Detection of extracellular vesicles: size does matter
van der Pol, E.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Discussion and outlook

The topic of this thesis is the detection of extracellular vesicles as biomarkers for disease. To give an example of biomarkers, Table 10.1 shows normal hematology values of blood cells. Such tables are used in hospitals to recognize abnormal values and diagnose patients. In the future, extracellular vesicles will become part of hematology tables, as their concentration correlate with disease. For this future to materialize, vesicle science will need a solid foundation. Prerequisites for the development of vesicle biomarkers are: (1) knowledge of physical properties of vesicles, (2) insight into capabilities and limitations of detection techniques, (3) availability of techniques with the capability of deriving the cellular origin and function of vesicles and with improved sensitivity compared to current state-of-art technology, and (4) standardization of measurements.

10.1 Impact of the physical properties of vesicles on their detection

From a biological and clinical perspective, the most relevant properties of extracellular vesicles are their cellular origin and function (Chapter 2). However, the cellular origin can only be measured indirectly, whereas a functional test, such as the fibrin generation test, requires isolation of numerous vesicles. Physical detectable properties of vesicles are size, concentration, refractive index, composition (e.g. lipids, DNA, RNA, antigens, and other proteins), morphology, density, and zeta potential (Chapter 3). From these properties the cellular origin of vesicles may be deduced. In this thesis we focus primarily on the size, concentration, and RI of vesicles, since these three properties play a key role in vesicle detection.

Table 10.1: Normal hematology values of the most abundant blood cells in male adults [4].

<table>
<thead>
<tr>
<th>Hematology parameter</th>
<th>Concentration (cells mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count</td>
<td>$1.50 - 4.00 \cdot 10^8$</td>
</tr>
<tr>
<td>Erythrocyte count</td>
<td>$4.50 - 5.50 \cdot 10^9$</td>
</tr>
<tr>
<td>Reticulocyte count</td>
<td>$2.5 - 10.0 \cdot 10^7$</td>
</tr>
<tr>
<td>Leukocyte count</td>
<td>$4.0 - 10.5 \cdot 10^6$</td>
</tr>
<tr>
<td>Total cell count</td>
<td>$4.7 - 6.0 \cdot 10^9$</td>
</tr>
</tbody>
</table>
Chapter 10. Discussion and outlook

10.1.1 Particle size distribution and concentration

Similar to others, we show that vesicles are heterogeneous in size and that their diameter ranges from 30 nm to 1 µm. We found concentrations in the order of $10^{10}$ vesicles mL$^{-1}$ in urine (diameter 70–800 nm, Chapter 4). We demonstrate that the concentration of vesicles decreases with increasing diameter, and that this relationship can be accurately described by the power-law function. From tunable resistive pulse sensing (TRPS) and nanoparticle tracking analysis (NTA) measurements it follows that > 80% of the vesicles is < 100 nm in diameter. At a concentration of $10^{10}$ vesicles mL$^{-1}$, the total volume of vesicles is approximately 85-fold less than of all leukocytes in 1 mL of blood, whereas the total surface area is comparable. We speculate that the number of receptors exposed on a vesicle is proportional to the surface area, whereas the amount of cargo a vesicle can transport is proportional to the volume. Given the equal total surface area and small total volume of vesicles relative to leukocytes, we hypothesize that surface receptors are important candidates to reveal the cellular origin and potential functions of vesicles.

Several techniques, such as dynamic light scattering and small-angle X-ray scattering, measure scattering from multiple vesicles simultaneously. Due to the broad particle size distribution (PSD) of vesicles, these techniques are prone to artifacts, since scattering is dominated by the presence of a few larger vesicles (Fig. 3.1E). In addition, the distinction between coexisting vesicle types is difficult if not impossible with such bulk measurements (Chapter 2). To resolve coexisting vesicle types, single vesicle detection is preferred.

Flow cytometers detect single particles, but given their limited sensitivity and the power-law distribution of the vesicle PSD, the majority of vesicles are below the detection limit of common flow cytometers. However, due to the high vesicle concentration, multiple vesicles are simultaneously illuminated by the laser beam and erroneously counted as a single event signal (Chapter 3). We christened this phenomenon swarm detection to distinguish it from coincidence detection. With swarm detection, tens or hundreds of vesicles are continuously present in the detection volume, while coincidence detection refers to the occasional presence of two cells in the detection volume.

The most important implication of the power-law function is the strong dependence of the detected vesicle concentration on the minimum detectable vesicle size (Chapter 4). For example, a decrease in the minimum detectable vesicle size of only 20 nm may result in a 2.4-fold increase in the obtained vesicle concentration (Fig. 4.1). This finding emphasizes the need to quantify and monitor the minimum detectable vesicle size. For four techniques we have determined the minimum detectable vesicle size, which is 70–90 nm for nanoparticle tracking analysis (NTA), 70–100 nm for TRPS, 150–190 nm for a flow cytometer dedicated to the detection of submicrometer particles, and 270–600 nm for conventional flow cytometry. These differences in minimum detectable vesicle size contribute for > 60% to the 300-fold difference in concentration between these techniques (Appendix B). In addition, the differences in the minimum detectable vesicle size explains why
the reported concentrations of vesicles in human plasma from healthy individuals range from $10^4$ to $10^{12}$ vesicles mL$^{-1}$ \cite{37, 314, 90, 833}. Thus, the vesicle size is not only important to distinguish vesicle types (Table 2.1), but is also an important quality parameter of vesicle concentration measurements. From now on, any reported vesicle concentration should include the detected size range.

10.1.2 Refractive index

The refractive index (RI) determines how efficiently a vesicle scatters light, which is essential to data interpretation and comparison. For urinary vesicles we obtained a mean RI of 1.37 at 405 nm, with an RI below 1.40 for 95% of vesicles (Chapter 6). This RI is much lower than the frequently and often unintentionally assumed values between 1.45 and 1.63 \cite{178, 311, 251}. To illustrate the optical impact of this finding: a 100 nm vesicle (RI=1.37) scatters approximately 90-fold less light than a similar-sized polystyrene bead (RI=1.63). The low RI of vesicles relative to water (1.34 at 405 nm) is consistent with their structure and falls within the range of estimates \cite{233, 252, 55} based on the RI of cells \cite{307, 31, 102, 47}. Due to the low RI, a 30 nm vesicle with a physical cross section of 700 nm$^2$ has an optical scattering cross section of only 0.02 nm$^2$. Needless to say, detecting scattering from vesicles demands a sensitive detector.

We used the vesicle RI to establish the relationship between the vesicle diameter and light scattering measured by flow cytometry, which is presently the most frequently applied technique for single vesicle detection. We demonstrated that the gating strategy proposed by the Scientific Standardization Committee (SSC) collaborative workshop of the International Society on Thrombosis and Haemostasis (ISTH) selects single vesicles and cells with diameters ranging from 800–2,400 nm instead of the envisioned 500–900 nm (Chapter 5). Thus, for over a decade, we and others have unintentionally studied particles with diameters $> 800$ nm. Our finding and recent cryo-electron microscopy data suggest that these particles are empty blood cells instead of extracellular vesicles \cite{19}.

10.1.3 Applicability to other vesicle samples

We have developed a toolbox to determine vesicle properties, which we applied to urinary vesicles, since urine contains a relatively high concentration of vesicles with low contamination of similar-sized non cell-derived particles, such as lipoproteins. In other samples, we have found vesicle properties comparable to urinary vesicles. For example, the power-law function was also applicable to fit the PSD of vesicles from plasma (Fig. B.2), blood bank concentrates, and cultured cell medium (data not shown). Nevertheless, a rigorous determination of vesicle properties in other samples is still needed but challenging, since samples like plasma contain different vesicle types and similar-sized non cell-derived particles. Tools that can determine the vesicle size and concentration, as well as identify different vesicles types and distinguish vesicles from other particles need to be developed.
10.2 Outlook

In this section, the future of vesicle detection will be discussed based on the physical properties of vesicles and insight into capabilities and limitations of detection techniques.

10.2.1 Novel detection techniques

Requirements

A biomarker based on vesicle enumeration should determine the concentration of a specific vesicle type within minutes. To realize this, detection techniques need to become sufficiently sensitive to identify the cellular origin of single vesicles with diameters of 50 nm and larger. In addition, detection techniques need to be able to distinguish vesicles from impurities. To provide valuable information on the vesicle concentration, the detection volume and minimum detectable vesicle size need to be quantified. Ultimately, single vesicles are sorted to perform functional tests on subpopulations of vesicles.

Cellular origin of vesicles

Current techniques will soon meet the requirements to determine the vesicle size and concentration. Therefore, the next step is to determine the cellular origin of single vesicles. The origin of vesicles could be derived by detecting their antigen expression, for example, erythrocyte vesicles with glycophorin A (CD235), platelet vesicles with glycoprotein IIIa (CD61), or tumor vesicles with Epithelial cell adhesion molecule (EpCAM; CD326).

The number of antigens per vesicle is unknown. We can provide an initial estimate by assuming that the antigen density in the membrane of a vesicle is similar to the density in the cell membrane. In this case, a 100 nm vesicle would have 300 glycophorin A molecules (erythrocyte [81]), 100 CD61 molecules (platelet [226]), or 0–20 EpCAM molecules (T24 and SKBR3 breast cancer cell lines [79]). For comparison, a 2 µm platelet typically has 50,000 CD61 molecules. Hence, the fluorescent signals from labeled vesicles are weak. A vesicle detection technique will require quantification of the minimum detectable number of dye molecules, and determination of the efficiency with which these few receptors are labeled. Potentially, labeling efficiency can be studied by optically trapping single vesicles in the evanescent field of an optical resonator (Optofluidics, Philadelphia, PA, USA) and imaging fluorescently labeled receptors with stimulated emission depletion microscopy.

A technology leap is still needed before current immunofluorescence techniques are sufficiently sensitive to differentiate between our hypothetical 100 nm vesicles. Alternative to the antigen based identification, we could pursue vesicle differentiation based on “bulk” chemical composition measured by Raman microspectroscopy, or differentiate based on morphology.
10.2. Outlook

Resistive pulse sensing

Raman microspectroscopy

Figure 10.1: Schematic representation of hybrid resistive pulse sensing - Raman microspectroscopy. Resistive pulse sensing consists of two fluid cells divided by a non-conductive membrane (blue horizontal line). A voltage is applied by two electrodes (yellow) to flow an electrical current ($I$) through the pore. When a vesicle (green dots) passes through the pore, this will result in an increase of the pore resistance and a decrease of the measured current. The change in current can be related to the vesicle size. After passing through the pore, the vesicle will be optically trapped by a focused laser beam to measure the elastic scattering intensity and Raman spectrum of the vesicle.

ExoFlow

The exosome flow cytometer, or “ExoFlow”, is a flow cytometer tailored to detect and characterize sub-micrometer vesicles. Due to the capability of measuring size, concentration, and multiple fluorescence signals on thousands of vesicles per second, flow cytometry is the workhorse for single vesicle detection. However, (1) size determination of vesicles by flow cytometry is inaccurate and imprecise because the vesicle RI is heterogeneous, (2) the presence of impurities reduces specificity of measurements, and (3) the sensitivity to detect vesicles < 150 - 190 nm is lacking. To overcome these problems we will integrate TRPS into the flow cell of the ExoFlow and improve the scatter sensitivity. We will use the combination of vesicle sizing by TRPS and the improved scatter sensitivity to determine the RI of all detected particles in the sample. Since the RI of urinary and plasma vesicles is 1.37±0.03 (Chapter 6) and 1.39±0.04 [169], respectively, we expect that this RI determination can be used to distinguish vesicles from protein aggregates (RI between 1.45 - 1.60) [328] and bulk proteins (RI between 1.61±0.02) [199].

Hybrid resistive pulse sensing - Raman microspectroscopy

TRPS can determine the size, concentration, and zeta potential of vesicles > 70 nm in suspension (Chapters 4 and 7). While these parameters are valuable, it is unlikely that the cellular origin of a vesicle can be deduced from its size and zeta potential. This can be overcome by complementing TRPS with Raman microspectroscopy. Raman microspectroscopy can provide chemical characteristics of single vesicles [9], such as the presence of proteins, lipids, organic compounds, and genetic content, which may identify the parent cell (Chapter 9).
Chapter 10. Discussion and outlook

Fig. 10.1 shows a schematic representation of the hybrid resistive pulse sensing - Raman microspectroscopy system. A vesicle is driven through the TRPS nanopore by electrophoresis to determine its diameter and zeta potential. Next, the vesicle is trapped by a focused laser beam, and its backscattering and Raman signature is probed. The backscattering and size of the vesicle are used to determine the vesicle RI from Mie theory.

We are in the process of constructing this setup using a customized TRPS device (qNano, Izon, Oxford, UK) and a custom-built Raman microspectrometer. The spectrometer has a spectral range of $-27$ to $3,490 \text{ cm}^{-1}$ and a spectral resolution of $4.0 \text{ cm}^{-1}$, which enables the identification of spectral features characteristic of phospholipid vesicles [63, 285]. After the Raman peaks that are characteristic of the origin of vesicles are known, coherent anti-Stokes Raman scattering (CARS) can be applied to increase measurement speed.

**Blinking tubes measured with NTA**

A study of plasma vesicles by cryo-electron microscopy [19, 143, 332] demonstrated the existence of a vesicle sub-population ($<5\%$ of total) that is not round but tubular. The tubular morphology may contain information on the cellular origin of these vesicles. Since the scattering cross section of tubular particles depends on the particle orientation, and these vesicles will rotate freely in suspension, the scatter signal from these tubular vesicles will appear to blink when observed by NTA [54].

### 10.2.2 Standardization

In addition to novel detection techniques, clinical studies on vesicles require data comparison. Currently, data comparison on vesicles in clinical samples is hampered by the application of varying vesicle isolation procedures and different instruments. Since the minimum detectable vesicle size and thus the detected vesicle concentration is instrument dependent, there is a high demand for standardization of vesicle measurements.

**Flow cytometry standardization through vesicle size approximation**

We are developing a standardization procedure to improve the reproducibility of the measured concentration of vesicles by flow cytometry. Without standardization, the reproducibility of vesicles concentration measurements has an $80\%$ coefficient of variation (CV). The proposed standardization strategy by the SSC collaborative workshop of the ISTH improved the reproducibility to a CV of $60\%$, but excluded the worst performing instruments. The main reasons for the poor performance of current standardization strategies are that differences between optical configurations of flow cytometers and differences between the RI of vesicles and beads are not taken into account.

Our solution encompasses a mixture of traceable reference beads [www.metves.eu](http://www.metves.eu) and software [www.exometry.com](http://www.exometry.com). The reference beads need to be measured...
10.2. Outlook

Figure 10.2: Screenshot of the software developed for flow cytometry standardization through vesicle size approximation.
Table 10.2: Fictive hematology values of the most abundant extracellular vesicles in male adults.

<table>
<thead>
<tr>
<th>Hematology parameter</th>
<th>Concentration (vesicles mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet vesicle count</td>
<td>$2.3 - 6.2 \cdot 10^9$</td>
</tr>
<tr>
<td>Erythrocyte vesicle count</td>
<td>$7.0 - 8.6 \cdot 10^{10}$</td>
</tr>
<tr>
<td>Reticulocyte vesicle count</td>
<td>$3.9 - 15.6 \cdot 10^8$</td>
</tr>
<tr>
<td>Leukocyte vesicle count</td>
<td>$6.2 - 16.4 \cdot 10^7$</td>
</tr>
<tr>
<td>Total vesicle count</td>
<td>$7.3 - 9.4 \cdot 10^{10}$</td>
</tr>
</tbody>
</table>

once a day and are automatically recognized by the software. The software establishes the relationship between particle diameter, RI and light scattering, which is specific for the optical configuration of the flow cytometer. Next, the software provides a vesicle size gate, which the user can apply to its own data analysis application to determine the concentration of vesicles within a predefined size range. Fig. 10.2 shows a screenshot of the software.

The standardization project is sponsored by the SSC of ISTH and involves 62 different flow cytometers in 32 clinical laboratories worldwide. The final results are expected at the ISTH 2015 congress.

10.2.3 Vesicle-based diagnostics have high potential

Our ambition is to add vesicles to the reference hematology values of blood cells in Table 10.1. An important question is how much sample would need to be analyzed to perform a vesicle-based hematology test. To make a first assessment, we assumed that the relative frequency of hematological vesicles is the same as the relative frequency of their cells of origin. Combined with a total concentration of $7 \cdot 10^{10}$ vesicles per mL blood, this resulted in the fictive hematology values of blood vesicles in Table 10.2. To count 1,000 vesicles (Poisson error $\leq 3\%$) of the least frequent vesicle type, we would need to analyze 100 nL blood and thus count 7.3 million vesicles. This sample volume can be perfectly handled by microfluidic chips, which may shift our single step isolation of vesicles from plasma (Chapter 8) to single step isolation of vesicles from whole blood [76], thereby solving several pre-analytical issues [331]. A major advantage of the vesicle hematology test would be the small sample volume. The small sample volume would lighten the phlebotomy burden on sensitive patient groups, such as neonates [44], and in addition, it might enable continuous monitoring of hematology values.

Other rare vesicles of clinical interest are tumor-derived vesicles. For prostate cancer, the median concentration of circulating tumor cells (CTCs) is $7$ per $7.5$ mL blood. Under the assumption that CTCs have a diameter of $30 \mu$m [73] and break up in vesicles with a PSD that resembles the power-law distribution, the concentration of tumor-derived vesicles is $\sim 10^{7}$ mL$^{-1}$. Blood of these patients contained 250 tumor microparticles with a diameter of $2-4 \mu$m per $7.5$ mL [73]. Extrapolating the power-law PSD from $2-4 \mu$m particles to tumor-derived vesicles $< 1 \mu$m results in a concentration of $\sim 10^{8}$ mL$^{-1}$. Although these numbers warrant further
investigation, they are in the same order of magnitude as the estimated leukocyte-derived vesicles in Table 10.1.

In conclusion, we have obtained a solid insight into the physical properties of vesicles and the capabilities of current detection techniques. We are developing techniques that are capable of deriving the cellular origin and function of single vesicles. These techniques will have improved sensitivity compared to current state-of-art. Standardization and rigorous characterization of the measurement techniques are essential. These are important steps towards vesicle-based diagnostics, allowing us to live healthier ever after.