Preparation of biomimetic reference nanoparticles for the characterization of extracellular vesicles

Final report (PD121326), Dr. Zoltán Varga

1. Introduction

Extracellular vesicles (EVs), including exosomes, microvesicles and other membrane surrounded structures released from cells, are in the forefront of biomedical research. Because EVs contribute to many physiological processes, EVs may serve as biomarkers for diseases, including cancer, neurological diseases, and thrombosis. **Despite the potential of EVs for diagnostic applications, gold standard techniques and reference materials for EV detection are lacking.** A recent international survey showed that optical methods are widely used to characterize single EVs. Of all respondents who specified their single EV detection method, 80% used nanoparticle tracking analysis (NTA), 18% used tunable resistive pulse sensing (TRPS), and 29% used flow cytometry (bead capture assays excluded). Because only **flow cytometry can identify single EVs at high throughput** (>5,000 events/s) in a reproducible manner, flow cytometers **hold most promise for clinical applications**.

A flow cytometer detects light scattering and fluorescence of single particles in a hydrodynamically focused fluid stream. The light scattering signals of two sizes of **polystyrene beads are commonly used to gate EVs**. However, light scattering depends on the size, refractive index (RI), shape and structure of the particle, the RI of the medium, and the optical configuration of the flow cytometer. At a wavelength of 405 nm, which is used in modern flow cytometers to illuminate particles, polystyrene has an RI of 1.63 whereas EVs have an effective RI below 1.40. Due to this RI mismatch, 200 nm EVs scatters 40 to 300-fold less light than 200 nm polystyrene beads, as illustrated in Figure 1. Also 200 nm silica beads, which have an RI between 1.44 and 1.47, scatter 5 to 50-fold more light than similar-sized EVs. Thus, the use of **solid synthetic reference beads to standardize optical measurements of EVs leads to false size assignment.**

Besides the correct size characterization of EVs, the **determination of the particle concentration** in EV samples is also an important, but not yet solved challenge in the field. EV concentrations are **currently incomparable between most clinical laboratories**. Therefore, EV reference materials and methods are urgently needed to calibrate flow rate, light scatter intensity and fluorescence intensity in the sub-micrometer size range.

2. Aims

The main aim of this 3-year post-doctoral project was to prepare and characterize **novel reference materials for EV** size and concentration determination. **Two different approaches** were attempted: First, synthetic nanoparticles were prepared with hollow shell structure, which resembles the light scattering properties of EVs. For this purpose, **hollow organosilica beads (HOBs)** were prepared, and were thoroughly characterized. Their applicability for flow cytometry measurements of EVs was also investigated.

Liposomes may serve an ideal reference system for EVs, as their **basic building block**, namely the phospholipid bilayer is the same. On the other hand, **determination of the number concentration of liposomes from first principles was not attempted so far.** In this project we also planned to prepare monodisperse liposome samples and determine the concentration of liposomes with well-defined size and composition via counting the number of phospholipid molecules in these “nanospheres”.

The preparation of the novel EV reference materials also connected to the **development of new analytical methods** for the characterization of EVs. These investigations aimed at (1) the quantification and phenotyping of EVs by using **chromatographic techniques** combined with fluorescence detection, (2) development of **infrared spectroscopy-based** method for the determination of EV concentrations, and (3)

the use of **Microfluidic Resistive Pulse Sensing** (MRPS) to accurately determine the size distribution and concentration of EVs.

3. Results and Discussion

3.1 Hollow Organosilica Beads (HOBs)

HOBs were synthesized by combining a basic amino acid catalysis route with a hard template approach using solid silica nanoparticles as templates. The organosilica shell on the template particles was prepared with 1,2-bis(triethoxysilyl)ethane (BTEE), which was followed by etching the core of the particles at high pH. The preparation protocol was optimized to achieve homogeneous shell particles. Fig. 1 shows the TEM images of the HOBs prepared by using 200 nm and 400 nm diameter solid silica particles as templates (HOB 200 and HOB 400, respectively). The mean size and the width of the size distribution of the particles were also determined by various techniques, which is summarized in Fig. 2.

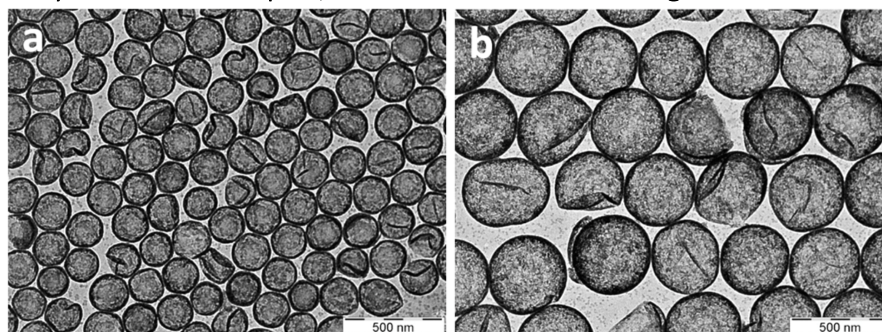

Fig. 1. Transmission electron microscopy (TEM) images of HOB 200 (a) and HOB 400 (b) particles.

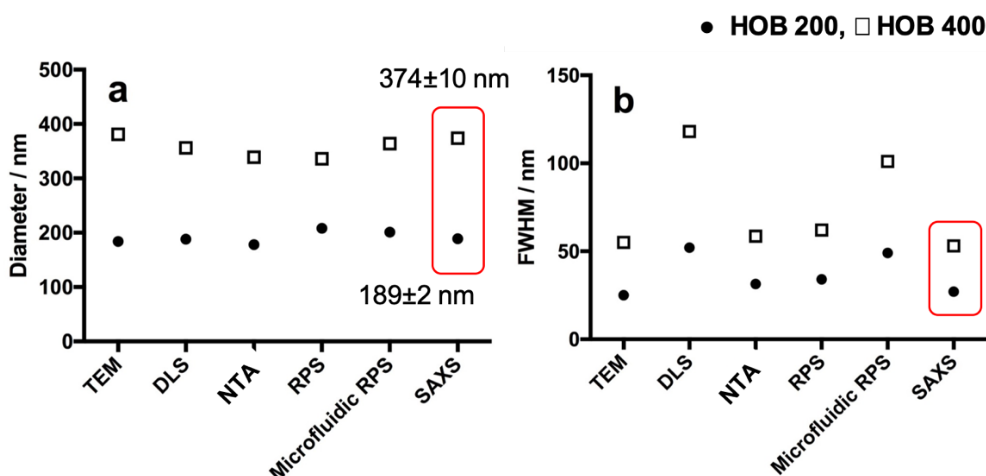

Fig. 2. Mean diameter and full width at half maximum (FWHM) values of the size distribution of HOB 200 (a) and HOB 400 (b) particles measured by TEM, dynamic light scattering (DLS), nanoparticle tracking analysis(NTA) , tunable and microfluidic resistive pulse sensing (TRPS and MRPS) and small-angle X-ray scattering (SAXS).

The applicability of HOBs in flow cytometry measurements was investigated with a comparison of the light scattering properties of HOBs and platelet-derived EVs (Fig. 3).

Thorough characterization of the prepared HOBs revealed narrow size distributions, colloidal stability, and homogeneous hollow core-shell structure of HOBs. Compared to potential biological reference particles [6], which like HOBs resemble the light scattering properties of EVs, safety, monodispersity and stability of HOBs are superior. The performed flow cytometry investigations confirm that HOBs have similar light scattering properties as EVs and therefore are more suitable as reference beads for flow cytometry characterization of EVs than solid polystyrene or silica beads. HOBs can be used to set size gates in nanometers independent from the optical configuration of a flow cytometer.

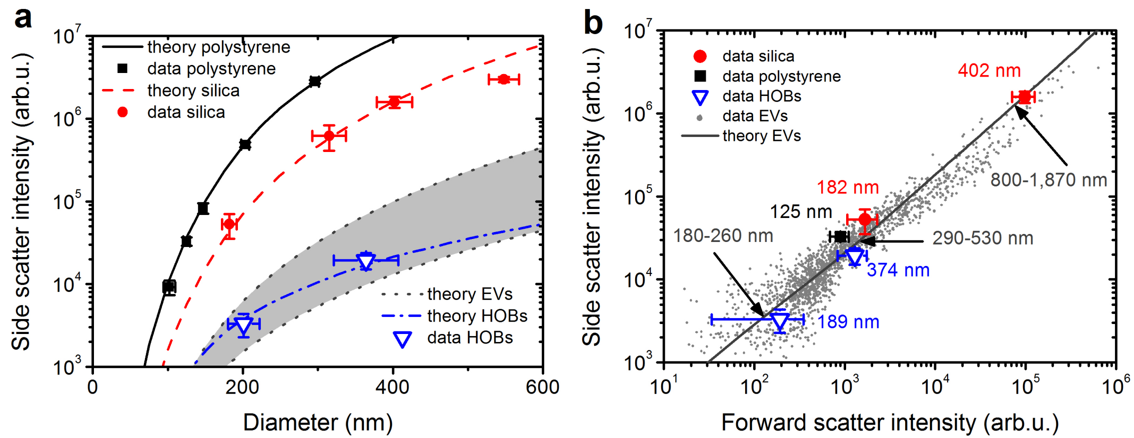

Fig. 3. Light scattering properties of polystyrene beads (squares), silica beads (circles), hollow organosilica beads (HOBs; triangles), and platelet-derived (CD61+) extracellular vesicles (EVs; dots) from human plasma measured (symbols) by flow cytometry and calculated (lines) by Mie theory. (a) Side scatter versus size (diameter). Whereas polystyrene and silica beads scatter orders of magnitudes more light than similar-sized EVs, HOBs resemble the expected side scatter properties of EVs (gray area). (b) Side scatter versus forward scatter. In contrast to polystyrene and silica beads, HOBs have forward and side scatter intensities similar to EVs of the same size.

Fluorescent labelling of HOBs was also carried out, for which a patent application is being prepared. HOBs and the fluorescently labelled HOBs are included in the METVES II project (co-founded by H2020 and EURAMET participating countries).

These results were presented at the CYTO2018 and ISEV2018 conferences (presentations) and published in the Journal of Thrombosis and Hemostasis (Z. Varga et al. 2018).

3.2 Sterically stabilized liposomes

The idea of determining the particle concentration of liposomes from first principles is based on the determination of the number of lipid molecules in the liposomes. If the phospholipid bilayer is in the gel-phase, the area-per-lipid within the bilayer of liposomes can be measured by wide-angle X-ray scattering (WAXS). Using this value, the size of the liposomes, and the molar concentration of phospholipids enables the calculation of the particle number concentration (Fig. 4)

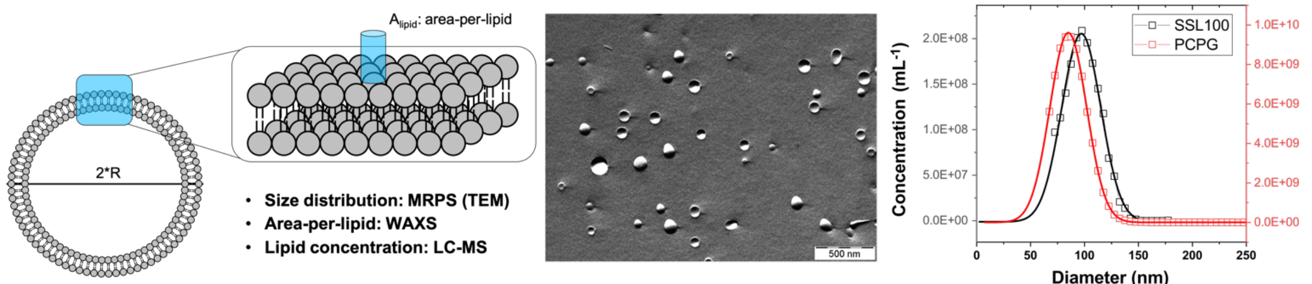

Fig. 4. Concept of the particle number concentration determination of liposomes (left), TEM (middle) and MRPS characterization of the investigated liposome samples.

Sterically Stabilized Liposomes (SSL) and Electrostatically Stabilized Liposomes (PCPG) were prepared by the lipid film hydration and extrusion method. TEM was used to characterize morphology of liposomes, while their size distribution was determined by **microfluidic resistive pulse sensing (MRPS)**. MRPS technology has been developed by Spectradyne LLC (California, USA) in the recent years, and one of the first devices in Europe was installed in our research lab. WAXS was used to measure the area-per-lipid value, and mass spectroscopy to determine the accurate concentration of lipids in the samples.

The prepared liposomes proved to be unilamellar with narrow size distributions (85 nm and 97 nm for PCPG and SSL100, respectively) as obtained by MRPS and TEM. DSC and IR measurements confirmed that the phospholipid bilayer of these liposomes is in the liquid-ordered phase, hence the area-per-lipid was

determined from WAXS measurements. Using the concentration of phospholipids from LC-MS measurements, the number concentration of liposomes was determined ($7.5*10^{13}$ mL^{-1} and $9.5*10^{13}$ for PCPG and SSL100 liposomes).

These results were presented at the ISEV2019 conference (poster and presentation) and a manuscript about the results is under preparation.

3.3 Detection and phenotyping of extracellular vesicles by size exclusion chromatography coupled with on-line fluorescence detection

Size exclusion chromatography (SEC) has been broadly adopted as a convenient and accessible technique for the purification of EVs. SEC is a size-based method that effectively separates nanoscale particles such as EVs from smaller molecules and impurities. Liquid chromatography systems with on-line UV-visible absorption, fluorescence, or light scattering detectors are rarely used in EV research, though fluorescence detection in particular would be especially valuable because it enables specific measurements of fluorescently labelled EVs. Labelling with fluorescent antibodies is widely used for quantifying EVs by flow cytometry, immunoassays and recently with nanoparticle tracking analysis (NTA), but has not yet been tested in conjunction with SEC.

Within this research project, the development of a new methodology combining size exclusion chromatography (SEC), a commonly used EV purification technique, with fluorescence detection of specifically labelled EVs was performed. The resulting platform, **Flu-SEC**, demonstrates a linear response to concentration of specific EVs and could form the basis of a system with phenotyping capability. Flu-SEC was validated using red blood cell derived EVs (REVs), which provide an ideal EV model with monodisperse size distribution and high EV concentration. MRPS was used to accurately determine the size distribution and concentration of REVs. Anti-CD235a antibody, specific to glycophorin A, and the more general wheat germ agglutinin (WGA), were selected to label REVs. The results show the quantitative power of Flu-SEC: a highly linear fluorescence response over a wide range of concentrations. Moreover, the Flu-SEC technique reports the ratio of EV-bound and free-antibody molecules, an important metric for determining optimal labelling conditions for other applications. These results were presented at the ISEV2018 conference (poster) and a manuscript summarizing the results was submitted and it is currently under review (Kitka et al. 2019).

3.4 Reagent-free total protein quantification of intact extracellular vesicles by attenuated total reflection - Fourier transform infrared (ATR-FTIR) spectroscopy

The protein composition and quantity of EVs is closely related to their functions, therefore its quantitative analysis can be used as EV-based biomarkers of various diseases. Within this research project, a reagent-free method based on attenuated total reflection - Fourier transform infrared (ATR-FTIR) spectroscopy was developed to quantify the protein content of EV samples without any sample preparation.

After calibration with bovine serum albumin, the protein concentration of red blood cell derived EVs (REVs) were investigated by ATR-FTIR spectroscopy. The integrated area of the amide I bands was calculated from the IR spectrum of REVs, which is proportional to the protein quantity in the sample regardless of its secondary structure. Spike test and dilution test were performed to determine the ability of using ATR-FTIR spectroscopy for protein quantification in EV samples, which resulted linearity with R-Square values as high as 0.992 over the concentration range of 0.07 to 1 mg/mL. The results indicate that ATR-FTIR measurements provide a reliable method for reagent-free protein quantification of EVs. The publication summarizing these results is currently under review (Mihály et al. 2019). Application of the FTIR method for various EV samples from single cell-origin was performed in collaboration with Tamás Visnovitz which was published in the Journal of Extracellular Vesicles (Visnovitz et al. 2019).

3.5 Size characterization of liposomes and EVs by novel methods

Small angle neutron scattering measurements were performed at the KWS-3 station of the Jülich Center for Neutron Science (JCNS) on liposomes and extracellular vesicles. The primary purpose of the measurement was to determine the size distribution of red blood cell EVs using the SANS technique, a method not yet used in the field. Comparison of the size distributions determined from neutron scattering experiments with those obtained by light scattering methods (DLS and NTA) provided an opportunity to estimate the thickness of the hydration layer of EVs. In addition to the above-mentioned methods, the EV samples were characterized by microfluidic resistive pulse sensing (MRPS). The combined evaluation of SANS and MRPS measurements made it possible to estimate the thickness of the protein crown covering the EVs. The publication summarizing the results is currently under review (Varga et al. 2019).

3.6 Characterization of red blood cell EVs by linear dichroism (LD), Flu-SEC and MRPS

For the first time we used linear dichroism (LD) technique for characterization of red blood cell-derived EVs in collaboration with the Biomolecular Self-Assembly Research Group of the TTK. We found that hemoglobin is located in red blood cell-derived vesicles in a well-characterized orientation linked to the phospholipid bilayer (Szigyártó et al. 2018). As a continuation of these experiments, the effect of membrane-active peptides on vesicles was studied using LD, Flu-SEC and MRPS techniques developed in this research project. Melittin was shown to remove the protein crown of vesicles from the peptides tested. A publication summarizing the results is currently under review (Singh et al. 2019).

4. Achievements and Outlook

Results of this research project were summarized in several manuscripts, three of which were already published in peer-reviewed journals, and another three are currently under review. The results were presented on 4 international conferences (4 oral and 3 poster presentations).

Several students joined the research, including Kata Szántay (BSc, ELTE, “Characterization of extracellular vesicles”) graduated in 2018, and Veronika Szentirmai (MSc, 2019, BME, “Characterization of extracellular vesicles: determination of protein concentration based on IR spectroscopy”) graduated in 2019. In the first semester of the 2019/2020 academic year, Luca Boncz (BSc, BME, "Isolation of extracellular vesicles from human blood by chromatographic methods") and Zita Csákó (BSc, BME, "Determination of size distribution of liposomes and extracellular vesicles by microfluidic resistance") will graduate, and two PhD students (Diána Kitka, ELTE, and Mohammed Aqueel, ELTE) are pursuing their PhD research under my supervision, who are expected to graduate in 2022.

Several domestic and international collaborations on the isolation and characterization of EVs have started in connection with this research project. The developed Flu-SEC technique was used to characterize plasma and serum EVs in international collaboration with the University of Helsinki's EV Laboratory (led by Pia Siljander). Flu-SEC results confirmed the results obtained by flow cytometry and proteomics, which showed that serum contains significantly increased levels of platelet-derived EVs. A publication summarizing the results is currently being written. In collaboration with Ádám Vannay (1st Pediatric Clinic, Semmelweis University) we investigate the effect of EVs isolated from peritoneal dialysis effluent on fibrosis, for which the necessary EV isolation steps are based on research conducted in the framework of this project.

The main achievement of this research project is that the result on HOB particles led to an international project called **METVES II** (“Standardisation of concentration measurements of extracellular vesicles for medical diagnoses”, www.metves.eu) launched in June 2019 (co-funded by the H2020 program and EURAMET participating countries), coordinated by Rienk Nieuwland (University of Amsterdam) The international consortium includes German, French, British, and Finnish national metrology institutes and three business associations (including BD Becton Dickinson AG). In this project, I lead the work package on the "Development of reference materials".