HLT02 METVES

Task 4.2. Development and distribution of biological microvesicles (MV) reference materials

Deliverable 4.2.9: Report on the stability over time of biological MV reference materials based on liposomes and purified MV

Participants: AMC, REG1(AMR), EJPD, PTB, SMD, VSL
Lead participant: AMC and REG1(AMR)
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Contents

Summary

Within the European Metrology Research Programme (EMRP), the 3 year project “Metrological characterisation of microvesicles (MV) from body fluids as non-invasive diagnostic biomarkers” (METVES) has started in June 2012. The aim of the project is to develop traceable measurement techniques for the characterisation of MV as biomarkers. Within the work package 4, in particular task 4.2, we need to produce and characterise three different biological materials, which are stable for at least 3 months and have physical properties similar to MV, such as size (diameter) or refractive index. There were four biological materials tested: Intralipids, erythrocyte MVs, purified lipoproteins (high-density and very-low density lipoproteins), and liposomes. They were characterised using different techniques such as SAXS, AFM, TSEM, RPS and NTA. We have evaluated the results and found that most of these biological materials have a broad size distribution (polydisperse). Thus, they are unsuited as a reference standard for size measurement. Other issues are stability, purity, cost, and feasibility to be measured by certain techniques. Based on this evaluation, these biological materials are not selected as biological MV reference material for inter-laboratory comparisons of task 4.3.

Introduction

Within the European Metrology Research Programme (EMRP), the 3 year project “Metrological characterisation of microvesicles (MV) from body fluids as non-invasive diagnostic biomarkers” (METVES) has started in June 2012. The aim of the project is to develop traceable measurement techniques for the characterisation of MV as biomarkers. To date, no suitable reference materials are available to perform traceable measurements of MV in body fluids by different techniques. Within the work package 4, efforts have been done in the development of synthetic (task 4.1) and biological (task 4.2) MV reference materials. This report (deliverable 4.2.9) will focus on task 4.2.

MV have a refractive index of 1.37 [1]. Therefore, for example, polystyrene beads with refractive index of 1.61 [2] are not suited as a reference material for MV characterisation. The aim of task 4.2 is to produce and characterise three different biological materials, which are stable for at least 3 months and have physical properties similar to MV, such as size (diameter) or refractive index. We have tested different biological materials such as Intralipids, erythrocyte MV, purified lipoproteins (high-density and very-low density lipoproteins), and liposomes in different sizes. These biological materials were characterised using SAXS, AFM, TSEM, RPS and NTA. Parameters such as polydispersity, stability, purity, cost, and feasibility were evaluated. Based on this evaluation, we have to select the possible candidates of MV reference material for inter-laboratory comparisons of task 4.3.

In this report, results of measurements using SAXS, AFM, TSEM, RPS and NTA are presented and compared. Finally, we conclude this task 4.2 and provide discussions on the possible biological MV reference materials for the future development.

Materials and Methods

Materials

Intralipids

Intralipids® 20% produced by Fresenius Kabi (Zeist, The Netherlands) were obtained from Pharmacy of Academic Medical Centre of University of Amsterdam (The Netherlands). This product was maintained in a sterile infusion bag in a volume of 100 ml (Figure 1) and stored at room temperature (~25 °C) for up to 18 months. According to the manufacturer, Intralipids® 20% is stable for 24 hours at 20-25 °C or up to 7 days at 2-8 °C after opening the container. Each participant received the sealed container of Intralipids® 20%.

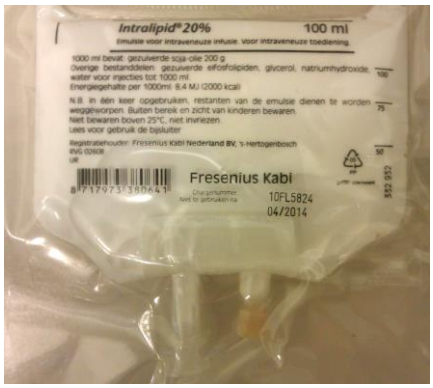


Figure 1. Intralipids® 20%

Lipoproteins

Purified high-density lipoprotein (HDL; Cat. Nr. 437641) and very-low density lipoprotein (VLDL; Cat. Nr. 437647) were purchased from Merck Millipore (Darmstadt, Germany). Composition of each lipoprotein is presented in Figure 2. According to the manufacturer, purified HDL and VLDL are stable up to 6 months after the production's date. These lipoproteins were stored at 2-8 °C upon arrival. Freezing-thawing was avoided. Aliquots (10 µL) were made under sterile conditions and distributed to all participants.

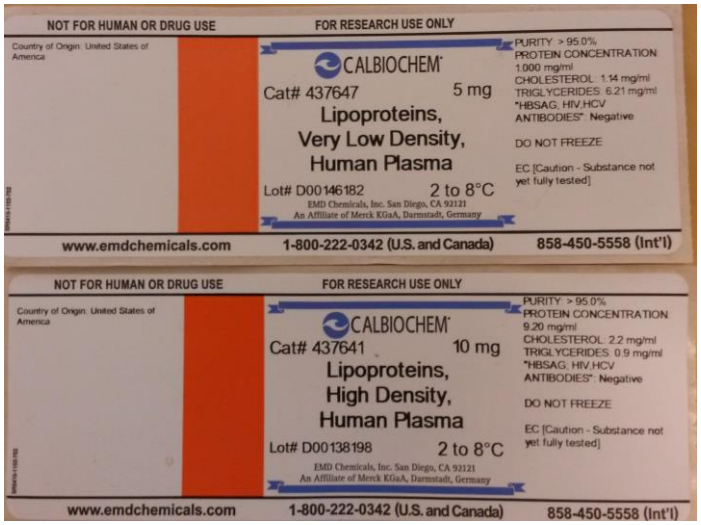


Figure 2. Composition of HDL and VLDL

Liposomes

PCPGlip-80, PCPGlip-200, PCPGlip-400, and PCPGlip-600 were liposomes suspensions prepared by REG(RCNS HAS). They were stored in 2 mL crimp top glass vials containing approximately 1 mL suspension (Figure 3). The liposomes were prepared by the lipid film hydration and extrusion method using polycarbonate filters with various pore sizes (80, 200, 400 and 600 nm). The nominal total lipid concentration of the samples is 18.55 mg/mL in pH 7.4 Tris-HCl buffered saline solution (10 mM Tris, 150 mM NaCl) containing 0.02% sodium azide. All liposome samples were stored between 2 °C and 8 °C and

freezing-thawing was avoided. All participants received one vial of each liposome suspension to be measured.

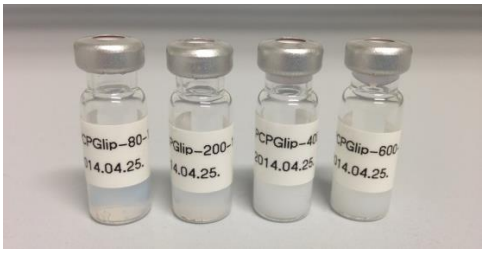


Figure 3. PCPGlip-80, PCPGlip-200, PCPGlip-400, and PCPGlip-600

Erythrocyte MV

Erythrocyte MV were prepared by REG1(AMR) from outdated erythrocyte concentrate (Sanquin, Amsterdam, The Netherlands). This erythrocyte concentrate was diluted 2-fold in Hepes buffer pH7.4 composed of Hepes (10 mmol/L), NaCl (137 mmol/L), and KCl (4 mmol/L) and then centrifuged at 1,560 × g for 20 min (twice) at 20 °C to deplete erythrocytes. All chemicals were from Merck Millipore. The supernatant containing erythrocyte MV was collected and further centrifuged at 100,000 × g, 20 °C for 2 hours to concentrate MV. Afterwards, the concentrated erythrocyte-derived MV were applied to the Sepharose CL-2B column prepared by AMC (according to protocol published by Böing et al [3] with some minor modification) for purification (Figure 4). Fractions 7-10 (total volume: 2 mL) were collected, pooled, and filtered through 0.2 μm nuclepore filter (GE Health Care Life Sciences, Buckinghamshire, UK). The flow through containing purified and filtered erythrocyte MV was concentrated using a 3K Amicon ultrafiltration unit (Merck Millipore) following the manufacturer's instructions by centrifuging at 14,000 × g for 30 minutes. These erythrocyte MV were frozen in liquid Nitrogen and stored at -80 °C. Each participant received frozen erythrocyte MV and it was thawed in melting ice before measurements.

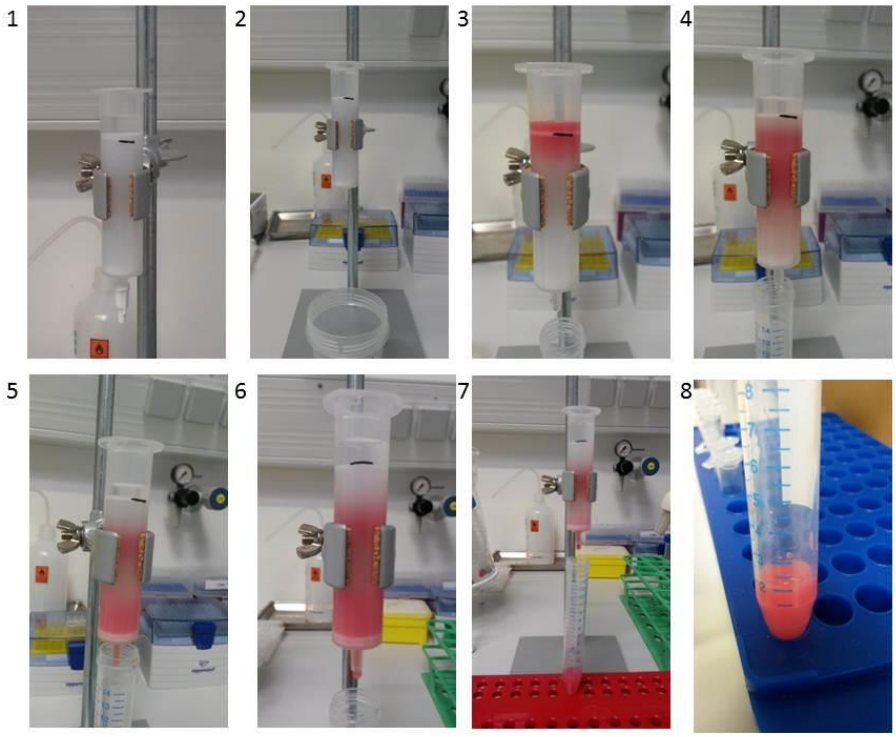


Figure 4. Purification of erythrocyte MV using Sepharose CL-2B column. Column was placed up right (1). The white cap was removed and the column was washed 3 times with buffer (2). Erythrocyte MV sample was added (3) and the eluate was directly collected (4). After the sample passed the filter top, buffer was added (4-5). The first 3 mL of eluate was discarded. The next 2 mL eluate which contained erythrocyte MV was collected (5-8).

Methods

RPS (tunable resistive pulse sensing)

RPS measurements were performed in AMC using qNano from Izon Science Ltd., Christchurch, New Zealand as described previously [4]. This instrument was operated with three different nanopores, namely NP100 with a particle detection range from 70 nm to 200 nm, NP200 from 100 nm to 400 nm, and NP400 from 200-800 nm. The nanopore was selected based on the expected size distributions of the samples (Table 1). The device was operated using a single pressure protocol at a pressure of 7 or 15 mBar. The voltage was set between 0.34 V and 0.5 V for all experiments, and the nanopore stretch was adjusted until the current of the signal trace approaches 100 nA. All samples were diluted 1000- to 10,000-fold in buffer and measured for 10 minutes or until 500 counts were reached. Measurement and analysis were performed with Izon Control Suite v2.2.2.44 software. The size distributions were then fitted with a Gaussian distribution to determine the mean.

NTA (nanoparticle tracking analysis)

A NS500 (NanoSight Limited, London, UK) equipped with an EMCCD camera (Andor Technology, Tokyo, Japan) and with a 405 nm laser was used in AMC for measurements of biological materials. Measurements were done as described previously [5]. All samples were 10,000- to 1,000,000-fold diluted with buffer before measured. The temperature is kept at 22 °C during measurement. Videos were recorded at 10 different positions at different camera levels depending on the scattering intensity of the particles in the samples (Table 1) for around 30 seconds and analysed using threshold 10 by NTA v2.3.0.17 (Nanosight). The obtained size distributions were then fitted with a Gaussian distribution to determine the mean.

Table 1. Nanopore type and camera level setting used for the measurement of biological materials

Samples	RPS Nanopore type	NTA Camera level
Intralipids	NP200	10
HDL	n.a.	n.a.
VLDL	NP100	14
PCPGlip-80	NP100	14; 15
PCPGlip-200	NP200	14
PCPGlip-400	NP200; NP400	10
PCPGlip-600	NP200; NP400	10
Erythrocyte MV	NP100	14

n.a. not analysed

SAXS (small-angle X-ray scattering)

The experiments are performed at the four-crystal monochromator (FCM) beam line of PTB at BESSY. A sample holder containing the samples sealed in vacuum tight containers is placed into a vacuum chamber equipped with a six axes manipulator for sample movement.

The measurements were performed at photon energies of 10 keV, 8 keV and 4 keV, in order to cover a large range of the momentum transfer q. For the measurements at 8 keV and 10 keV, the samples were filled into disposable glass capillaries made of borosilicate glass with an inner diameter of 1 mm and a wall thickness of 10 µm. The capillaries are closed by welding the upper end in the flame of a propane oxygen torch. For measurements at 4 keV, the samples were filled into a holder specifically designed for liquid samples at low energies (D2.1.4). The liquid in this holder is enclosed between to silicon nitride windows with a thickness of 500 nm, and the sample thickness is approximately 0.1 mm.

The synchrotron radiation is collimated using pinholes to a size smaller than 0.5x0.5 mm² and focused on the sample. The incident photon flux is measured using a transmission diode located in front of the sample and before the guard pinhole. A removable, calibrated diode behind sample is used to measure the transmission of the sample. The scattered radiation is collected by a PILATUS 1M detector at a variable distance between 2.3 m and 4.6 m behind the sample for the liposome and erythrocyte MV samples. For lipoproteins, a different setup was chosen with a fixed distance of 414 mm .

In case of the variable setup, the distance between the sample and the detector is measured using optical encoders and was set close to the nearest (2.3 m) and farthest (4.3 m) position available at the highest and lowest photon energy, respectively, in order to cover a large range of *q*. At each setting, several images are recorded and averaged pixel-by-pixel for each sample. The resulting images are averaged azimuthally about the beam centre to get the scattering curve. A transmission measurement with the same condition is performed for all samples including the blank.

AFM (atomic force microscopy)

AFM measurements were done at VSL, SMD, and EJPD. In VSL and SMD, AFM Veeco Dimension 3100 working in tapping-mode was used. EJPD used an AFM Dimension 3500 equipped with a metrological head working in tapping-mode. All the experiments were performed on a mica substrate. The mica surface was modified/coated before deposition of samples using: (i) poly-L-lysine or (ii) purified human CD235a antibody (for capturing erythrocyte MV). Samples were characterised under wet and/or dry conditions. For measurement under wet condition, AFM equipped with liquid cell was used and about 80 µL of buffer was added on top of the sample. For experiment under dry condition, the sample was rinse with ultrapure water and dry under nitrogen (N_2) flow.

(i) Poly-L-lysine

Muscovite mica discs were freshly cleaved several times using adhesive tape in order to have flat surface. About 20 µL of poly-L-lysine was applied on the mica during several minutes, giving rise to a positively-charged surface. Substrates were subsequently rinsed with ultrapure water and dried under air flow. Twenty µl of diluted sample (Table 2) was applied on modified-substrate during few minutes and rinsed with either pure water or phosphate-buffered saline (PBS) solution.

(ii) Purified human CD235a antibody

To attach CD235a antibody, the surface of mica was modified as previously described [6]. Briefly, freshly cleaved muscovite mica was first incubated in a 55% ethanolamine in DMSO at 70°C for more than 10 hours. Subsequently, the Amine-modified mica were rinse using DMSO and ethanol, and dried under nitrogen (N_2) flow. Prior to protein coupling, the modified mica was incubated for 3 hours with 1 mg/mL EGTA in chloroform containing 0.5% (v/v) triethylamine, dried under N2, and glued onto steel disks. Fifty µL of CD235a antibody (0.01 mg/mL) was applied to the modified mica and incubated for 30 minutes at room temperature. Excess of antibody was removed by rinsing with PBS. Twenty µL of undiluted

erythrocyte MV sample was then applied and incubated for 60 minutes. To remove unbound MV, the mica was rinsed with PBS.

Table 2. Dilution was performed prior to measurement of biological materials

Samples	Dilution
HDL	40-fold in HEPES
VLDL	40-fold in HEPES
PCPGlip-80	100- to 1000-fold in TRIS
PCPGlip-200	50- to 100-fold in TRIS

TEM (transmission electron microscopy) and STEM (Scanning transmission electron microscopy)

Samples were fixed at room temperature overnight by 0.1% (weight/volume; w/v) paraformaldehyde (Electron microscopy sciences, Hatfield, PA). Next, a 200 mesh EM copper grid with formvar coating (Electron microscopy sciences) was put on top of a sample (10 µL), and incubated for 7 minutes at room temperature. The grids were transferred to 1.75% uranyl acetate (w/v) for negative staining. The grid was imaged using a Tecnai 12 transmission electron microscopy (TEM, FEI Company, Eindhoven, The Netherlands), operated at 80 kV. The same grids were also imaged using transmission scanning electron microscopy (TSEM), which provides 3D information enabling better description of the shape of vesicles [7]. We used a commercial Zeiss Leo Supra 35 VP equipped with a transmission detector which is built by K. E. Development and distributed by Zeiss as an add-on (Carl Zeiss, Oberkochen, Germany). TSEM was operated at 30 kV.

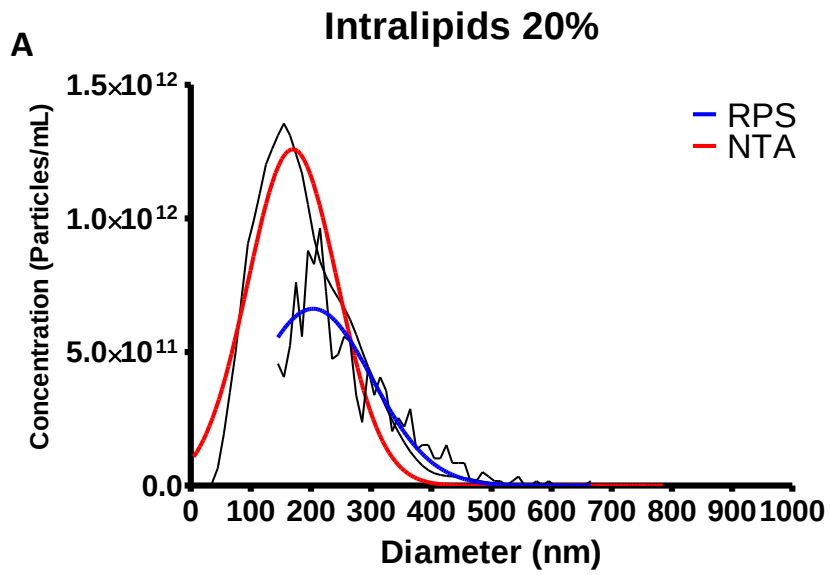
Results

Intralipids

Intralipids have a broad size distribution. As shown in Figure 5A, the size distribution ranges from 145-600 nm as measured by RPS (mean 203.3 ± 98.7 nm) and from 35-800 nm as measured by NTA (mean 169.6 ± 74.30 nm). These results also were confirmed by AFM measurement (Figure 5B). SAXS was not performed because of a broad size distribution of Intralipids.

Using TEM, round dense particles with an unapparent membrane border were detected. This is unexpected as the application of negative staining should give contrast on the membrane border and the particles should appear lighter than the membrane border. We investigated by comparing freshly prepared and overnight prepared samples under TEM. We did not find the round dense particles in the

freshly prepared sample, but only in the overnight preparation (Figure 6). Thus, Intralipids is apparently unstable during overnight preparation.



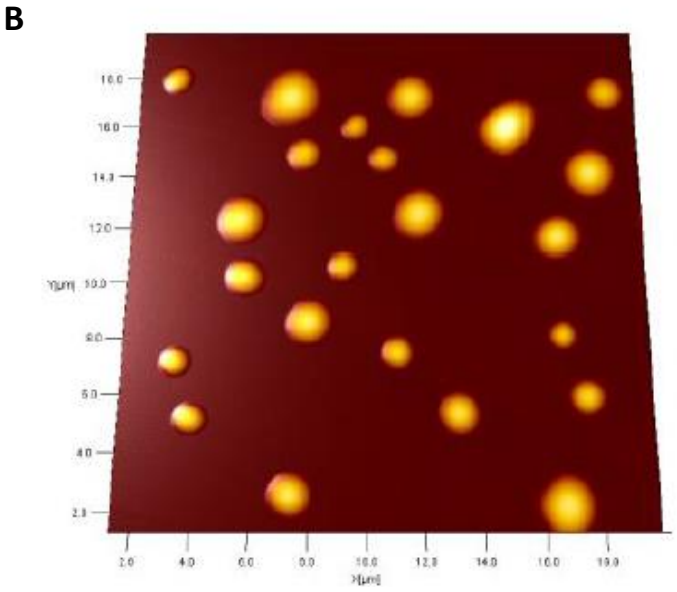


Figure 5. RPS, NTA, and AFM measurements of Intralipids 20%. Size distribution of Intralipids analysed by RPS and NTA (A). The red and blue lines represent the Gaussian distribution of size distribution measured respectively. AFM measured Intralipids 20% in tapping mode (B). Intralipids were disposed on mica coated with poly-L-lysine and imaged under dry condition.

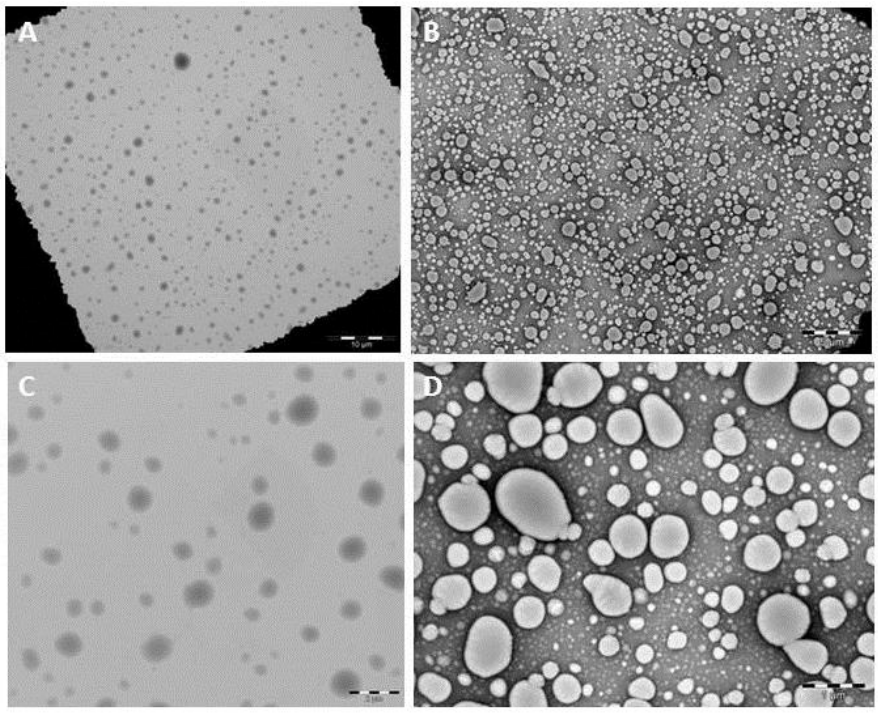


Figure 6. TEM analysis of Intralipids 20%. Intralipid was fixed overnight (A, C) or 30 minutes (B, D) in paraformaldehyde, stained using uranyl acetate, and imaged using TEM. The scale bars are 10 µm for A, 5 µm for B, 2 µm for C, and 1 µm for D.

Lipoproteins

HDL was successfully measured by SAXS and TEM (Figure 7A and B respectively). These HDL particles are homogenous with a size around 10 nm according to SAXS measurement. Other techniques such as AFM, RPS, and NTA were not able to measure HDL particles. The difficulties of imaging HDL particles using AFM are salt contamination present in the sample buffer and the small size of the HDL particles. RPS and NTA were not sensitive to detect HDL particles because their size is below the detection limit of these techniques.

VLDL was apparently more polydisperse than HDL. As shown in Figure 8A, the size distribution ranges from 45-365 nm (mean: 63.98 ± 13.10 nm) as measured by RPS and from 16-615 nm (89.64 ± 32.29 nm) as measured by NTA. These results have been confirmed by AFM analysis (Figure 8B and C) which showed that VLDL particles range from diameter of 10 to 100 nm (mean: 48 nm). These particles tend to agglomerate and show a round-shaped structure with a collapsing or even empty centre (Figure 8B). Furthermore, the particles exhibit lower phase signal (darker region in the phase image) compared to the substrate (lighter region), probably indicating that the VDL particles are soft material (Figure 8B). Similar to observations using RPS, NTA, and AFM, we also observed the polydisperse VLDL particles using TEM (Figure 8D). SAXS was not performed due to polydispersity of VLDL particles.

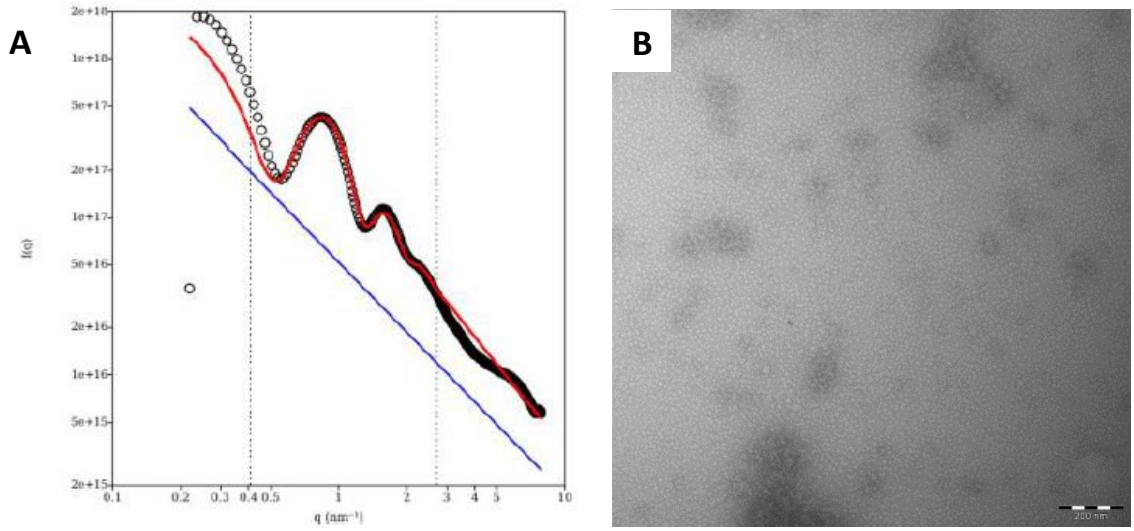
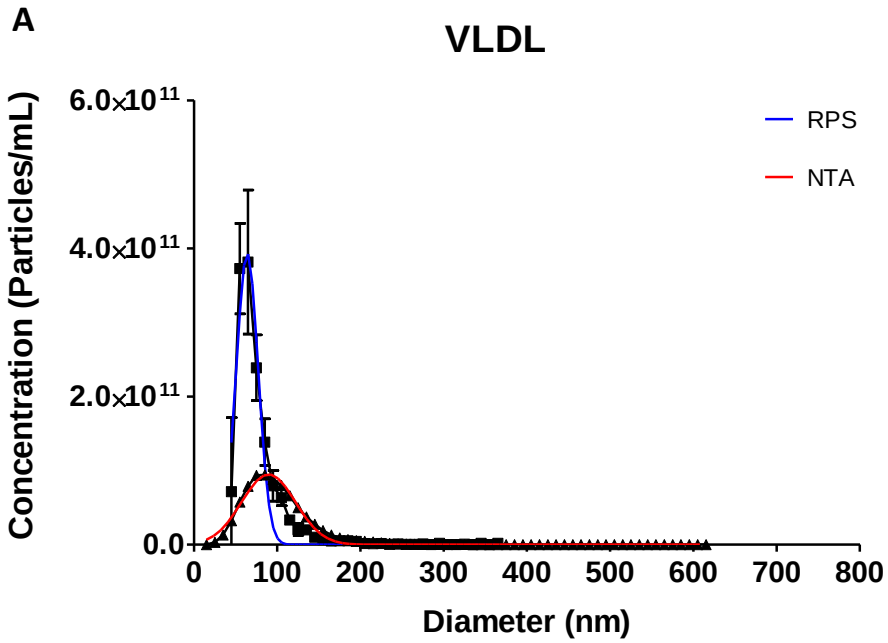
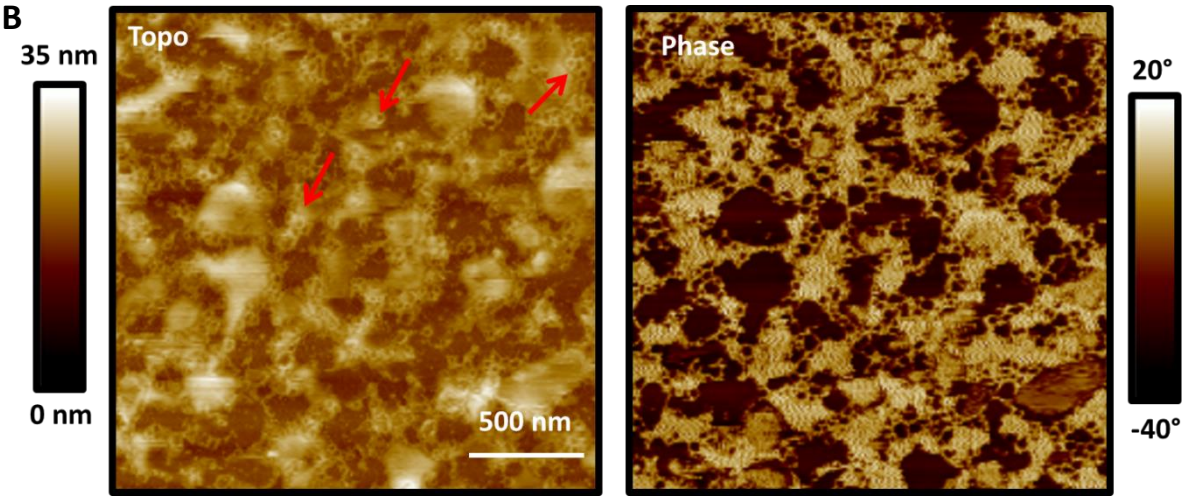


Figure 7. Measurements of HDL using SAXS and TEM. The scale bar of EM image is 200 nm.



B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0 nm
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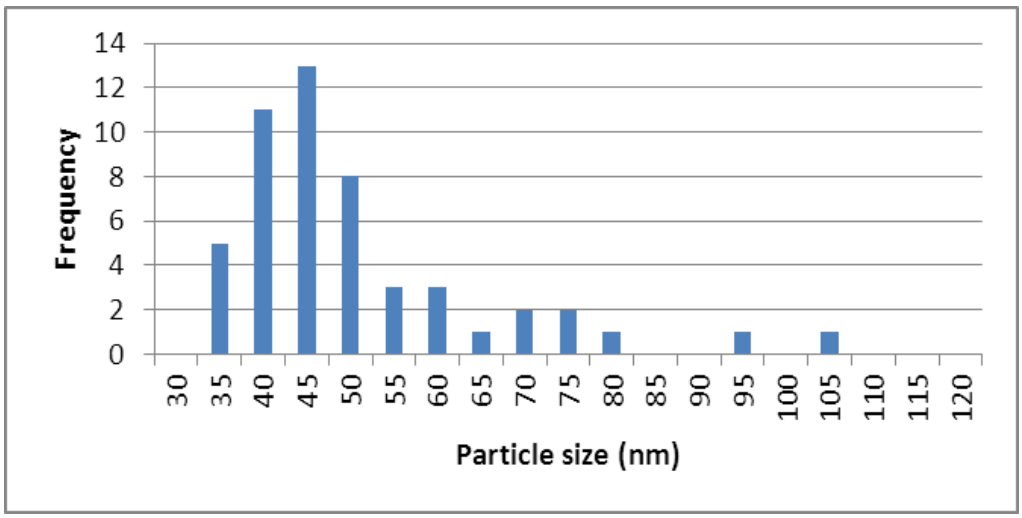
C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Particle size (nm)

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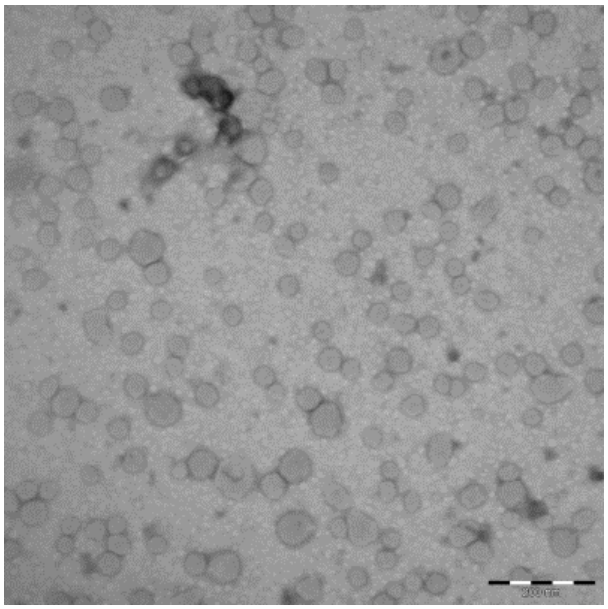

Figure 8. VLDL measurements using RPS, NTA, AFM, and TEM. Concentration and size measurements of VLDL by RPS and NTA were depicted (A). AFM measurements of VLDL have been done using tapping mode under dry condition (B). The particle size distribution is from 10 to 100 nm (x/y dimension) with a few nanometers high (C), as pointed out by the red arrows (B). The particles exhibit lower phase signal (darker region) compared to the substrate (lighter region) in the phase image (B). TEM image shows VLDL particles stained by uranyl acetate (D). The scale bar in TEM image is 200 nm.

Liposomes

PCPGlip-80, PCPGlip-200, PCPGlip-400, and PCPGlip-600 have been measured by RPS, NTA, SAXS, AFM, and TEM. We have observed by RPS and NTA that PCPGlip-80 and PCPGlip-200 are more monodisperse than PCPGlip-400, and PCPGlip-600 (Table 2). SAXS analysis of PCPGlip-80 also shows that this type of liposomes gave a sufficiently narrow size distribution (Figure 9).

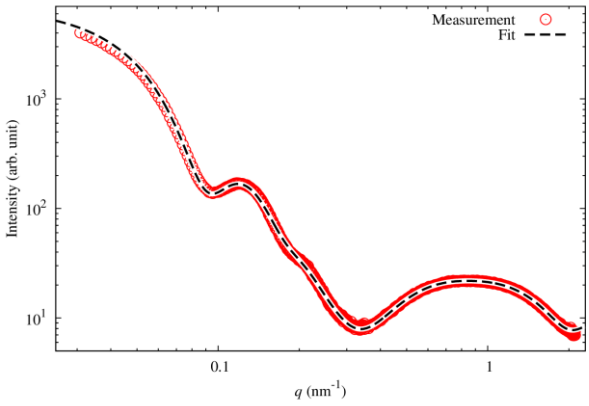

Figure 9. PCPGlip-80 was measured using SAXS. The scattering curve could be fitted using a model of lipid bilayer with an outer diameter of 72 nm and a CV of 20%

The measurement of liposomes using AFM was difficult due to the changes in morphology of liposomes during adhesion on the mica surface coated with poly-L-lysine. Liposomes spread out when they adhere on the mica and the lipid bilayer was also visible (Figure 10). The mean diameter of PCPGlip-80 measured using AFM is estimated around 40 nm with a broad size distribution between 20-70 nm. Contamination of salt crystals from the sample buffer which have the same size as the liposomes was an issue. Although the sample has been rinsed with water before imaging, the salt crystals still were present. Also, liposomes seem to fuse when they were measured in a high concentration. Thus, titration is necessary before AFM measurement.

All liposomes samples were measured by RPS and NTA to check their stability after 1 year storage at 4 °C. We found that results obtained by RPS and NTA were not comparable (Figure 11). To check these results, we performed measurements on PCPGlip-80, PCPGlip-400, and PCPGlip-600 using a W130i DLS (dynamic light scattering) apparatus (Avid Nano Ltd., High Wycombe, UK). We found that after 1 year storage, the mean particle size of PCPGlip-80 is stable (90.9 nm versus 90.64 nm), whereas this is increased in other liposome samples.

Table 3. PCPGlip-80, PCPGlip-200, PCPGlip-400, and PCPGlip-600 measured by RPS and NTA

Liposomes	RPS		NTA	
	Mean size distribution (nm)	CV (%)	Mean size distribution (nm)	CV (%)
PCPGlip-80	71.55 ± 7.93	11.1	85.46 ± 29.52	34.5
PCPGlip-200	135.6 ± 24.01	17.7	130.8 ± 37.03	28.48
PCPGlip-400	170.6 ± 94.65	55.5	297 ± 110.5	37.2
PCPGlip-600	199.1 ± 78.40	39.4	337.4 ± 131.5	37.2

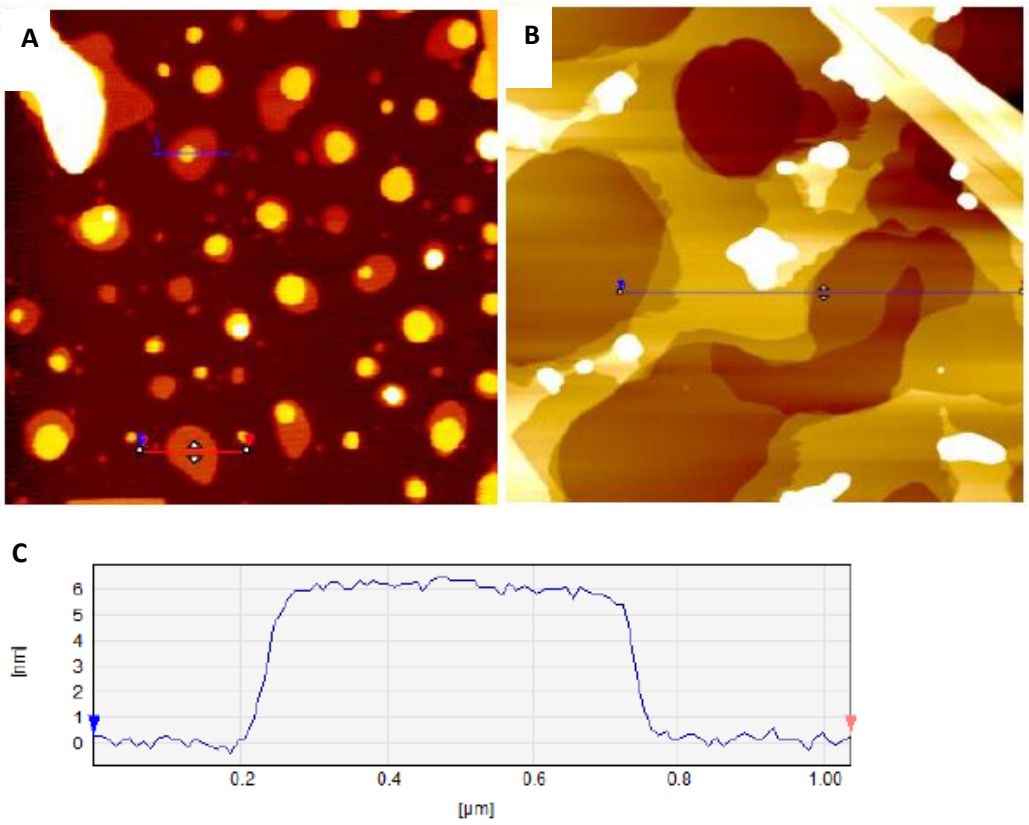

Figure 10. PCPGlip-80 was measured using AFM. The topography images were presented (A, B). The scan size of image A is 5 µm and image B is 8 µm. Liposome particle is flattened/spread out when adhering on the mica surface coated with poly-L-lysine (C).

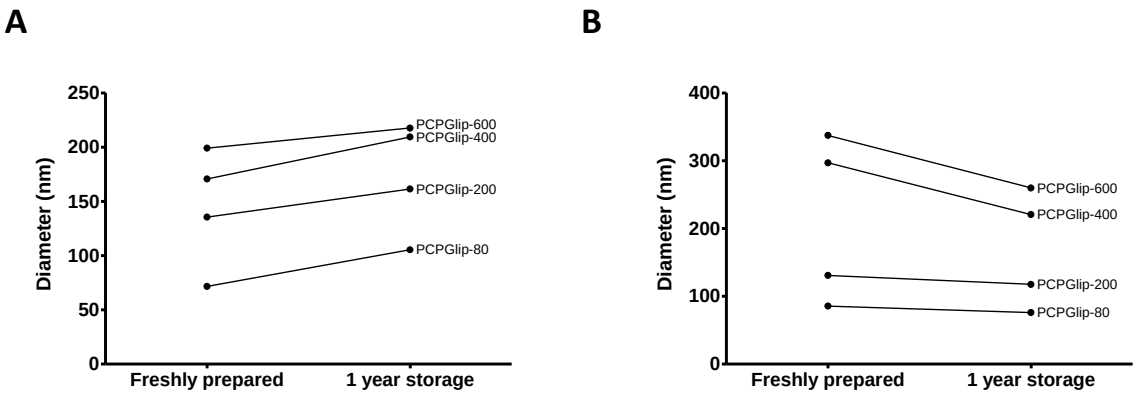

Figure 11. PCPGlip-80, PCPGlip-200, PCPGlip-400, and PCPGlip-600 were measured using RPS (A) and NTA (B) after 1 year storage at 4 °C.

Erythrocyte MV

Erythrocyte MV are naturally occurring vesicles which can be isolated from different type of biological fluids. We have isolated erythrocyte MV from outdated erythrocyte concentrate. We performed

purification using Sepharose CL-2B to remove proteins and filtration using 0.2 µm filter to obtain a narrow size distribution of erythrocyte MV. These MV were measured by RPS, NTA, SAXS, AFM, and TEM/STEM. To be noted, we have produced and analysed several batches of erythrocyte MV samples. We have seen differences between batches which are likely caused by the variations in the erythrocyte concentrates and in the preparation of erythrocyte MV samples from these concentrates. When precipitation occurred which might happen occasionally after thawing the sample, this sample was discarded. Results presented on Table 3 are the representative of mean diameter size measured by different techniques in erythrocyte MV samples. Based on these results, erythrocyte MV (i) could be measured by different techniques and (ii) are stable during measurement under wet or dry conditions. However, the size distributions of all batches of erythrocyte MV samples are still too polydisperse to allow for a traceable measurement of the particle size, despite the purification and filtration step with a 0.2 µm filter. For example, the calculated diameter ranges from 20 to 120 nm in a very broad distribution has been measured by AFM.

Effect of storage has been investigated on erythrocyte MV by using RPS, NTA, and AFM. Mean particle size was analysed in the erythrocyte MV samples during storage at ambient temperature using AFM (Figure 12A) and at -80 °C using RPS and NTA (Figure 12B). We found that erythrocyte MV are stable during storage at ambient temperature up to 7 days and after storage at -80 °C up to 6 months (Figure 12B).

Table 4. Mean diameter (nm) of purified erythrocyte MV

RPS	NTA	SAXS	AFM		STEM
			Wet	Dry	
176	155	145	74	70	219

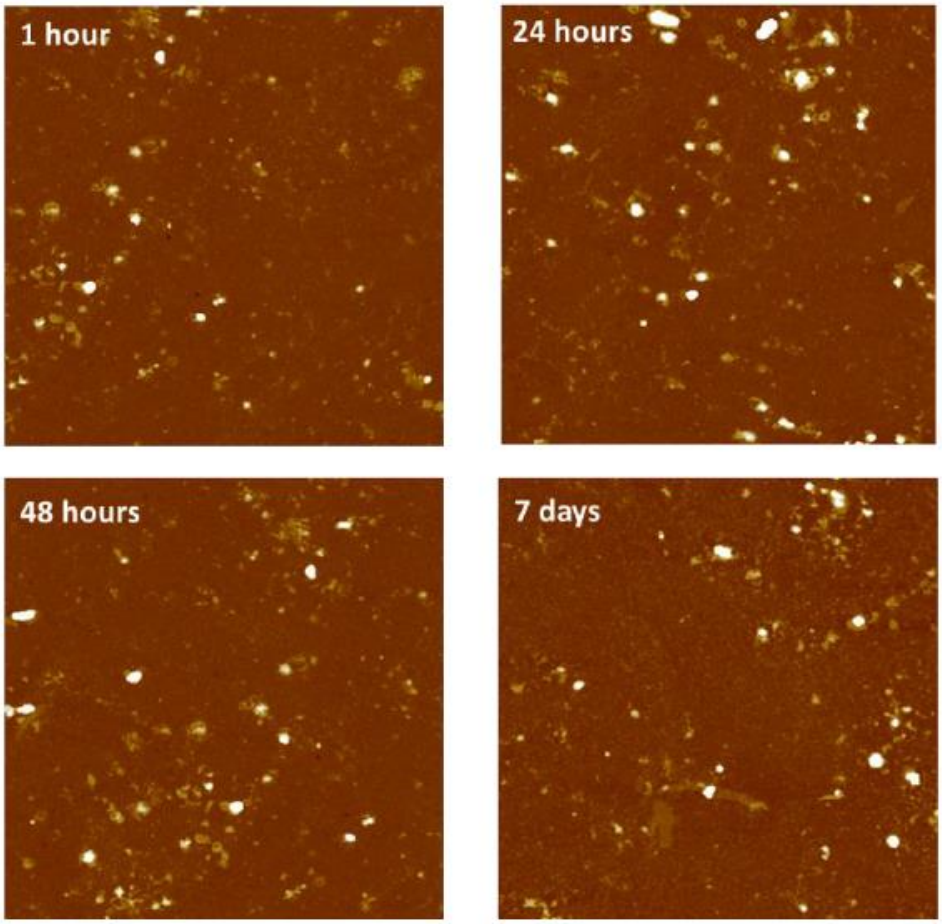

Figure 12. Erythrocyte MV sample from the same preparation was analysed using AFM after storage at ambient temperature at different time points. Height images were scanned at 10µm².

Table 5. Quantification of mean particle size (nm) by AFM, RPS, and NTA

Storage	AFM		RPS	NTA
	Mean height	Mean calculated diameter	Mean diameter	Mean diameter
1 hour	29 ± 8	65 ± 18	n.a.	n.a.
24 hours	30 ± 9	62 ± 15	175 ± 42	147 ± 39
48 hours	31 ± 9	62 ± 21	n.a.	n.a.
7 days	34 ± 13	61 ± 21	n.a.	150 ± 42
6 months	n.a.	n.a.	138 ± 36	139 ± 39

n.a.: not analysed

Discussion

Several biological materials (Intralipids, lipoproteins, liposomes, and erythrocyte MV) have been tested to select the candidate(s) for biological MV reference material. Based on the evaluation of several parameters such as polydispersity, stability, purity, cost, and feasibility using RPS, NTA, SAXS, AFM, and TEM/STEM, none of these tested biological materials is suited (Table 5). Thus, inter-laboratory comparisons of task 4.3 using biological MV reference material cannot be preceded.

Table 5. Parameters evaluated for selecting biological MV reference material

Material	Polydispersity	Stability	Purity	Cost	Feasibility
Intralipids® 20%	+++	-	++	+	++
Lipoproteins	+	-/+	+	+++	+
Liposomes	+	++	+	++	+
Erythrocytes MV	++	+	+	++	++

As shown in Table 5, poldispersity (broad size distribution) and feasibility are strongly correlated. This means that in the future biological materials with a narrow size distribution or known size distribution should be produced. Microfiltration has been done in this current task to produce more homogeneous population of liposomes and erythrocyte MV. However, this effort fails especially to produce particle size around 200 nm or larger. To obtain a narrow size distribution, other microfiltration techniques such as tangential flow filtration and flow field flow fractionation and size exclusion chromatography may be more suitable for separation of particles with different sizes in the future.

Liposomes are the most stable in comparison to other biological materials tested. Perhaps, the presence of sodium azide in the sample buffer and packing sample using a crimp top vial may help in maintaining the stability of liposomes. Thus, these applications should be investigated in the near future for preparing biological MV reference materials.

Salt crystals contamination is the major problem in measuring MV in physiological solutions using AFM under dry conditions. Rinsing with water before imaging was apparently not sufficient to overcome the problem. Therefore, proper controls should be used in performing AFM analysis.

In conclusion, the current tested biological materials are unsuited as a reference material. Thus, we still need to find or produce and evaluate other type of biological materials.

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