Detection of extracellular vesicles: size does matter
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CHAPTER 3

Methods for detection and characterization of extracellular vesicles

Abstract

Microparticles and exosomes are cell-derived vesicles present in body fluids that play a role in coagulation, inflammation, cellular homeostasis and survival, intercellular communication, and transport. Despite increasing scientific and clinical interest, no standard procedures are available for the isolation, detection and characterization of microvesicles and exosomes, because their size is below the reach of conventional detection methods. Our objective is to give an overview of currently available and potentially applicable methods for optical and non-optical determination of the size, concentration, morphology, biochemical composition and cellular origin of microvesicles and exosomes. The working principle of all methods is briefly discussed, as well as their applications and limitations based on the underlying physical parameters of the technique. For most methods, the expected size distribution for a given vesicle population is determined. The explanations of the physical background and the outcomes of our calculations provide insights into the capabilities of each method and make a comparison possible between the discussed methods. In conclusion, several (combinations of) methods can detect clinically relevant properties of microvesicles and exosomes. These methods should be further explored and validated by comparing measurement results so that accurate, reliable and fast solutions come within reach.

This chapter has been published as:
3.1 Introduction

Cells release vesicles that function as vehicles for the transport and delivery of cargo between cells [268, 215]. In addition, vesicles promote coagulation and inflammation. Throughout this chapter, we will use “vesicles” as a generic term for all types of cell-derived extracellular vesicle, unless stated otherwise. Although the clinical interest and relevance of vesicles is increasingly recognized [244], their isolation and detection is still cumbersome [157]. At present, novel detection methods are being explored [333, 183, 159, 225, 338]. This chapter is an assessment of the accuracy and practicability of methods for the detection of vesicles.

3.1.1 Microparticles and exosomes

The best studied types of vesicle are exosomes and microvesicles. Although a generally accepted definition is lacking [74, 50], there are several features characterizing exosomes and microvesicles. Exosomes are released from cells containing multivesicular bodies when the membranes of multivesicular bodies fuse with the plasma membrane. By transmission electron microscopy (TEM), exosomes appear with characteristic doughnut morphology, and their diameter ranges between 30 and 100 nm [69]. Their density ranges from 1.13 to 1.19 g mL$^{-1}$ [292], and proteomes contain characteristic but not unique protein families, including heat shock proteins and tetraspannins [295]. The main function of exosomes is to modulate the immune response [291].

Microparticles are released from the plasma membrane during “budding” or “shedding”. Most, if not all, eukaryotic cells release microvesicles, especially during conditions related to stress, such as activation and apoptosis [106]. Microparticles are larger and more heterogeneous in morphology than exosomes, with reported diameters ranging between 100 nm and 1 µm [66]. Microparticles are best known for binding coagulation factors and exposing tissue factor [269, 213, 112]. Their absence is associated with a bleeding tendency [270], and their (increased) presence is associated with disseminated intravascular coagulation and thrombosis [212, 37]. There is increasing evidence that exosomes and microvesicles are “multipurpose carriers” facilitating the intercellular exchange of transmembrane receptors, mRNA, microRNA, and signaling molecules [304, 83, 13]. Furthermore, they promote cellular survival by removing dangerous or redundant intracellular compounds [109, 7, 256].

3.1.2 Vesicle isolation, detection, and characterization

Currently, progress in vesicle research is hampered by several factors. Because of the biological complexity of body fluids, isolation of vesicles has proven to be extremely difficult. For instance, isolation of vesicles from blood is affected by venepuncture, time between blood collection and handling, the anticoagulant, centrifugation and washing procedures, the presence of lipoprotein particles and small platelets within the size range of vesicles, the high viscosity of blood, and the pres-
ence of sticky proteins, including fibrinogen and albumin. Because of their small size, vesicles are below the detection range of conventional detection methods. As a consequence, recovery and contamination cannot be reliably quantified, and isolation protocols have not been standardized. For example, conflicting results were reported on the procoagulant properties of vesicles from sickle cell disease patients [27, 265]. Both studies attributed the procoagulant features to “microvesicles”, but they used markedly different isolation protocols, involving centrifugation at 18,890 g [27] or 100,000 g [265], presumed to result in isolation of microvesicles or microvesicles and exosomes, respectively.

### 3.1.3 Clinically relevant properties of vesicles

In this chapter, currently available and potentially applicable methods for the detection and characterization of vesicles are presented. Clinically relevant properties of vesicles are size, concentration, morphology, biochemical composition, and cellular origin. From the size information of individual vesicles, a relative size distribution can be obtained, providing insights into the number of vesicles of one particular size relative to those of another size. We define concentration as the number of vesicles per unit volume. If both the relative size distribution and concentration are known, an absolute size distribution can be obtained, which gives the number of vesicles of one particular size per unit volume. By morphology, we mean shape and ultrastructure. Ultrastructure is illustrated in Fig. 3.1A, where vesicles differ not only in shape but also in contrast and surface pattern. The biochemical composition refers to the biological and chemical components of which vesicles are composed. The cellular origin refers to the cell type from which the vesicles originate.

### 3.1.4 Standard population and outline

For each detection method, the working principle is briefly explained and the measurement time is estimated, assuming the detection of 10,000 particles, a number that is common in flow cytometry. In addition, we give a prediction of the performance of each method in detecting size, concentration, morphology, biochemical composition, and cellular origin, by considering the underlying physical parameters of the methods. To compare the performance of the methods for size detection, we made a model predicting the size distribution for a given population of vesicles (see Appendix A). As outlined previously, isolation of vesicles from blood is a challenge. Therefore, we arbitrarily chose vesicles from urine to create a standard population as a realistic input for our model. Urine can be used to prepare a relatively high concentration of vesicles without excessive contamination with, for example, platelets or proteins.

To create the standard population, we isolated vesicles from fresh cell-free urine of a healthy male individual by highspeed centrifugation (Fig. 3.1A; 30 min at 18,900 g), followed by ultracentrifugation of the supernatant (Fig. 3.1B; 1 h at 154,000 g). We imaged vesicles by TEM and measured the diameter of 500
Figure 3.1: Transmission electron microscopy (TEM) of vesicles from fresh cell-free human urine. (A) vesicles isolated from cell-free human urine by centrifugation (30 min at 18,900 g). (B) vesicles isolated from microparticle-depleted urine by ultracentrifugation (1 h at 154,000 g). (C) Concentration vs. diameter for vesicles as measured by TEM, and referred to as the standard population. The plot shows a broad distribution between 20 and 440 nm, with a single peak at 45 nm. (D) Scattering cross-section vs. diameter (logarithmic scale) for vesicles, calculated using Mie theory of a sphere ($n_p = 1.38$) surrounded by a membrane (10 nm; $n_s = 1.48$). The medium is water ($n_m = 1.33$) and the wavelength of the laser is 532 nm. The scattering cross-section, and thus the quantity of light scattered by a vesicle, strongly decreases with decreasing diameter. (continued)
3.2 Optical methods

Figure 3.1: (E) Scattering coefficient vs. diameter (logarithmic scale) for the standard population. The scattering coefficient, which is the average number of scattering events that light encounters per unit length, is given by the product of the concentration of the standard population and the scattering cross-section. The scattering coefficient strongly increases with increasing diameter, indicating that the contribution of light scattered by vesicles smaller than 100 nm is relatively small.

vesicles in each fraction. The combined size distributions are shown in Fig. 3.1C. As different size distributions of vesicles in blood have been reported [333, 183, 267, 251], it is difficult to compare our standard population with its plasma counterpart. Nevertheless, our standard population corresponds well with recent data on the size distribution of plasma vesicles [333]. One has to bear in mind that the reported absolute size distributions are affected by isolation procedures. In the literature, vesicle concentrations in plasma range from $10^7$ to $10^{12}$ L$^{-1}$ [333, 338, 251, 261, 211, 124]. As our simulations demand an absolute size distribution as input, we arbitrarily multiplied our relative vesicle size distribution by $10^9$ L$^{-1}$, as this concentration is usually reported in plasma.

The outline of this chapter is as follows. The first part describes optical detection, and is subdivided into methods based on light scattering or fluorescence. The second part describes non-optical detection methods. Table 3.1 provides an overview of all detection methods.

3.2 Optical methods

Optical methods have the potential to accurately obtain all clinically relevant properties of single vesicles at a high speed. Two important parameters in optics are the wavelength of light and the refractive index of particles relative to the suspending medium. Optical phenomena, including reflection and refraction, depend on the refractive index $n$ of the material. The refractive index depends on the wavelength $\lambda$, and is defined as the ratio of the speed of light in vacuum to that in the material. In practice, the higher the difference between the refractive index of a vesicle and its surroundings, the more light will be scattered.

3.2.1 Light scattering

Light that illuminates a vesicle is partly absorbed and partly scattered. As many optical setups are based on the detection of scattered light, it is important to know how much light is scattered by a single vesicle. The quantity of light scattered by a single vesicle is proportional to the scattering cross-section $\sigma$. When the diameter
### Table 3.1: Assessed capabilities of (potential) methods for the detection of microvesicles, based on the underlying physical parameters

<table>
<thead>
<tr>
<th>Method</th>
<th>Resolution (nm)</th>
<th>Detection limit (nm)</th>
<th>Size Distribution Requirements</th>
<th>Assumptions/Information demands</th>
<th>Method Specific Assumptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical methods</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scattering</td>
<td>200</td>
<td>&gt;10 nm</td>
<td>−</td>
<td>+</td>
<td>Calibration with beads</td>
</tr>
<tr>
<td>Optical microscopy</td>
<td>≥300 nm</td>
<td>1 mm</td>
<td>+</td>
<td>−</td>
<td>Calibration with beads</td>
</tr>
<tr>
<td>Scattering flow cytometry</td>
<td>1 nm to 6 µm</td>
<td>+/−</td>
<td>T, η, n, Vd, model</td>
<td>+</td>
<td>Calibration with beads</td>
</tr>
<tr>
<td>Raman spectroscopy</td>
<td>350</td>
<td>To be investigated</td>
<td>+</td>
<td>−</td>
<td>Calibration with beads</td>
</tr>
<tr>
<td>Fluorescence microscopy</td>
<td>300</td>
<td>200 nm</td>
<td>Single QD</td>
<td>+</td>
<td>Calibration with beads</td>
</tr>
<tr>
<td>Fluorescence microscopy</td>
<td>600</td>
<td>500 nm</td>
<td>Single QD, Cd</td>
<td>+</td>
<td>Calibration with beads</td>
</tr>
<tr>
<td>Non-optical methods</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-ray microscopy</td>
<td>12 ++</td>
<td>&gt;100 µm</td>
<td>No shrinkage</td>
<td>+</td>
<td>I, λ, νc</td>
</tr>
<tr>
<td>TEM</td>
<td>∼1</td>
<td>~1 nm</td>
<td>+</td>
<td>−</td>
<td>T, η, n, Vd, model</td>
</tr>
<tr>
<td>AFM</td>
<td>1 µm</td>
<td>1 nm</td>
<td>+</td>
<td>−</td>
<td>T, η, n, Vd, model</td>
</tr>
<tr>
<td>STED microscopy</td>
<td>600</td>
<td>200 nm</td>
<td>+</td>
<td>+</td>
<td>Calibration with beads</td>
</tr>
<tr>
<td>Optical microscopies</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confocal microscopy</td>
<td>600</td>
<td>200 nm</td>
<td>+</td>
<td>+</td>
<td>Calibration with beads</td>
</tr>
<tr>
<td>Digital microscopy</td>
<td>12</td>
<td>10 nm</td>
<td>+</td>
<td>+</td>
<td>Calibration with beads</td>
</tr>
</tbody>
</table>

Abbr. abbreviations and symbols are clarified on page 35.

Chapter 3: Methods for detection and characterization of extracellular vesicles.
3.2. Optical methods

AFM, atomic force microscopy; DLS, dynamic light scattering; F-CS, fluorescence correlation spectroscopy; F-NTA, fluorescence nanoparticle tracking analysis; NTA, nanoparticle tracking analysis; QD, quantum dot; STED, stimulated emission depletion; TEM, transmission electron microscopy. For each method, the resolution, detection limit, ability to measure the size distribution and concentration, ability to provide biochemical information, and the measurement time are estimated. Requirements of the method and/or assumptions that have to be made to determine the size distribution and concentration are also listed. \( d \) is the microvesicle diameter, \( d_c \) is the channel diameter, \( l_c \) is the channel length, \( \eta \) is the viscosity of the solvent, \( n_v \) is the refractive index of the vesicle, \( n_m \) is the refractive index of the medium, \( Q \) is the flow rate, \( T \) is the temperature of the solvent, \( V_d \) is the detection volume, and \( \nu_{av} \) is the average particle transport velocity. A method that is incapable, capable but with limitations, or capable of providing information on the size distribution, particle concentration or biochemical composition is indicated by \(-\), \(+/-\), and \(+\), respectively. The measurement time is indicated by S, M, and H, which mean shorter than 1 min, between 1 min and 1 h, and longer than 1 h, respectively. Assumptions which are not explained in this chapter are provided in Appendix A.

is at least 10 times smaller than the wavelength, the Rayleigh approximation can be applied to calculate the scattering cross-section,

\[
\sigma \propto \frac{d^6}{\lambda^4} \left( \frac{m^2 - 1}{m^2 + 2} \right)^2
\]  

(3.1)

where \( \propto \) denotes “proportional to”, \( d \) is the particle diameter, and \( m = n_v/n_m \) is the refractive index ratio of the vesicle and the medium \[140\]. At a wavelength of 532 nm, which is commonly used in optical devices, the Rayleigh approximation can be applied to particles of \( 532/10 \approx 50 \text{ nm} \) and smaller, which is typically the size of the smallest exosomes. From Eqn 1, it follows that if a vesicle is only 10-fold smaller than another vesicle, the scattering cross-section and thus the scattered amount of light decreases \( 10^6 \)-fold.

Mie theory provides exact predictions of the absorption and scattering of light from spheres with arbitrary diameter and refractive index \[40\]. The solid line in Fig. 3.1D shows the scattering cross-section vs. the diameter for a sphere that contains a high refractive index shell, for example a phospholipid membrane, as calculated with Mie theory. The calculation parameters are chosen to be as realistic as possible for the case of vesicles. Fig. 3.1D, in a semi-logarithmic representation, shows that the scattering cross-section drops rapidly for smaller vesicles. To illustrate how this decrease affects light scattered from all vesicles of the standard population, the concentration (Fig. 3.1C) is multiplied by the scattering cross-section (Fig. 3.1D) to obtain the scattering coefficient per diameter (Fig. 3.1E). The scattering coefficient, depicting the mean number of scattering events of the light per unit length, is a measure of the amount of light scattered by all vesicles per diameter. Please note that the contribution of light scattered by vesicles smaller than 100 nm is surprisingly small (Fig. 3.1E), given their high concentration (Fig. 3.1C). Consequently, smaller vesicles require more sensitive
optical detection than larger vesicles, and scattering of small particles can easily be overwhelmed by scattering of large particles.

**Optical microscopy**

In a bright-field optical microscope, the sample is illuminated by visible light. Scattered light from the sample is collected by a microscope objective and focused on a charge-coupled device (CCD) camera. The resolution is the shortest distance between two adjacent points that can be distinguished by an optical microscope. The best achievable resolution $R$ is given by the Rayleigh criterion,

$$R = \frac{1.22\lambda}{2NA}$$

where NA is the numerical aperture of the microscope objective. NA characterizes the range of angles over which the microscope objective accepts light. Oil-immersion microscope objectives have an NA up to 1.4. Assuming a wavelength of 532 nm, the best resolution of a standard optical microscope is approximately 200 nm. So, it is impossible to measure the size and morphology of vesicles smaller than 200 nm by optical imaging. Despite this limitation, gold particles down to 10 nm in diameter have been detected, because gold particles scatter light very efficiently [87]. They appear as bright spots, but their true particle size is hard to determine by optical microscopy. For vesicles with light scattering higher than the detection limit, an estimation of the concentration can be made from the count of the number of scatter events if the detection volume $V_d$ is known. The time needed to measure 10,000 vesicles with a standard optical microscope is in the order of hours, and no information on the biochemical composition or cellular origin is provided.

**Scattering flow cytometry**

Flow cytometry is well known for counting and separating single cells (diameter $>1 \mu m$) in fluids at a rate of thousands per second. Most flow cytometers can detect scattered light and fluorescence. In this section, we consider only light scattering.

A flow cytometer guides cells and vesicles through a laser beam in a hydrodynamically focused fluid stream. One detector is placed in line with the laser beam and measures the forward scattered light (FSC). A second detector measures the side scattered light (SSC) perpendicular to the beam. From light-scattering theory, the following approximate results can be expected. Particles larger than the wavelength of light, such as cells, predominantly scatter light in a forward direction. Hence, FSC is associated with particle size. Particles smaller than the wavelength, such as organelles, scatter relatively more light in a perpendicular direction, so SSC is associated with the complex anatomy of cells. In reality, however, light scattering is a complex process. Therefore, light scattering of biological particles is an active research field [134, 135].
3.2. Optical methods

Figure 3.2: Calculated size distribution for optical detection methods (open black) relative to the standard population (transparent red), based on the underlying physical parameters of each method. (A) Scattering flow cytometry determines concentration vs. diameter. Vesicles larger than 300 nm in diameter are detected, but vesicles smaller than 300 nm are detected with low efficiency. (B) Dynamic light scattering (DLS) determines the relative size distribution. The distribution is normalized to 1, and shows a shift to larger diameters. (C) Nanoparticle tracking analysis (NTA) determines the absolute size distribution with high precision for vesicles larger than 100 nm, but is currently not sensitive enough to detect vesicles smaller than 50 nm. (continued)
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Figure 3.2: (D) Stimulated emission depletion (STED) microscopy determines counts vs. diameter. The distribution is normalized to a total count of 10,000 vesicles, and shows a high correlation with the standard population. (E, F) Both fluorescence correlation spectroscopy (F-CS) and fluorescence NTA (F-NTA) determine the absolute size distribution, which shows a high correlation with the standard population. The Pearson correlation coefficients between the calculated distribution and the standard population are 0.61 (scattering flow cytometry), 0.40 (DLS), 0.53 (NTA), 1.00 (STED), 0.98 (F-CS), and 1.00 (F-NTA).

A flow cytometer performs well in the distinction of cell types, but has major drawbacks in determining the size of vesicles. First, the lower detection limit of commercial flow cytometers for polystyrene beads is 300–500 nm [225, 267, 276]. Consequently, only a small fraction of vesicles can be detected. Second, only particles that differ by approximately 280 nm or more in size can be resolved with flow cytometers [225, 251]. Third, quantitative size information is obtained by comparing the scattering intensity of vesicles with that of beads of known size. The scattering intensity, however, depends not only on size but also on shape, refractive index, and absorption. The refractive index and absorption are even interconnected via Kramers-Kronig relationships [94]. For example, according to Mie calculations, a spherical gold particle 200 nm in diameter scatters 27 times more light than a polystyrene sphere of a similar size, which, in turn, scatters 15 times more light than a vesicle, owing to refractive index differences (see Appendix D.1). Furthermore, for non-spherical geometries, complex computer simulations are required [302].

Fig. 3.2A shows an impression of the size distribution as calculated for a flow cytometer in scattering mode, using the standard population as input. As the detection limit is approximately 300 nm, smaller vesicles are detected with low efficiency [225, 251]. Consequently, the measurements do not reflect the standard population. The poor capability to resolve size differences results in a smooth curve. The concentration of vesicles can be estimated when the flow rate \( Q \) is known. No specific information on morphology is obtained from the light-scattering intensity. Biochemical information is obtained by correlating the FSC with the SSC signal. As vesicles have a size in the order of the wavelength of visible light or smaller, they scatter light substantially in a perpendicular direction. Side scatter from a large vesicle therefore overwhelms side scatter from smaller structures inside. As a consequence, distinguishing vesicles with different cellular origins by correlating FSC and SSC signals is difficult, but it can be improved by analyzing the polarization of sideward scattered light [117].

Dynamic light scattering (DLS)

DLS, also known as photon correlation spectroscopy or quasi-elastic light scattering, determines the relative size distribution in a fluid of particles ranging in size between 1 nm and 6 \( \mu \)m [63, 85]. Particles in a fluid continuously move in random
directions, owing to continuous collisions with solvent molecules. This causes a random motion of particles called Brownian motion. The velocity distribution of particles depends on the temperature $T$, viscosity $\eta$, and (hydrodynamic) particle diameter $d$. The smaller the particle, the faster the Brownian motion. Particles undergoing Brownian motion cause intensity fluctuations of scattered light, which is measured typically in 30 s. The relative size distribution is obtained from the intensity fluctuations by applying a mathematical algorithm following from light-scattering theory. Light scattering theory requires the refractive index ratio $m = n_v/n_m$ of the vesicles and the medium, which is currently unknown. DLS performs well in the size determination of monodisperse samples, i.e. samples containing particles of one particular size, and monitoring a change in a sample such as aggregation [48, 101, 137]. Detection of the size distribution of polydisperse samples, i.e. samples containing different-sized particles, is less accurate, as the measured size distribution is influenced by the presence of small numbers of larger particles, such as platelets or other contaminants, which scatter more light than small vesicles, as shown in Fig. 3.1D [101, 137]. Furthermore, the result depends on the applied mathematical algorithm [48, 101], and two populations can only be resolved if the particle diameter differs at least two-fold [48, 101, 137, 170].

Fig. 3.2B shows the calculated relative size distribution for DLS relative to the standard population. The maximum value of the distribution is arbitrarily set to 1, as the concentration is unknown. Because larger vesicles scatter light more efficiently than smaller ones, the smallest vesicles become undetectable, and the distribution shifts to larger diameters. Our calculations closely fit the size distribution measurement of vesicles from fresh frozen plasma obtained with the N5 Submicron Particle Size Analyzer [183]. DLS does not provide information on the biochemical composition or cellular origin.

**Nanoparticle tracking analysis (NTA)**

NTA measures the absolute size distribution of particles ranging in size from 50 nm for biological particles to 1 µm. Particles in a fluid are illuminated by a laser beam and therefore scatter light, which is collected by a conventional optical microscope. NTA visualizes the scattered light from single particles in the field of view of the microscope. The scattered light shows up as small bright spots moving because of Brownian motion. The movements of individual particles are followed through a video sequence acquired over one to several minutes, and the mean velocity of each particle is calculated with image analysis software [124]. Because the velocity of Brownian motion depends on the temperature $T$, viscosity $\eta$, and (hydrodynamic) particle diameter $d$, it is possible to obtain an absolute size distribution after system calibration with beads of known size and concentration.

Fig. 3.2C shows the calculated absolute size distribution for NTA. On the basis of our assessment, NTA performs well for vesicles larger than 50 nm, but detection of vesicles smaller than 50 nm is not possible, owing to the detection limit of the microscope. NTA does not detect biochemical composition or cellular origin.
Raman spectroscopy

Raman spectroscopy is an inelastic light-scattering technique used to reveal the structure and biochemical composition of macromolecules inside single living cells. The sample is illuminated by monochromatic laser light. Molecular vibrations in the sample cause an energy loss or gain during a scattering event, resulting in a change in wavelength of the scattered light, which can be detected by specialized, sensitive spectrometers. The pattern of molecular vibrations is molecule-specific. As vesicles are composed of many different biomolecules, which all have unique Raman spectra, the chemical composition can be investigated without labeling. A confocal Raman microspectrometer can detect the Raman spectrum of volume elements of approximately $0.3 \mu m^3$, which overlaps with the dimension of vesicles, such that the chemical composition of single vesicles can potentially be detected without labeling. Furthermore, Raman microspectroscopy is a quantitative technique. The signal strength is linearly proportional to the number of molecules. For a vesicle that fits within the probe volume, the magnitude of the Raman signal strength is proportional to the volume of a single vesicle, and therefore estimates the relative size; this is a method that warrants further investigation before a reliable comparison with the standard population can be made. The concentration can be determined if the detection volume $V_d$ is known. The estimated measurement time is 3 h.

3.2.2 Fluorescence

Fluorescence is the property of a material whereby the material absorbs light of a particular wavelength and re-emits it at a usually longer wavelength. Most cells and vesicles exhibit no intrinsic fluorescence by which they can be distinguished. Therefore, vesicles are labeled with conjugates of antibodies or proteins with fluorophores. Commonly used fluorophores are organic dye molecules and quantum dots. Quantum dots have a typical diameter of 2–20 nm, and have been used as an artificial light source with which a vesicle can be labeled. In general, quantum dots are brighter and more stable than organic dye molecules or fluorescent proteins. As vesicles usually expose antigens from the parental cells, all methods based on fluorescence detection potentially provide information on the biochemical composition and cellular origin of vesicles. Fluorescence also offers opportunities to acquire additional chemical information, as the fluorescence intensity, wavelength and average time for which light is absorbed (fluorescence lifetime) depend on the molecular environment.

Fluorescent multilabeling analysis is not easy to perform, and there are several practical problems. For example, antibodies usually bind not only to the antigen of interest but also to Fc receptors. Furthermore, antibodies adhere nonspecifically or form aggregates, interfering with quantitative optical methods. In addition, other optical difficulties limit the feasibility of fluorescence detection. For example, it may be difficult to distinguish the fluorescence signal of interest from background radiation caused by autofluorescence, or irreversible photobleaching of fluorophores.
may occur \cite{128}. In the case of multilabeling, fluorophores can spectrally overlap, such that fluorescence associated with one fluorophore is detected by more than one detector \cite{25}.

**Fluorescence microscopy**

A fluorescence microscope is an optical microscope optimized for fluorescence detection. Usually, the fluorescence emission is separated from the excitation light with a spectral filter, before detection by a CCD camera. Modern fluorescence microscopes are able to detect fluorescence from a single fluorophore. For example, Zhang et al. \cite{335} loaded a synaptic vesicle with a single quantum dot (approximately 15 nm) to monitor membrane fusion and retrieval by high-speed imaging fluorescence microscopy.

In the case of autofluorescence, the size of vesicles can conceptually be determined, as the fluorescence signal may be proportional to the vesicle volume. However, in the case of fluorescent labeling, it is highly unlikely that the fluorescent amplitude will be proportional to the volume, so no size information can be obtained. Fluorescence microscopy allows an assessment of the concentration of vesicles with a certain property under the assumption that all vesicles with that property are indeed labeled and that the detection volume $V_d$ is known. A typical measurement time is approximately 1 h.

**Stimulated emission depletion (STED) microscopy**

In practice, STED microscopy is high-resolution fluorescence microscopy with better spatial resolution than described by Eqn. \ref{eq:3.2} for diffraction-limited optics. A resolution of 16 nm in diameter was successfully demonstrated, and this is sufficiently small to size vesicles \cite{320,323}. Sizing vesicles by STED, however, requires labeling of the entire surface of vesicles. Under the assumption that such labeling can be performed, Fig. \ref{fig:3.2D} shows the calculated size distribution. The predicted distribution correlates well with the standard population. Not only is STED microscopy promising for determining the size of and locating fluorescently labeled vesicles, but the high resolution can potentially be used to gain information on morphology and to determine the distribution of labeled receptors at the surface of larger vesicles, just as is presently done for organelles inside living cells \cite{130}. The concentration can be determined if the detection volume $V_d$ is known, and the measurement time for probing 10,000 particles is in the order of hours.

**Fluorescence flow cytometry**

In a fluorescence flow cytometer, the fluorescence from single particles present in a hydrodynamically focused fluid stream is measured at a rate of thousands of particles per second. With fluorescence flow cytometry, it is possible to distinguish vesicles on the basis of the spectral properties of the fluorescence signal \cite{225}. For detection of nanometer-sized particles by flow cytometry, the signal-to-noise ratio for fluorescence is higher than the signal-to-noise ratio for scattering, so
fluorescence flow cytometry is likely to be more sensitive than scattering flow cytometry. Flow cytometers with confocal optics can detect single fluorophores with an efficiency of approximately 10% by minimizing background fluorescence. As in fluorescence microscopy, the size distribution can, in principle, be determined when the amplitude of the fluorescence signal is proportional to the vesicle volume, a method that warrants further investigation. Fluorescence flow cytometry can estimate the concentration if the flow rate $Q$ is known, again under the assumptions that all vesicles are labeled and have a fluorescence intensity above the detection limit and threshold of the flow cytometer.

**Fluorescence correlation spectroscopy (F-CS)**

F-CS was originally introduced to measure parameters of molecular diffusion. It can determine the absolute size distribution and fluorescence signal of particles in a fluid. The size distribution is obtained from fluorescence intensity fluctuations caused by particles moving by Brownian motion through a well-characterized illuminated volume. Unlike DLS, F-CS detects single fluorescent molecules, and is therefore more sensitive for vesicles smaller than 50 nm; the size distribution can be more accurately determined in the presence of larger vesicles, and the concentration can be measured if the detection volume $V_d$ is known. Fig. 3.2E shows the calculated absolute size distribution, under the assumption that all vesicles are labeled. Although good correlation with the standard population can be simulated, we should be aware that small numbers of larger particles may influence the size distribution substantially. The measurement time is in the order of minutes.

**Fluorescence NTA (F-NTA)**

F-NTA determines the absolute size distribution and fluorescence signal of particles in a fluid. The method is similar to NTA, but is based on tracking of fluorescent particles. F-NTA is an extremely sensitive method for vesicles in the size range of exosomes, because the signal-to-noise ratio for fluorescence is expected to be considerably higher than signal-to-noise ratio for light scattering. With F-NTA, individual quantum dots can be detected. However, bleaching of the fluorescent marker may limit the tracklength. The good size and concentration detection properties are illustrated in Fig. 3.2F. Here, the simulations of the absolute size distribution of vesicles by F-NTA show an excellent correlation with the standard population.

### 3.3 Non-optical methods

**X-ray microscopy**

X-ray microscopy is a relatively new method in biomedical science for imaging the intact structure of a biological sample at a resolution of 12 nm. For example,
3.3. Non-optical methods

Figure 3.3: Calculated size distribution for non-optical detection methods (open black) relative to the standard population (transparent red), based on the underlying physical parameters of each method. (A) X-ray microscopy and atomic force microscopy (AFM) determine counts vs. diameter. The methods can resolve size differences in the order of a few nanometers, and the results show a high correlation with the standard population. (B) Absolute size distribution of a commercial impedance-based flow cytometer with a channel diameter of 25 µm. Vesicles smaller than 300 nm are detected with low efficiency. The Pearson correlation coefficients between the calculated distribution and the standard population are 1.00 (X-ray microscopy, AFM) and 0.27 (impedance-based flow cytometer).

the surface of macrophages was analyzed in their natural state and cryo-fixed eukaryotic cells were imaged in three dimensions by X-ray tomography. The high resolution of X-ray microscopy is achieved by reducing the wavelength to a few nm. According to Eqn. 3.2, a shorter wavelength lowers the diffraction limit. Considering the high resolution, X-ray microscopy is a promising method for detecting the size and possibly the morphology of vesicles in their physiological state. Fig. 3.3A shows the calculated size distribution of vesicles using an X-ray microscope with 12 nm resolution. Due to the high resolution there is a high correlation with the standard population.

An interesting feature of X-ray microscopy is the potential to detect the biochemical composition without labeling, since X-rays induces fluorescence of most substances. Furthermore, with X-ray microscopy the localization of chemical elements within cells can be visualized when using X-ray absorption near-edge structure spectra.

Presently, a major drawback of X-ray microscopy is that X-ray sources are scarce (synchrotron radiation) or impractical for clinical use (laser plasma x-ray contact microscopy). However, the field is rapidly evolving, which is exemplified by the increased availability of the required instrumentation.
Transmission electron microscopy (TEM)

TEM uses electrons instead of photons to create an image. The best achievable imaging resolution of TEM is given by Eqn. 3.2 and depends largely on the spatial stability of the electron beam in combination with the chemical stability of the sample. As the wavelength of electrons is more than three orders of magnitude shorter than the wavelength of visible light, the resolution of TEM can be lower than 1 nm. Because of this high resolution, it is possible to determine the size and morphology of vesicles [227].

As TEM is performed in a vacuum, biomaterials require fixation and dehydration, which affect size and morphology. Furthermore, the concentration of vesicles has to be increased by (ultra)centrifugation. As a consequence, the size distribution depends upon preanalytical conditions, and the concentration of vesicles cannot be determined. With immuno-gold labeling, it is possible to provide biochemical information [227]. The measurement time is in the order of hours.

Atomic force microscopy (AFM)

AFM was developed in 1986 by Binnig et al. [36], and provides subnanometer resolution topography imaging. An atomic force microscope consists of a cantilever with a sharp tip at its end that scans a sample surface without physical contact. Movements of the tip are measured, and a three-dimensional image is created by software.

Owing to a lateral resolution of 3 nm and a vertical resolution < 0.1 nm [36], AFM is suitable for size detection and performs better than DLS on polydisperse samples [137]. Siedlecki et al. and Yuana et al. showed that AFM can be used to measure the relative size distribution of vesicles in their physiologic state [333, 267]. Because of the high resolution of AFM, vesicles must be bound to an extremely flat surface, such as mica. Antibodies can be used to bind vesicles to the surface, so that biochemical information can also be obtained [333]. Because the efficiency of vesicle binding to a surface using antibodies is unknown, the concentration of vesicles cannot be determined with certainty. Furthermore, the surface binding may affect the morphology of vesicles, and this may hamper the determination of the real diameter.

Fig. 3.3A shows the calculated relative size distribution for 10,000 counts as measured with AFM, assuming isovolumetric particle deformation and equal surface binding. Under these assumptions, there is excellent correlation with the standard population, owing to the high resolution. The measurement time is in the order of hours.

Impedance-based flow cytometry

The Coulter principle is employed in an impedance-based flow cytometer to count and measure the size of single particles in a fluid within seconds. An impedance-based flow cytometer consists of two chambers divided by an insulating membrane containing a single channel. In each chamber, an electrode is immersed in an
electrolyte to drive an ionic current through the channel. Particles driven into the channel cause a reduction in current. A relative size distribution can be calculated from the change in current when the channel length $l_c$ and diameter $d_c$ are known. The concentration can also be determined if the average particle transport velocity $v_{s,av}$ is known.

The sensitivity of impedance-based flow cytometry depends on the channel size with respect to the vesicle size. The vesicle diameter must be between approximately 0.1 and 0.7 times the channel diameter [144]. In practice, at least two impedance-based flow cytometers, each with a different channel diameter, are required to cover the whole size range of vesicles. The lower detection limit of commercial impedance-based flow cytometers is currently 300 nm [338]. Consequently, only a small fraction of vesicles can be detected.

Fig. 3.3B shows the calculated absolute size distribution for a commercial impedance-based flow cytometer with a channel diameter of 25 µm [338]. As the detection limit is approximately 300 nm, smaller vesicles are detected with low efficiency. An impedance-based flow cytometer does not provide information on the morphology, biochemical composition, or cellular origin, but the method can be combined with light scattering and fluorescence flow cytometry.

### 3.4 Discussion and conclusion

This chapter gives an overview of (potential) methods for the detection and characterization of vesicles. Table 3.1 lists the assessed possibilities and limitations of each method, based on the underlying physical parameters of each technique. Considering the optical methods based on light scattering, DLS and NTA are potentially capable of measuring relative and absolute size distributions, respectively, of vesicles within minutes. Except for Raman spectroscopy, methods based on light scattering cannot distinguish vesicles from similar-sized lipoprotein particles or small platelets, as no biochemical information is obtained. Raman spectroscopy could potentially detect the size, concentration, and biochemical composition of single vesicles without labeling, but the measurement time is in the order of hours.

Of the optical methods based on fluorescence, STED microscopy, F-NTA, and F-CS are potentially capable of measuring the absolute size distribution and obtaining biochemical information by the application of fluorescent antibody labeling. Fluorescent antibody labeling which is not easy to perform, and involves several practical and optical problems. Real size distribution measurements may be less accurate, as optical detection can be influenced by many factors, such as age of the light source, cleanliness of the cuvette or flow channel, stability of the building and supporting table, and preanalytical conditions.

Among the non-optical methods, X-ray microscopy, TEM and AFM have high ($\leq 12$ nm) imaging resolution as compared with optical methods. Size and morphology information can be obtained by imaging, and biochemical information can also be obtained. However, measurements are based on many assumptions, and
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the measurement time is more than 1 h per sample. A fast non-optical method is impedance-based flow cytometry, which can resolve small size differences but only within a limited size range. This technique provides no biochemical information unless combined with fluorescence flow cytometry.

From Table 3.1 F-NTA seems to be the most suitable method for the detection of size, concentration, biochemical composition, and cellular origin of vesicles at high speed, especially as the method can determine the relevant characteristics of vesicles directly in body fluids. Nevertheless, the other methods mentioned in this chapter are being rapidly developed, and this might lead to new possibilities and shorter measurement times.

3.4.1 Combining methods

Methods that have been successfully combined for vesicle detection are flow field-flow fractionation (F-FFF) with multiangle light scattering (MALS) or DLS [159]. F-FFF can fractionate 27 nm diameter vesicles from 36 nm diameter vesicles [170]. Subsequently, DLS or MALS can accurately determine the size, as the fractionated sample is monodisperse. MALS is based on angle resolved light scattering, and is used for molar mass and mean particle size determination. We did not discuss MALS earlier, because the technique does not provide a size distribution for polydisperse samples. Another method that is practically extendable is Raman spectroscopy, which was recently successfully combined with both Rayleigh scattering and fluorescence microscopy for intracellular chemical analysis [237]. Raman spectroscopy can also be extended with electron microscopy to correlate detailed biochemical information with the relative size distribution and morphology [305]. Finally, Raman spectroscopy can be integrated with optical coherence tomography to obtain quantitative information on the concentration-dependent scattering coefficient [95, 224].

3.4.2 Improving methods

A conventional method that can be optimized for the detection of vesicles is flow cytometry. By reducing flow chamber dimensions, optimizing the flow chamber shape, reducing the flow velocity, and using large-aperture optics, the sensitivity can be increased tremendously. Steen extended a commercial flow cytometer with dark-field illumination and detection to improve the detection limit to 70 nm for polystyrene spheres [276]. Single quantum dots can be detected with 99 % accuracy by flow cytometry when a submicrometer fluidic flow channel combined with a confocal microscope is used [275]. NTA is a relatively new method, and is currently showing a high degree of development. Increasing the detector sensitivity and decreasing the wavelength may lower the detection limit to 30 nm for biological particles, so that even the smallest vesicles come within reach without the need for fluorescent labeling. In specialized laboratories, two impedance-based flow cytometers have been optimized for the detection of submicrometer particles by reducing the channel diameters to 500 and 132 nm [144, 257]. In combination with
3.4. Discussion and conclusion

a commercial impedance-based flow cytometer, this covers the whole size range of vesicles, but centrifugation or filtration of the sample is required to prevent frequent problems with blocking of the flow channel.

3.4.3 Recently obtained results

Recently, some of the methods discussed have been applied to vesicles \[107\]. Here, we give an interpretation of these results based on our analysis. The concentration of vesicles in platelet-free plasma was reported to be $200-260 \times 10^9 \text{ L}^{-1}$ by NTA \[124\] and $3-702 \times 10^9 \text{ L}^{-1}$ by AFM \[333\]. It is possible that the real concentration is higher, as the detection efficiency of both methods is $<100\%$. However, if we consider false positives such as lipoprotein particles, the real concentration may also be lower. With flow cytometry, Yuana obtained a 1,000-fold lower CD41\(^+\)-vesicle concentration, of $11-291 \times 10^6 \text{ L}^{-1}$, than was obtained with NTA and AFM. The discrepancy in results between flow cytometry and NTA and AFM can be explained by considering the detection limit of commercial flow cytometers, which is insufficient to detect vesicles smaller than 300 nm. As most vesicles are smaller than 300 nm and are therefore not detected (Fig. 3.3A), the detection efficiency is $<2\%$. In addition, different results have also been obtained with the same method. Lawrie et al. used DLS equipment from two companies, and obtained different size distributions for the same vesicles in fresh frozen plasma \[183\]. Sources of these differences could be the detection angle and the applied mathematical algorithm. In conclusion, several (combinations of) methods can correctly detect clinically relevant properties of vesicles. These methods should be further explored and validated by comparing measurement results, so that accurate, reliable and fast analyses of extracellular vesicles come within reach.