Invited lecture/Research

Validation of Interferometric Light Microscopy for Assessment of Extracellular Particles in 250 samples of Diluted Plasma: Preparing the Path for Future Clinical Practices

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Abstract:
In recent years, the analysis of extracellular particles (EPs) has become instrumental in deciphering intercellular communication and disease biomarkers. Interferometric light microscopy (ILM) has emerged as a powerful label-free technique for real-time characterization of nanoscale particles. This contribution presents for the first time measurements of EPs directly in diluted plasma by ILM on populations of samples. The study involves the measured number density of EPs, the corrected number density of EPs (taking into account dilution of the sample) and the size of EPs. Analysis involves plasma of multiple species—canine, equine, and human. Most of the D_h in all three species were confined to the interval between 130 nm and 200 nm. We found no statistically significant correlation between the corrected n and D_h indicating good performance of the method. The correlation between the measured n and D_h was statistically significant indicating hindered movement of EPs due to their higher number density. These results showed that high throughput measurement of number density and size of EPs in plasma is feasible.

Keywords: Extracellular vesicles; Interferometric Light Microscopy; Plasma analysis; Nanoparticle characterization; Videodrop technology; Nanoparticles

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1. Introduction

In recent years, the study of EPs has emerged as a crucial avenue for understanding intercellular communication and disease biomarkers (Welsh et al., 2024). Among various analytical techniques, ILM has proven to be a powerful tool for real-time, label-free characterization of nanoscale particles of different types (Romolo et al., 2022). This technology already proved its utility by quantifying viruses in a river (Roose-Amsaleg et al., 2017) or by analysing aquatic biotic nanoparticles (Boccara M et al., 2016).
In this contribution, we present the application of ILM to the measurement of extracellular particles in diluted plasma. ILM enables assessment of number density and size of particles in the size range of 80 to 500 nm. A few microliter sample is needed. The aim of this contribution is to analyse populations of samples of plasma from different species by ILM.

2. Material and methods

2.1. Preparation of the samples

Animal blood samples were taken from expired transfusion bags. Three bags of canine blood and 1 bag of equine blood were used. Human blood samples were donated by the authors. Collection was established in the morning after fasting for a minimum of 12 h overnight. A G21 needle (Microlance, Becton Dickinson, Franklin Lakes, NJ, USA) and 2.7 mL evacuated tube with trisodium citrate (BD Vacutainers, 367714A, Becton Dickinson, Franklin Lakes, NJ, USA) were used. Blood samples were centrifuged (centrifuge Centric 400/R, Domel, Železniki, Slovenia) at 18°C and different centripetal accelerations (between 50 × g and 1000 × g) for different times (from 5 min and 30 min) to obtain plasma. We analysed 250 samples (81 canine, 113 equine and 56 human). For assessment with ILM, plasma was diluted to such extent to enable saturation of the interferred light. As the medium we used saline for injections 9 mg/mL (B Braun, Melsungen AG, Melsungen, Germany). The probe dilution was 50 ×.

2.2. Interferometric light microscopy

The number density of EPs in the sample (n) and the average hydrodynamic diameter of EPs in the sample (D_h) were determined by ILM using Videodrop (Myriade, Paris, France). Signals from the medium (saline for injections) was under the detection limit. The threshold value of 4.2 was used. Seven microliters of sample were placed between cover glasses and illuminated by 2 W of blue LED light. The light scattered on the particle was imaged by a bright-field microscope objective and allowed to interfere with the incoming light. The image was recorded by a complementary metal-oxide-semiconductor high-resolution high-speed camera. The number density of the particles is the number of detected particles within the detected volume, which depends on the microscope characteristics and the particles' size. The typical detection volume was 15 pL. D_h was estimated by tracking the position of the imaged particle within the recorded movie. It was assumed that particles undergo Brownian motion due to collisions with surrounding particles. The diffusion coefficient D of the motion of the particle is taken to be proportional to the mean square displacement d of the particle between two consecutive frames taken in the time interval Δt, $\langle d^2(\Delta t)\rangle = \langle 4D\,\Delta t\rangle$, while D_h was estimated by assuming that the particles were spherical and using the Stokes–Einstein relation $D_h = kT/3\pi\eta D$. Each particle that was included in the analysis was tracked and processed individually, and the respective incident light signal was subtracted from each image. Processing of the images and the movies was performed by using the associated software, QVIR 2.6.0 (Myriade, Paris, France). More details are given in Romolo et al., (2022).

2.3. Design of the study

We assume that centrifugation of blood within the above range of centripetal accelerations and times of centrifugation would not sediment EPs. However, larger centripetal accelerations and longer times more effectively sediment erythrocytes than smaller centripetal accelerations and shorter times of centrifugation. Motion of mutually interacting erythrocytes pushes plasma carrying EPs upwards into the compartment that is gathered for analysis (Božič et al., 2022). We do not expect changes in the average size of the EPs but we could expect that there would be different number densities of EPs in plasma obtained

by different centrifuge settings. Recommendations of the producer of the ILM (Myriadelab, Paris, France) indicate that measurements of number density between 5×10^8 /mL and 5×10^9 /mL are reliable. We therefore hypothesized that the dependence of n on D_h in the range where the measurements are reliable would be constant. We considered measured n of the diluted plasma sample and n corrected for dilution. The corrected values give information on the EP content of plasma while the measured values are of interest to analyse the performance of the instrument.

3. Results

Figure 1 shows dependence of EP D_h on n in canine, equine and human plasma. Different colors represent different species (orange – dog, blue – horse, black – human). In upper panels (A and B) n were corrected for dilution and lower panels (B and D) show measured n. Most of the D_h in all three species were confined to the interval between 130 nm and 200 nm **(Figure 1)**. There were four points considerably out of this range in Panels A and C, therefore we performed analysis also by excluding these 4 outliers (Panels B, D). While corrected n of EPs in human samples extended more or less evenly over a wide interval, clusters of blue and orange points pertaining to a particular animal can be distinguished in Panels A and B.

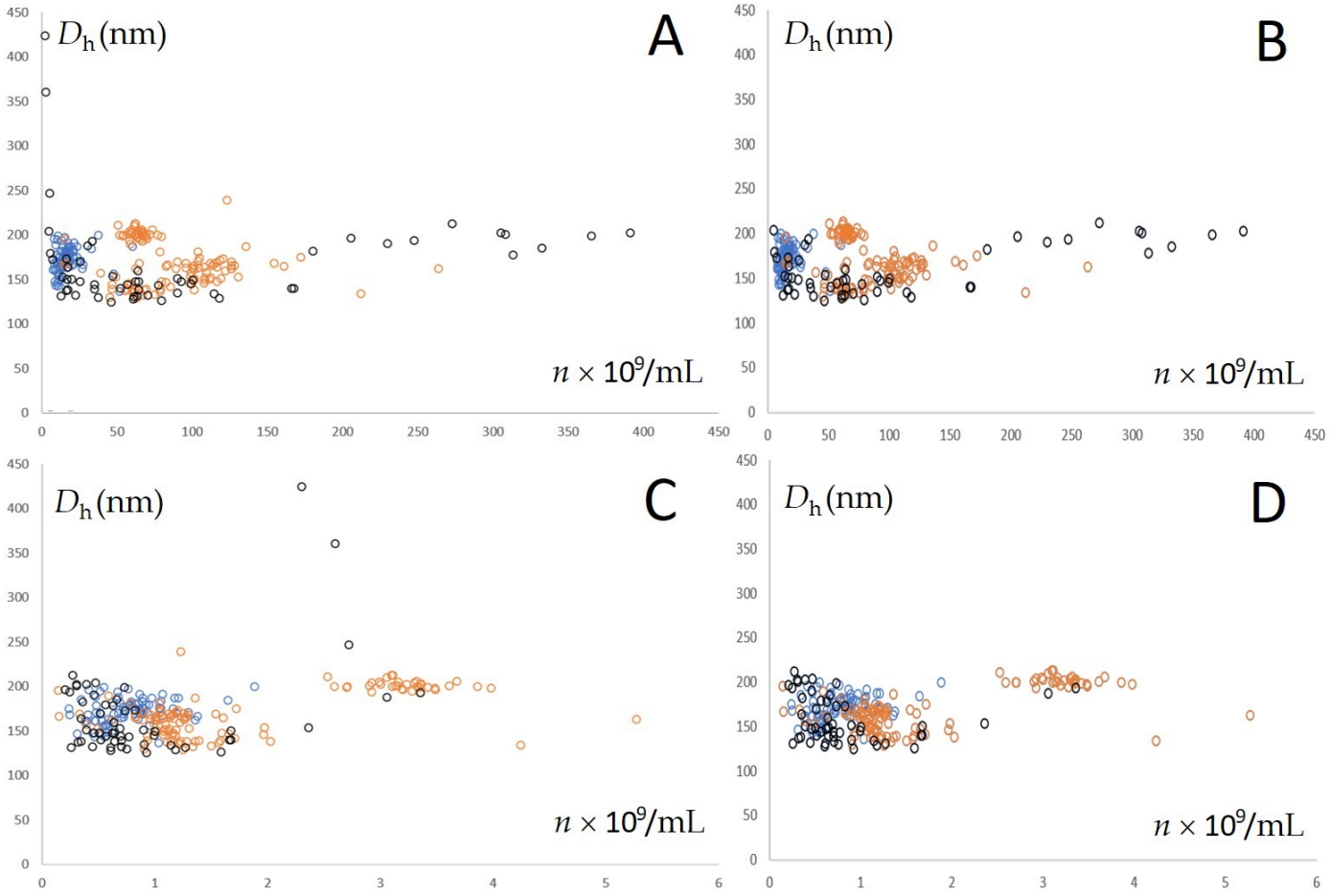

Figure 1. Hydrodynamic diameter of EPs in plasma (D_h) in dependence on the number density of EPs (n). Panels A and B: corrected n. Panels C and D: measured n. In Panels B and D, 4 outliers with D_h over 230 nm have been removed from respective Panels A and C. Orange circles: canine samples, blue circles: equine samples, black circles: human samples.

Table 1 shows Pearson correlation coefficient between n and D_h. We found no statistically significant correlation between the corrected n and D_h while the correlation between the measured n and D_h was statistically significant. Removing 4 outlier points from the graph did not considerably affect the statistical analysis results.

Table 1. Pearson coefficients of the correlation between the corrected n and D_h and between the measured n and D_h.

	All data		4 outliers removed	
	Corrected D_h/n	Measured D_h/n	Corrected D_h/n	Measured D_h/n
Pearson coefficient	<0.001	0.38*	0.08	0.41*
p	>0.05	$<10^{-4}$	>0.05	$<10^{-4}$

Asterisk denotes statistically significant result.

4. Discussion

We have for the first time performed measurement of EPs directly in diluted plasma in a large number of samples. We observed that the hydrodynamic diameter of EPs was in all species between cca 130 and 200 nm (**Figure 1**). The number densities of EPs in the samples varied and seemed to be characteristic to a donor (**Figure 1**). We observed no correlation of the corrected n of EPs in plasma and D_h (**Table 1**) which we assumed to be in agreement with the dynamics of the sedimentation of particles during centrifugation. In contrast, we observed a statistically significant positive correlation between measured n and D_h (**Table 1**). As D_h is determined by using the Einstein equation and the diffusion coefficient of the medium is assessed from the record of the movement of the particles, it is possible that the structure of plasma may hinder the movement of the particles depending on their concentration. If there are more particles in the plasma, they are restricted in their motion which is reflected in an overestimated size. However, in practice we are interested in concentration of EPs in blood or in plasma, both of which are relevant for therapeutic purposes. ILM performed well (**Figure 1**) as D_h and corrected n showed no correlation.

It was reported that the number density of extracellular vesicles in ex-vivo samples of human blood plasma were estimated at 10^{10} particles/mL but may be increased as a result of various pathological conditions (Božič et al., 2019). We have obtained n values up to the order of 10^{11} EPs per mL. Estimating viscosity of plasma by the viscosity of water, hydrodynamic diameter D_h remained between 130 and 200 nm, which agrees with the results on extracellular vesicles isolated from plasma obtained by dynamic light scattering (150 nm) (Božič et al., 2019).

5. Conclusions

We report on the first measurements of EPs in diluted plasma by ILM. The estimation of size and number density are in agreement with the data from the literature. It is indicated that high throughput measurement of number density and size of EPs in plasma is feasible. The hydrodynamic diameter of EPs in plasma did not depend on the concentration of EPs in the sample while number density of EPs measured in diluted plasma positively correlated with the hydrodynamic radius indicating hindered movement of EPs due to higher number density. Further research is needed to explore the potentials and limitations of ILM, yet our results indicate that it is a method that should be reckoned with in the study of EPs and extracellular vesicles.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, blood was donated voluntarily by the authors of the study.

Conflicts of Interest: The authors declare no conflict of interest.

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