

Research

Measuring Density and Viscosity of Samples as an Improvement of Determination of Hydrodynamic Diameter of Extracellular Particles by Interferometric Light Microscopy

Mercaldo Beatrice^{1,*}, Romolo Anna², Di Loria Antonio^{1,3}, Alterisio Maria Chiara¹, Esposito Sergio¹, Ciaramella Paolo¹, Kralj-Iglič Veronika²

¹ University of Naples Federico II, Department of Veterinary Medicine and Animal Production, Naples, Italy

² University of Ljubljana, Faculty of Health Sciences, Laboratory of Clinical Biophysics, Ljubljana, Slovenia

³ Immunoveg s.r.l. c/0 Dipartimento di Agraria Parco Gussone, Portici, Napoli, Italy

* Correspondence: Beatrice Mercaldo: beatrice.mercaldo@unina.it

Citation: Mercaldo B, Romolo A, Di Loria A, Alterisio MC, Esposito S, Ciaramella P, Kralj-Iglič V. Measuring Density and Viscosity of Samples as an Improvement of Interferometric Light Microscopy. *Proceedings of Socratic Lectures*. 2026, 14, 95-100.
<https://doi.org/10.55295/PSL.14.2026.III9>

Publisher's Note: UL ZF stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2026 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract:

We present technological advancement of the method for assessment of the hydrodynamic diameter D_h of nanoparticles suspended in liquid, by interferometric light microscopy (ILM). In ILM, D_h is determined by Einstein-Stokes equation which includes dynamic viscosity η of the sample as an input. Hitherto, η was estimated by the respective value for water at given temperature. It was of our interest to see how the results obtained by direct measurement of η differ from this estimate. We measured the density of the sample by oscillating U-tube Density Meter and used this value as an input to measure η by a rolling and falling ball Micro viscometer. We determined D_h by interferometric light microscope. An overestimation of D_h by using the “water” estimation was found to range from 1% to 7%. Furthermore, a significant difference was observed between D_h obtained by the respective data ($t = 25.985$; $p < 0.001$). The percentage difference between the two methods of determining D_h showed a moderate and statistically significant negative correlation with plasma density (Spearman coefficient $\rho = -0.365$, $p < 0.001$) and a very strong and significant negative correlation with dynamic viscosity ($\rho = -0.968$, $p < 0.001$). The inherently negative Spearman coefficients demonstrate that the overestimation error introduced by the measurement using water dynamic viscosity increases significantly with increase of the density and dynamic viscosity of the sample. The correction method based on direct measurement of η offers a more accurate, scientifically sound measurement of D_h of extracellular particles derived from different biological matrices (e.g., plants, micro-organism cultures, bodily fluids, etc.).

Keywords: Extracellular vesicles; Interferometric Light Microscopy; Nanoparticles; Density; Viscosity; Biophysical Properties

1. Introduction

Nanomedicine and innovative biotechnologies have recently introduced as focus of research the development of nanoparticle-based systems for diagnostic and therapeutic purposes. Among these, particular attention has been paid to extracellular particles (EPs), including extracellular vesicles (EVs) (Welsh et al., 2024).

Research is focusing on the isolation and use of these particles as biomarkers and carriers derived from biological fluids (blood, plasma), but also on the study, within the field of plant biology, of so-called plant-derived extracellular particles (PEVs) (Wiklander et al., 2019; Welsh et al., 2024). These structures, in particular, are of great interest due to their biocompatibility, stability and lack of immunogenicity (Cui et al., 2024).

Both for plant and animal-derived particles, a careful assessment of their physical characteristic is the key to studying them properly. This is because there is a strong correlation between physical properties of nanoparticles and their bio-functional properties (bioavailability, pharmacokinetics, etc.) (Welsh et al., 2024). The hydrodynamic diameter (D_h) of nanoparticles, in particular, is a variable on which the ability to cross biological barriers, load-carrying capacity, bioavailability and pharmacokinetics strictly depend. Among the techniques developed to study the physical characteristics of these structures, research has focused on the development of innovative technologies such as Interferometric Light Microscopy (ILM). This is an optical technology within the field of single-particle tracking (SPT), which aims to overcome the limitations of other mass analysis techniques, such as Dynamic Light Scattering (DLS) (Filipe et al., 2010; Boccara et al., 2016; Berry et al., 2024; Korenjak et al., 2024).

D_h is calculated in ILM system following the Stokes-Einstein equation. The equation requires the viscosity of medium and the temperature value. To accurately translate Brownian motion into a metric dimension, it is essential to have viscosity of the medium examined. In experimental practice, it is generally assumed that the viscosity of the matrix—since most cases involve dilute solutions—is comparable to that of water. However, when working with complex biological matrices, such as plasma or plant extracts—even when diluted—it must be considered that these are complex colloidal suspensions rich in proteins and lipid fractions that increase their viscosity (Windberger et al., 2003). Failure to apply a specific viscosity correction result in an incorrect evaluation, and the instrument, interpreting the kinetic slowdown as an effect due to mass rather than medium friction, generates a systematic overestimation of the particles D_h . For this reason, the aim of this study is to verify, using correct measurements of canine plasma density and dynamic viscosity (dynVis), whether the dimensional determination of nanoparticles using ILM is more accurate.

2. Material and Methods

2.1. Samples Management

A total of 79 canine plasma samples were used in the study. Samples were randomly collected from dogs of various ages, sexes, breeds, and health statuses at the Veterinary Teaching Hospital of the Department of Veterinary Medicine and Animal Production, University of Naples “Federico II.” 3 ml of blood sample were drawn from the jugular vein and collected in EDTA-containing tubes. The samples were subsequently centrifuged at 1500 g for 15 minutes at 4°C to obtain the plasma, which was transferred to Eppendorf tubes, stored at -80°C, and sent to the Laboratory of Biophysics at the Faculty of Electrical Engineering, University of Ljubljana, Slovenia, for further analysis. All the analyses were performed for diluted 10x plasma sample, and all the following results are considered for diluted 10x sample.

2.2. Assessment of density and viscosity

Density evaluation was performed using an oscillating U-tube Density Meter (DMA 1002, Anton Par, Graz, Austria). The sample was placed in the measuring cell, and the tube was mechanically oscillated. The oscillation frequency changes according to the internal mass, so this variation allows the density of the sample to be calculated with very high precision. The minimum sample quantity required to perform the analysis is 1 ml. Measurements were performed setting the temperature at 20°C. For the same samples, dynamic viscosity

was measured, using a rolling and falling ball Micro viscometer (Lovis 2000 M, Anton Par, Graz, Austria). A small metal ball rolls and falls inside an inclined glass or quartz measuring capillary tube with a known internal diameter filled with the sample. Gravity causes the ball to move, but the viscosity of the liquid slows it down, and the instrument measures the rolling and falling time of the ball. Capillary Lovis 1.59 Short was used, temperature and measurement angle have been set at 20°C and 45° respectively, and density was entered for each sample.

2.3. Interferometric Light Microscopy (ILM)

For evaluating EPs, samples were processed with ILM using Videodrop (Myriade, Paris, France). All samples were 10 times diluted with saline solution. Measurements were performed at room temperature, recorded at the time of measurement and entered manually for each sample (20-22°C) to achieve the optimal EPs concentration and dimension for detection between 5×10^8 and 5×10^9 particles/ml. A total of 7 μ l of sample was placed between cover glasses and illuminated by a 2 W blue LED light, using a threshold value of 4.2. The average concentration (ILMc) and D_h of EPs were determined for each sample. Particle number density was estimated as the ratio between the number of particles detected and the scanned volume. The D_h was determined by tracking the position of the particle captured in the recorded video. A limit of 20 movies or 300 detected particles was set for each sample. Image and movie processing was performed using the associated software QVIR 2.6.0 (Myriade, Paris, France). The D_h was determined by setting both the dynVis of water, generated automatically by the system (Water viscosity: 0.954 mPas), and the actual dynVis of the sample, which had been measured previously. For statistical purposes, the measurement based on the dynVis of water is defined as "estimated D_h " (eD_h), while the measurement based on the actual dynamic viscosity of the sample is defined as "corrected D_h " (cD_h), and the absolute difference (in nm) and the percentage difference between the two measurements were calculated.

2.4. Statistical Analysis

All data were analysed using descriptive statistics. All measurements were performed in triplicate and are presented as median and interquartile range. Shapiro-Wilk test was used to perform the normality of data. Paired samples T-test was used to highlight differences between eD_h and cD_h , considered statistically significant for a p-value < 0.05. The correlation between variables was performed using Spearman's rank correlation coefficient (ρ). A significant correlation was observed for $p < 0.05$ and classified according to the ρ value as follows: i) $\rho < \pm 0.1$ no correlation; ii) ρ from ± 0.1 to ± 0.3 weak; iii) ρ from ± 0.3 to ± 0.5 moderate; iv) ρ from ± 0.5 to ± 0.7 strong; v) $\rho > \pm 0.7$ very strong. Graphical analysis with Bland-Altman plots were used to show the agreement between variables (Bland & Altman, 1986).

3. Results

3.1 Data distribution and comparisons between methods

The data followed normal distribution, except for the values relating to plasma density and the percentage difference between the methods ($p = 0.001$). A summary of values for eD_h , cD_h , absolute difference in nm, percentage difference, density and dynamic viscosity is shown in **Table 1**. When examining the values obtained from measurements using the method with measured viscosity, an overestimation of particle size by using the data for water was found to range from 1% to 7%. Furthermore, a significant difference was observed between measurements based on the dynamic viscosity of water and those based on specific dynamic viscosity of the samples ($t = 25.985$; $p < 0.001$).

Table 1. Average, Standard Deviation, Minimum, and Maximum Value for estimated and corrected hydrodynamic diameter, absolute and percentage differences between the two measures, and density and dynamic viscosity of samples.

Variable	Average	Standard Deviation	Minimum value	Maximum Value
Estimated D_h	145.06 nm	± 19.40	107.33 nm	194.00 nm
Corrected D_h	139.15 nm	± 18.55	103.74 nm	189.05 nm
Absolute Difference ($cD_h - eD_h$)	-5.90 nm	± 2.02	-11.40 nm	-1.04 nm
Percentage Difference ($cD_h - eD_h$)	-4.13%	± 1.30	-7.00%	-1.00%
Density	1.00693g/cm ³	± 0.00053	1.00499g/cm ³	1.00859g/cm ³
Dynamic Viscosity	0.994 mPas	± 0.012	0.960 mPas	1.026 mPas

D_h : hydrodynamic diameter; nm: nanometres; mPas: millipascal second

3.2 Correlation between measurement differences and the biophysical properties of the sample

To verify if the differences in the observed measurements were due to biological variability in plasma, Spearman's correlation (ρ) was applied. The percentage difference between the two methods of determining particle size showed a moderate and statistically significant negative correlation with plasma density ($\rho = -0.365$, $p < 0.001$) and a very strong and significant negative correlation with dynVis ($\rho = -0.968$, $p < 0.001$). Since percentage difference values are inherently negative, the negative Spearman coefficients demonstrate that the overestimation error introduced by the measurement using water dynamic viscosity increases significantly with increase of the specific density and dynamic viscosity of the sample. Furthermore, the Bland-Altman plot revealed a constant systematic error in the overestimation of the hydrodynamic diameter using the water-dynVis-based method, as can be seen in **Figure 1**. The 95% confidence intervals were wide, ranging from -1.95 nm to -9.85 nm, indicating considerable inter-individual variability in the degree of error.

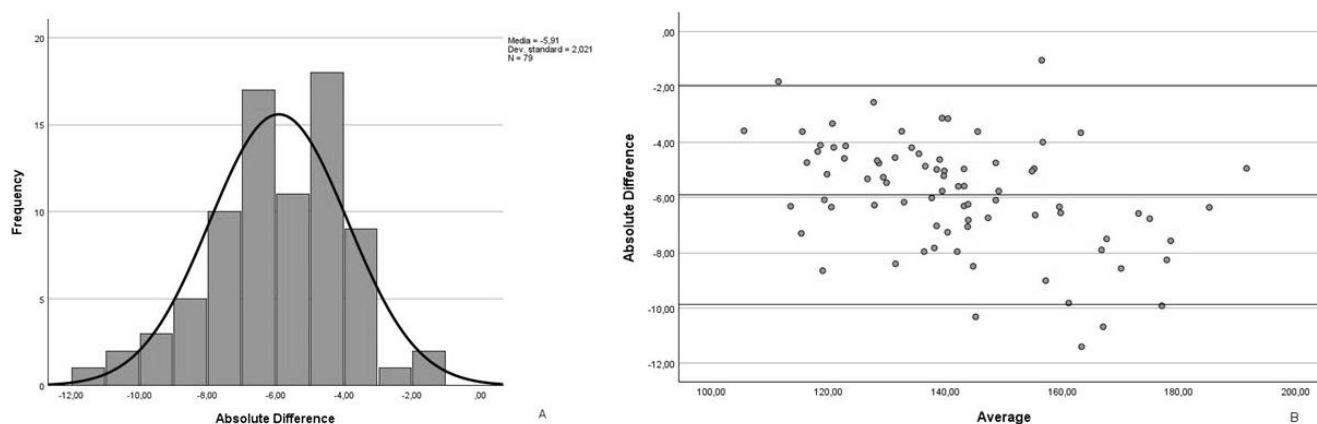


Figure 1. Analysis of the discrepancy between the hydrodynamic diameter measurement using water dynamic viscosity and the corrected measurement using the sample specific dynamic viscosity. (A) The histogram shown in the figure displays the distribution of absolute differences (expressed in nanometres) with a superimposed normal curve; the distribution appears symmetric and centred around a mean bias of -5.91 nm (SD = 2.021). (B) The Bland-Altman plot highlights the agreement between the two methods, showing the presence of a systematic error of overestimation in the measurement based on water viscosity and an error trend proportional to the increase in average particle size.

4. Discussion

The aim of this study was to assess the impact of the biophysical properties of canine plasma (density and viscosity) on the determination of the hydrodynamic diameter of nanoparticles using ILM, by comparing the approach based on the theoretical viscosity of water with a correction protocol which takes into account the actual viscosity of the individual sample. The results clearly show that the uncritical assumption of water viscosity introduces a significant systematic error, leading to a constant overestimation of the actual particle size. This phenomenon is not an artefact, but is governed by precise

physical laws and directly reflects the intrinsic biological variability of the plasma matrix of the samples examined (Boccara et al., 2016). The explanation for this systematic bias, quantified by the Bland-Altman plot as an average error of -5.91 nm, can be found in the theoretical foundations of Brownian motion as tracked individually by the ILM. The instrument calculates the root-mean-square displacement of individual particles based on the variation in their interference pattern over time, and then calculates their hydrodynamic diameter using the Stokes–Einstein equation (Young & Kukura, 2019). In the equation, the hydrodynamic diameter is inversely proportional to the viscosity of the medium. As plasma has a density and viscosity that are inherently higher than those of water—due to the complex suspension of macromolecular proteins such as albumin, globulin and fibrinogen, as well as lipid fractions—the free motion of the particles tracked by ILM is slowed down (Kwaan, 2010). Consequently, if the instrument’s algorithm processes the video tracking on the assumption of water viscosity, it interprets this reduced particle movement as the result of a larger size, leading to an overestimation (Filipe et al., 2010). The strong negative correlation observed between the percentage difference and plasma viscosity provides direct evidence of this mechanism. The negative correlation coefficient demonstrates that, as plasma viscosity and density increase, the magnitude of the error introduced by the method that takes water viscosity into account increases linearly. This finding has crucial biological significance: plasma composition is not constant, but varies drastically depending on age, nutritional status, and the presence of inflammatory, infectious or metabolic conditions (Geyer et al., 2016). Consequently, the wide range of agreement limits shown in the Bland-Altman plot demonstrates that it is impossible to apply a fixed or universal mathematical correction factor to all samples, making it essential to measure viscosity individually in order to obtain the actual particle sizes. Furthermore, an interesting finding is the evidence of a proportional error within the Bland-Altman plot. The distribution of the data points shows a negative slope, indicating that the overestimation caused by the method using water viscosity is not only present but becomes progressively more pronounced as the difference in the average size of the analysed nanoparticles increases.

Although a linear variation in diameter of between 1% and 7% may appear modest at first analysis, its true biological impact becomes clear when the three-dimensional geometry of the particles is considered. As these particles can be approximated as spherical structures, their volume (V) is related to the radius (r) by a cubic relationship ($V = 4/3\pi r^3$). An estimate of the total particle volume that differs by 7% from the actual value is a significant error, because when the cubic effect is taken into account, the percentage error rises to 22.5% (Gardiner et al., 2013).

The inclusion of the room temperature, recorded manually for each sample, is a key factor in the stability of the method. Temperature influences the Stokes-Einstein equation both directly and indirectly through the modulation of viscosity itself, which decreases as temperature rises. Actively tracking the thermal microfluctuations in the laboratory allows the pure effect of the biological matrix on the interferometric measurement to be isolated, eliminating environmental biases and conferring high reproducibility on the proposed method.

It is important to emphasise that the value of performing accurate measurements is not limited exclusively to animal-derived biological matrices, but also applies to the study of plant-derived materials. In recent years, the isolation of natural nanoparticles derived from plant matrices – in particular PEVs or those derived from fruit – has become central to nanomedicine, nutraceuticals and drug delivery, thanks to their biocompatibility and lack of toxicity (Cui et al., 2024). The plant extraction fluids, cell juices or apoplastic fluids from which these nanoparticles are isolated are characterised by a high degree of biochemical complexity. The massive presence of soluble sugars, organic acids, polyphenols and structural polymers such as pectins drastically alters the rheological properties of the medium, resulting in density and viscosity values significantly higher than those of water. The proposed approach therefore constitutes a transferable, cross-disciplinary methodological standard, indispensable for the accurate characterisation of nanoparticles across all fields of application.

5. Conclusions

Data collected demonstrate that the conventional approach based on water viscosity is not biometrically suitable for ILM analysis in canine plasma. The correction method tested offers a more accurate, scientifically sound and clinically necessary solution for standardising the characterisation of nanoparticles derived from different biological matrices.

Funding: This research was supported by European Union's Horizon 2020 research and innovation program FarmEVs under grant agreement No 109338, and by Slovenian Research and Innovation Agency ARIS P3-0388 and J3-60063.

Ethical Statement: The study was approved by the Ethical Animal Care and Use Committee of the University of Naples Federico II (Protocol n. PG/2025/0133615 dated 02/10/2025)

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

References

- Berry M, Arko M, Romolo A, Brložnik M, Mrvar Brečko A, Korenjak B, Iglič A, Kadunc Kos V, Kruljc P, Nemec Svete A, et al. Validation of Interferometric Light Microscopy for Assessment of Extracellular Particles in Diluted Plasma: Preparing the Path for Future Clinical Practices. *Proc. Socrat. Lect.* 10th 2024; 10:54–58. DOI:10.55295/PSL.2024.19
- Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet.* 1986; 1:307–310.
- Boccara M, Fedala Y, Bryan CV, Bailly-Bechet M, Bowler C, Boccara AC. Full-field interferometry for counting and differentiating aquatic biotic nanoparticles: from laboratory to Tara Oceans. *Biomed Opt Express.* 2016; 7:3736–3746. DOI: 10.1364/BOE.7.003736
- Cui L, Perini G, Palmieri V, De Spirito M, Papi M. Plant-Derived Extracellular Vesicles as a Novel Frontier in Cancer Therapeutics. *Nanomater* 2024; 14: 1331. DOI: <https://doi.org/10.3390/nano14161331>
- Filipe V, Hawe A, Jiskoot W. Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharm. Res.* 2010; 27:796–810. DOI: 10.1007/s11095-010-0073-2
- Gardiner C, Ferreira YJ, Dragovic RA, Redman CW, Sargent IL. Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis. *J Extracell Vesicles.* 2013; 2. DOI: 10.3402/jev.v2i0.19671
- Geyer P, Kulak N, Pichler G, Holdt L, Teupser D, Mann M. Plasma Proteome Profiling to Assess Human Health and Disease. *Cell Syst.* 2016; 2:185–195. DOI: 10.1016/j.cels.2016.02.015
- Korenjak B, Tratenšek A, Arko M, Romolo A, Hočevar M, Kisovec M, Berry M, Bedina Zavec A, Drobne D, Vovk T, et al. Assessment of Extracellular Particles Directly in Diluted Plasma and Blood by Interferometric Light Microscopy. A Study of 613 Human and 163 Canine Samples. *Cells* 2024; 13:2054. DOI: <https://doi.org/10.3390/cells13242054>
- Kwaan H. Role of plasma proteins in whole blood viscosity: A brief clinical review. *Clin Hemorheol Microcirc.* 2010; 44:167–176. DOI: 10.3233/CH-2010-1271
- Welsh JA, Goberdhan DCI, O'Driscoll L, Buzas EI, Blenkiron C, Bussolati B, Cai H, Di Vizio D, Driedonks TAP, Erdbrügger U et al. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *J. Extracell. Ves.* 2024; 13:e12404. Doi: 10.1002/jev2.12404
- Wiklander OPB, Brennan MÁ, Lötvall J, Breakefield XO, El Andaloussi S. Advances in therapeutic applications of extracellular vesicles. *Sci Transl Med.* 2019; 11:eaav8521. DOI: 10.1126/scitranslmed.aav8521
- Windberger U, Bartholovitsch A, Plasenzotti R, Korak KJ, Heinze G. Whole blood viscosity, plasma viscosity and erythrocyte aggregation in nine mammalian species: reference values and comparison of data. *Exp Physiol.* 2003; 88:431–40. DOI: 10.1113/eph8802496
- Young G, Kukura P. Interferometric Scattering Microscopy. *Annu Rev Phys Chem.* 2019; 70:301–322. DOI: 10.1146/annurev-physchem-050317-021247