch of PEVTES for the inter-laboratory comparison study, which was also distributed for traceable characterisation to BAM, Exometry, LGC, LNE, MTA TTK, UH, VSL, and VTT.

PEVTES was developed with the goal to resemble real EVs present in human plasma. EVs are known for their heterogeneity, and therefore this heterogeneity needed to be maintained to make PEVTES a clinically relevant test sample. For example, EVs have a broad size distribution which is largely maintained in PEVTES by isolating EVs using Sepharose 2B size-exclusion chromatography (SEC), which has a cut-off of about 70 nm. Thus, the size range of SEC-isolated EVs are expected to have a diameter ranging from approximately 70 nm to approx. 1 µm.

Furthermore, EVs also differ in their biochemical composition, which is mainly due to the fact that EVs originate from different cell types. Since plasma EVs originate mostly from blood cells, and blood contains multiple types of cells, only the EVs that expose the marker of interest should be pre- labelled. Consequently, PEVTES will also contain unlabelled EVs.

Finally, human plasma also contains non-EV particles such as lipoproteins, which outnumber EVs. Since the size distributions of EVs and lipoproteins overlap, PEVTES also contain unlabelled non-EV particles that

co- elute with EVs on SEC. Thus, although PEVTES contains partially purified and labelled EVs, PEVTES is still a highly complex sample containing a mixture of labelled and non-labelled EVs and non-EV particles with a broad size range. Thus, the composition of a plasma-derived PEVTES sample is much more complex than that of e.g., monodisperse reference materials, and this heterogeneity complicates or even impairs traceable characterisation of physical properties of the (pre-labelled) EVs in multiple manners, as indicated in the next sections.

Size distribution

The size distribution of particles including EVs in PEVTES was determined by PTA by LGC. The determined diameter size range was 40-500 nm, with a modal peak at 46.0 ± 2.4 (mean ± standard deviation, n=15).

LNE also used AF4-UV-MALS to determine the size distribution of particles in PETVES including EVs. Before the PEVTES measurements could be performed, LNE optimised the workflow using milk-derived EV-containing samples. During the measurement of PEVTES, LNE identified two distinct peaks, one of which was associated to bovine serum albumin that is present in PEVTES to improve the stability of EVs. The particle gyration radius of the second peak was in the range 58 – 247 nm, with a weight-averaged gyration radius of 135 ± 75 nm. Data interpretation should take into account that the measured size may represent not only EVs but also non-EV particles, such as lipoproteins.

Number concentration

The number concentration of EVs in PEVTES was determined with PTA by LGC. The total particle concentration >40 nm is 1.23 x 10^{10} particles/mL ± 3.34 x 10^{9} (mean ± standard deviation, n=15). However, data should be interpreted with caution, as PTA measures the concentration and size of all particles present in suspension, i.e. EVs as well as non-EV particles such as lipoproteins, milk fat globules and casein particles.

AMC used a calibrated flow cytometry (flow rate, light scattering, and fluorescence, A60-Micro, Apogee Flow Systems) to measure the number concentration of labelled EVs in PEVTES. The measurements performed using a commercial flow cytometer do not have a measurement uncertainty, because essential information such as sample stream width, sheath flow, and the exact optical alignment of the system, are not traceably measured and therefore negatively impact the uncertainty budget. Hence AMC determined the coefficient of variation (CV) of the concentration of labelled EVs that are present in PEVTES, which is part of the measurement uncertainty by dual angle scatter flow cytometry. Concentrations of labelled EVs (i.e., the EVs that also measured in the interlaboratory comparison study Objective 4) were determined within an EV size range of 180–1000 nm in diameter and a fluorescence intensity > 35 APC molecules of equivalent soluble fluorochrome (MESF), and > 133 PE MESF. The mean erythrocyte-derived EV concentration is 2.33 $E^{+05/}$mL, and the mean platelet-derived EV concentration 3.09 E^{+06}/mL. For the erythrocyte-derived EV concentration, the CV within one aliquot (12 replicates) was 4 – 6 %, and the CV% was 9 % when comparing all PEVTES measurements. For the platelet EV concentration, the CV within one aliquot (12 replicates) was between 4– 8 %. The CV % was 11 % when comparing all PEVTES measurements.

The CV % of the concentrations of (labelled) erythrocyte as well as platelet EVs shows that PEVTES has a repeatability score supporting its use as a relevant EV biological test sample and its use in the interlaboratory comparison study (Objective 4). The mean *total* particle concentration (n=21) in PEVTES measured by calibrated flow cytometry is 2.19 E^{+08} particles/mL, which clearly demonstrates that the labelled EVs originating from erythrocytes or platelets constitute only a small fraction of all particles present in PEVTES.

Fluorescence intensity

The fluorescence intensity could not be traceably determined because the instruments were either (i) not sensitive enough (flow cytometry at BAM, and High Performance Liquid Chromatography-SEC by TTK), (ii) in development (the project's metrological flow cytometry by VSL), or (iii) the required volume for each measurement could not be provided from one batch of PEVTES (calibrated fluorescence spectrometers and integrating sphere spectroscopy by BAM).

Refractive index (RI)

The RI of the diluted PEVTES was measured using goniometry at VSL. Table 4 shows the RI of different dilutions of the PEVTES sample.

Table 4. RI of PEVTES dilutions measured with goniometry at VSL

	405 nm	436 nm	480 nm	509 nm	546 nm	580 nm	644 nm
10x diluted PEVTES	1.347142	1.344855	1.342171	1.342171	1.339205	1.338170	1.335851
30x diluted PEVTES	1.345552	1.343165	1.340557	1.339172	1.337679	1.336594	1.334447
100x diluted PEVTES	1.344603	1.342221	1.339666	1.338264	1.336663	1.335598	1.333591
300x diluted PEVTES	1.344475	1.342059	1.339519	1.338138	1.336486	1.335493	1.333461

The goniometer measured the RI of the PEVTES dilutions with an expanded uncertainty of $1.4E^{-6}$. Furthermore, (as mentioned above) VSL is currently building a metrological flow cytometer. This instrument is almost operational, and will be capable of simultaneously measuring the RI, size and fluorescence of single particles in suspension. Once the metrological flow cytometer is fully operational it can be used to traceably characterise reference materials and biological particles such as EVs. Moreover, this metrological flow cytometer will be directly capable of measuring the physical properties of labelled EVs in suspension, which circumvents the problems encountered by the presence of unlabelled EVs and unlabelled non-EV particles.

Conclusions

To fulfil the aim of Objective 3, a stable biological test samples containing EVs from human body fluids was developed. Prior to the start of this project such a test sample resembling ex vivo EVs was non-existent, and thus this was exploratory and high-risk research. AMC and UH both independently developed biological test samples from different human body fluids and tested and compared the sample stability regarding the concentration of pre-labelled EVs for one year. AMC developed a biological test sample from human blood plasma, whereas UH developed such a sample from human urine.

The urine-derived pre-labelled EV-containing test sample faced reproducibility issues between aliquots of the same batch. Therefore, UH together with AMC decided that only the developed plasma-derived EV test sample (PETVES) should be used as a biological test sample in the organised inter-laboratory comparison study (Objective 4).

Regarding the development of traceable methods (including uncertainty evaluation) to characterise the developed PEVTES the project faced challenges regarding uncertainty evaluation, instrument sensitivity, and required volumes. Moreover, the complexity of the PEVTES sample itself makes it less suitable for high-end methods that are used for traceably particle characterisation. Furthermore, the presence of BSA in PEVTES, added for stability, hampered characterisation by TEM, PTA, and AF4-UV-MALS.

For some of the proposed techniques, e.g. commercial flow cytometry, an uncertainty budget cannot be determined because essential information that is required to determine the uncertainty, such as sample stream width, sheath flow, and the exact optical alignment of the system, have not been traceably measured. However, the CV of the concentration of labelled EVs that are present in PEVTES, was successfully measured instead.

To mitigate the above challenges, VSL is currently building a metrological flow cytometer. This instrument is almost operational and will be capable of simultaneously measuring the RI, size and fluorescence of single particles in suspension. Once the metrological flow cytometer is fully operation it can then be used to traceably characterise reference materials, and biological particles such as EVs.

4.4 Objective 4: To evaluate and validate the performance of the clinically relevant synthetic reference materials from objective 1 via an inter-laboratory comparison with an adequate number of clinical end users. This should include an assessment of the reproducibility of measurements of the concentration of EV from the biological test samples from Objective 3, across a range (≥ 20) of standard flow cytometers in clinical labs

In this objective, the overall goal was to perform an interlaboratory comparison study to test whether the developed infrastructure in 18HLT01 METVES II, i.e., the clinically relevant synthetic reference materials (Objective 1) and the biological test samples (Objective 3), can be used to calibrate flow cytometers, and, thus, enable the generation of comparable concentration measurements on EVs.

Flow cytometers collect light scattering and fluorescence data of single particles in suspension at high throughput. Prior to the start of this project there was no single flow cytometer that is capable of detecting all EVs that are present in a clinical sample. This is because most EVs have a diameter below 200 nm, and hence have weak light scattering and fluorescence signals. Moreover, flow cytometers differ in sensitivity due to the optical configuration, and hence the measured concentrations of EVs differ between instruments. Finally, flow cytometry data on both light scattering and fluorescence are reported in arbitrary units, thereby hampering direct comparison of flow cytometry measurement results.

Previously, interlaboratory comparison studies were performed to compare and standardise concentration measurements of EVs using flow cytometry. The last study was performed in 2018, as part of the preceding EMPIR project HLT02 METVES. These studies were important because they showed that there are multiple hurdles in EV flow cytometry. In a flow cytometry there are three aspects that can be calibrated: (i) the flow rate, (ii) light scattering, and (iii) fluorescence.

In the previous HLT02 METVES organised interlaboratory comparison study, for the very first time the flow rate was calibrated for flow cytometry. Although neither light scattering nor fluorescence were calibrated. Another major shortcoming in the previous interlaboratory comparison study was the EV-containing biological test sample; human plasma, which was frozen, distributed, and then EVs were labeled and measured in the thawed and diluted plasma sample. Each participating laboratory had to label plasma EVs using an elaborate protocol, and due to plasma impurities (possibly caused by formation of small fibrin clots during thawing) differences in concentration measurements could have been caused by the test sample itself.

Calibration of flow cytometers, i.e., calibration of flow rate, light scattering, and fluorescence, requires reference materials within the size range and with optical properties resembling EVs (e.g., similar RI). Prior to the start of this project such reference materials did not exist. Therefore, the METVES II project develop EV dedicated and clinically relevant reference materials (Objective 1), and in parallel, pre-labelled and ready to use biological test samples PETVES (Objective 3).

The METVES II project then used its EV reference materials and PETVES as part of a global interlaboratory comparison study in which, for the first time ever, *all* relevant aspects of flow cytometry (flow rate, light scattering, and fluorescence) are calibrated. By calibrating light scattering and fluorescence, arbitrary units of light scattering and fluorescence can be converted into the diameter of EVs and number of molecules of equivalent soluble fluorophore (MESF), respectively.

In June 2021, a total of 49 laboratories were invited by AMC to participate in the interlaboratory comparison study. Laboratories were invited from those participating in

1. the EV flow Cytometry working group (www.evflowcytometry.org)
2. the previous ISTH (International Society on Thrombosis and Haemostasis) collaborative workshop
3. METVES II stakeholders, and/or
4. the Reference material task force of the International Society for Extracellular Vesicles (ISEV; www.isev.org).

Participants had to provide a proven track record of EV detection by flow cytometry, and to have at least one flow cytometer capable of (1) differentiating 100 nm polystyrene beads from background noise by light scattering, (2) detecting APC, FITC, and PE fluorescence, (3) measuring EVs directly in diluted blood plasma at high throughput (> 1,000 events/s).

In total, 24 laboratories with 39 different sufficiently sensitive flow cytometers from 12 different countries were interested and fulfilled the requested criteria and were included in interlaboratory comparison study.

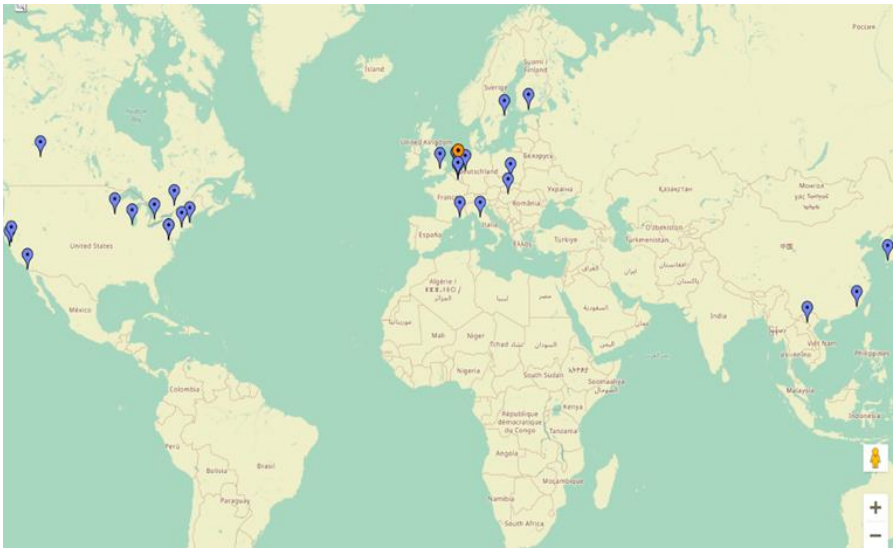


Figure 6. Laboratories participating in the 18HLT01 METVES II interlaboratory comparison to standardise concentration measurements of extracellular vesicles (Prepared using Google Maps).

AMC coordinated the selection, preparation and sending of control samples, calibrators, and test samples to participants in the interlaboratory comparison study. The control samples included essential buffers, vCalTM Nanorainbow Beads, and Becton Dickinson (BD) staining buffer.

BD APC/PE MESF beads, SNP, Rosetta calibration beads (mix of polystyrene beads of different sizes), and hollow organosilica beads of different sizes were selected as calibrators.

BD APC/PE MESF beads were used for fluorescence calibration, SNP beads for flow rate calibration, and Rosetta calibration beads for scatter calibration.

The project's PEVTES (Objective 3) and liposomes (BD, TTK) were selected as test samples.

AMC together with BD prepared the interlaboratory comparison measurement protocol, which involved multiple on-site tests to improve and optimise the protocol.

In May 2022, AMC organised an online webinar to introduce the project and protocol (https://www.metves.eu/output/videos), prior to samples being shipped to the participating laboratories.

Following the intercomparison study, the analysis of data was performed by Exometry, and in total complete data sets from 21 flow cytometers were received.

The instrument sensitivity of the flow cytometers, in terms of their smallest detectable polystyrene and EV size was determined by Exometry, using Exometry developed calibration beads and software. Rosetta Calibration relates the light scatter measured by forward scattering (FSC) and side scattering (SSC) to the effective scattering cross section and diameter of EVs. EVs were modelled as core-shell particles with a core RI of 1.38, a shell RI of 1.48, and a shell thickness of 6 nm.

Fluorescence calibration was performed using partner BD's custom-made MESF beads, and flow rate calibration was based on SNPs developed in Objective 1. For flow rate calibration, the concentration of SNPs was traceably determined by SAXS at PTB and spICPMS by LGC. Scatter calibrations were performed using Rosetta calibration beads and software. For each instrument, a serial dilution of the PEVTES was analysed to exclude swarm detection. Swarm detection is a special form of coincidence detection where multiple particles at or below the detection limit are simultaneously present in the laser beam of a flow cytometer, and hence are erroneously measured as single events such as EVs.

The RI of the PEVTES medium was traceably determined by a metrological goniometer developed by VSL (Objective 3) at a wavelength of 405 nm. The results are shown in Table 1. Light scattering depends on the contrast between the particle and the surrounding medium. In the Rosetta calibration software, Exometry assumed a medium RI of 1.345 for distilled phosphate-buffered saline (dPBS). Sample analysis was ongoing

when the RI results were provided. Together with the results from the swarm detection analysis and the requirement that samples at a given dilution need to have > 100 counts, we excluded the 10x diluted PEVTES sample based on goniometer results and selected the 30x diluted PEVTES sample for further analysis.

Calibration allowed the setting of instrument specific limits of detection sensitivity for APC/PE fluorescence (MESF) and EV size (nm) using custom-build software (MATLAB R2020b). The applied thresholds/gates were set based on the mean of all instruments multiplied by two. Figure 7 shows the instrument sensitivity for APC/PE fluorescence (MESF) and diameter (nm) of 18 instruments. All instruments below the applied threshold (red line) were used for preliminary results. Lower applied thresholds are 220 APC MESF, 50 PE MESF and 215 nm. Platelet EVs were also identified with a CD61-APC label and erythrocyte EVs with a CD235a-PE label.

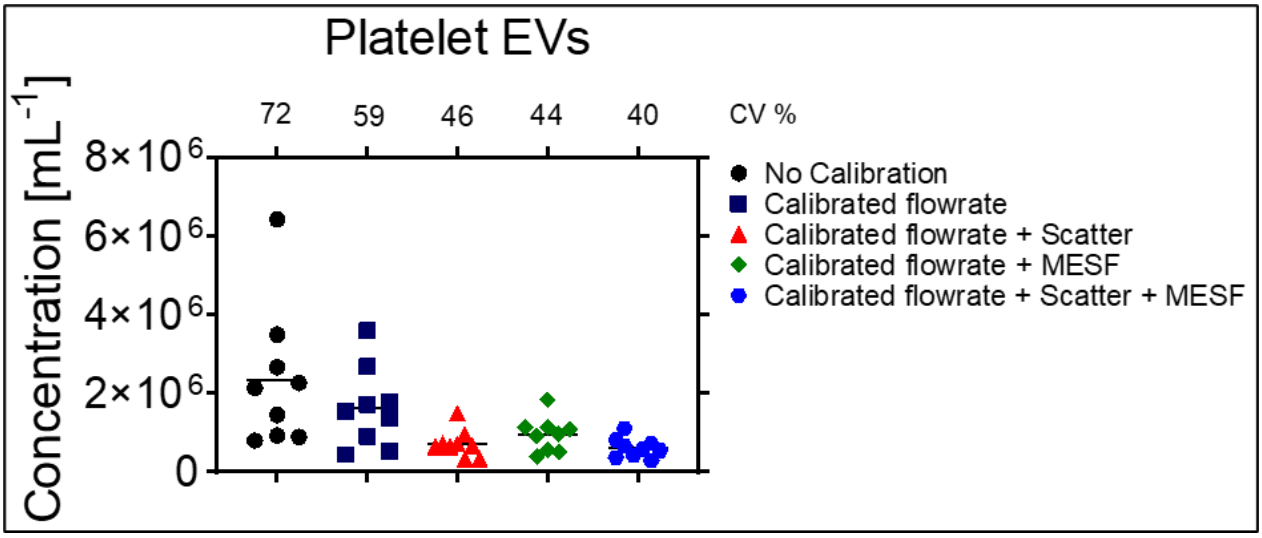


Figure 7. Preliminary results of concentration measurements of a biological test sample containing platelet-derived EVs measured on different flow cytometers, i.e., instruments that differ in their optical configuration) without calibration, and after calibration of flow rate, light scatter and fluorescence (MESF; mean equivalent of soluble fluorochromes). The CV(%) is defined as the standard deviation/mean x 100.

Conclusions

Due to the COVID-19 pandemic and unforeseen associated lockdowns, the interlaboratory comparison study including data analysis was delayed, and data analysis is still ongoing. However, the results already analysed are very promising and Figure 7 shows that combined calibration of flow rate, light scattering and fluorescence indeed reduces the CV of flow cytometry.

The main goal of 18HLT01 METVES II from a healthcare perspective was to demonstrate that instrument (flow cytometer) calibration is feasible and improves standardisation of concentration measurements of EVs in a clinically relevant EV-containing test sample. The project has achieved this and developed a traceable infrastructure to calibrate flow cytometers. This calibration can now enable the conversion of the flow cytometry data from arbitrary units of light scattering and fluorescence into SI units or MESF. In the long term, this should enable the comparison of concentration measurement results of dim submicron particles, present in complex bio fluids, between instruments that differ in their optical configuration. As well as traceable and reproducible flow cytometry results, in particular for EV research.

Due to the HLT02 METVES and 18HLT01 METVES II projects, measuring the concentrations of EVs within known size ranges has become feasible. This opens the door to measuring the concentrations of EVs, independently from their cellular origin, in different body fluids and for a variety of diseases. Moreover, since comparable EV concentration measurements are now possible, clinically relevant reference ranges in body fluids from healthy individuals can be established and hence future multi-center studies performed.

5 Impact

A stakeholder committee of 10 members was set up, including two members from industry, one clinician, one member from the National Institute of Standards and Technology (USA), four well-respected EV researchers from the EU and two from the USA. A website was also set up (www.metves.eu) for stakeholders to find out important information and events for the project. Further to this, 41 conference presentations and posters have been presented on the project's results 13 peer-reviewed open access scientific publications have been published, and a patent application has been filed for the production of liposomes.

Impact on industrial and other user communities

EVs in liquid biopsies behold the promise of becoming new biomarkers for common diseases. In 2022, the estimated liquid biopsy market size was expected to exceed $ 2.1 billion, with a compound annual growth rate of > 23 %. Consequently, there is a growing demand for biomarker research from the industry. One of the industrial partners in this project is BD, which is one of the leaders in the global flow cytometry market and due to the connections of the other project partners (AMC, Exometry, PTB, TTK, VSL, UH), it is expected that the metrological basis developed in this project will become a prerequisite for clinical acceptance and routine application of EV-based diagnostics. This acceptance will support the uptake and use of the project's developed EV reference materials (Objectives 1 & 3) and reference methods (Objective 2) in the future development of (i) reference materials for EV, virus or bacteria measurements, (ii) flow cytometers dedicated to nanoparticle detection, (iii) diagnostic kits, and (iv) drug-loaded therapeutic EVs or liposomes.

As one of the world leaders in measurement procedures, reagents and instruments for research & clinical cell analysis, partner BD is very interested in the commercialisation and dissemination of the project's outputs to industrial and clinical end users. Software developed by partner Exometry will be used by BD for flow cytometry calibration in conjunction with BD's FlowJo Plug-in that was developed to support this (Objective 3). In addition, BD has filed a patent application on the production of liposomes from Objective 1 will produce a report on potential interest in the commercialisation, licensing, and/or distribution of the project's reference materials (Objectives 1 & 3) and reference procedures (Objective 2).

Indeed, one of the reference particles (Objective 1) has already been commercialised by Exometry together with partner TTK as "verity shells" (https://www.exometry.com/products/verity-shells).

For industry, it is important that reliable and comparable data is generated with commercial equipment. Commercial flow cytometers were used by 24 participants in the project's interlaboratory comparison study, including 3 industrial laboratories.

Furthermore, comparing the preliminary results from this project METVES II interlaboratory comparison study (Objective 4) to the results of the previous METVES projects' comparison study, demonstrates that commercial flow cytometers have already become more sensitive for small particle detection and hence industry is investing in this area.

Impact on the metrology and scientific communities.

The METVES II project has developed new methods for traceable number concentration and diameter measurements of submicron particles. VSL together with AMC developed the first ever precision goniometer with an expanded uncertainty of $1.4E^{-6}$ (Objective 3). In METVES II the goniometer was used to determine the RI of fluids commonly used in clinical flow cytometry such as sheath fluid and physiological buffers such as phosphate-buffered saline. This information is critical because EVs have a low RI, and the RI contrast affects the size determination of EVs, and hence affects the measured concentrations of EVs.

VSL together with AMC also constructed a metrological flow cytometer (Objective 3). This metrological flow cytometer is nearly operational and has already gained considerable interest at international scientific meetings because this instrument, will (in principle) be capable of simultaneously measuring the RI, size and fluorescence of single particles in suspension. When operational, this metrological flow cytometer, will be the first of its kind and can be used as a metrological instrument to characterise single reference materials, biological particles as EVs, etc.

The PEVTES sample developed by this project (Objective 3) is unique as it expresses the complexity and heterogeneity of a real human plasma sample. The procedure to develop stable labelled EV-containing test

samples is also completely new, although future research is needed to explore its use in standardisation studies.

METVES II partners and stakeholders are prominent members or chairs of the EV Flow Cytometry Working Group (a collaboration between ISAC, ISEV and ISTH) and standardisation bodies of ISEV and ISTH. In these international organisations, results have been presented and disseminated. The developed infrastructure of METVES II is new to the EV field, and this knowledge has been shared with the scientific and medical communities e.g.

- at the “EV Club seminars” (ISEV initiative) https://www.youtube.com/watch?v=ghub6emZDAA)
- at an industry-sponsored Nature webinar (https://www.nature.com/webcasts)
- and at a METVES II organised workshop (https://www.metves.eu/downloads/workshop/Workshop_METVES_II.pdf).

A major element of generating impact is training and education. Two PhD students, one with an engineering background and one with a biomedical background, were employed and trained during METVES II to strengthen collaboration between NMI‘s (VSL) and hospitals (AMC, UH). Further to this, (bio) medical students were trained by AMC to calibrate flow cytometers, an annual summer course (starting 2023) is scheduled by AMC to teach (bio) medical students to calibrate flow cytometers using reference materials, test samples and software developed in the METVES II project. Furthermore, ISEV is setting up an online training course on EV detection and isolation methods, that will explain the relevance of metrology and calibration to an audience of clinicians and (bio) medical researchers using the knowledge and information developed in the METVES II project.

Impact on relevant standards

At present, no EU directives or appropriate measurement standards exist with regard to EVs. Partners in METVES II have provided input to both international ISO TC24 Particle characterisation including sieving and TC229 Nanotechnologies and national METSTA SR229 Nanotechnology standards development organisations that are related to methods used for characterising reference particles. Input was also provided by the consortium to existing technical reports, specifications and the revisions of existing technical specifications, including ISO 19430 “Determination of the particle size distribution and number concentration by the Particle Tracking Analysis (PTA) Method”, and ISO/TS 19590 “Nanotechnologies — Characterisation of nano-objects using single particle inductively coupled plasma mass spectrometry“. Furthermore, the project’s results were used in new standards and technical reports, including ISO 23484 “Determination of particle concentration by small-angle X-ray scattering (SAXS)”, and ISO/ TS 24672 “Nanotechnologies - Guidance on the measurement of nanoparticle number concentration'. Finally, the project has presented its results to the Scientific Standardisation Committee (SSC) of the ISTH Subcommittee on Vascular Biology and at the EV Flow Cytometry working group (Objective 4).

Longer-term economic, social and environmental impacts

In the long-term, the infrastructure and reference materials developed in this project to calibrate flow cytometers is expected to have a considerable impact on biomarker research. Firstly, the concentration of EVs in a body fluid depends on collection, handling and storage procedures. Until now, there was no method available to quantify and monitor the concentration of EVs in a reproducible and standardised manner. But thanks to the METVES II project, calibration of flow cytometers to standardise concentration measurements of EVs (and other submicron particles) has become possible, which will be used in future experiments to optimise pre-analytical procedures.

One of the hallmarks of clinically relevant biomarkers is the establishment of a reference range. A reference range is the normal concentration of a biomarker in a particular body fluid of healthy human. When the biomarker concentration (e.g. EV) is outside the reference range, this may be relevant for diagnosis, prognosis, and monitoring of disease. But to establish biomarker reference ranges, all instruments and laboratories must generate comparable data. This goal of establishing reference ranges for EVs in body fluids can become possible in future using the project’s outputs; (i) synthetic EV reference materials, (ii) traceable measurement methods for them, (iii) biological test samples (PETVES) and (iv) an interlaboratory comparison study with clinical end users, of the EV reference samples and methods.

Comparable concentration measurements of EVs, should pave the way towards clinically relevant multicentre studies of the concentration of cell-type specific EVs and their biomarker potential. Since all body fluids contain EVs from multiple cell types, the developed infrastructure will be broadly applicable, and is expected to find new and non-invasive disease biomarkers that may facilitate earlier diagnosis, reduce healthcare costs, and improve patient survival rates. Importantly, the reference materials and methods developed in METVES II could also be used in the future to standardise concentrations of non-EV particles, e.g. lipoproteins, viruses, bacteria and non-biological particles.

6 List of publications

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This list is also available here: https://www.euramet.org/repository/research-publications-repository-link/

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